Growth inhibitory efficacy of *Cornus officinalis* in a cell culture model for triple-negative breast cancer

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**Abstract.** Triple-negative breast cancer (TNBC) lacks the expressions of estrogen receptor-α, progesterone receptor and human epidermal growth factor receptor-2. The treatment options for TNBC include anthracyclin/taxol based conventional chemotherapy and small molecular inhibitor based targeted therapy. However, the therapeutic efficacy is limited by systemic toxicity and acquired tumor resistance; identification of less toxic testable alternatives is urgently required. Non-toxic nutritional herbs are commonly used in traditional Chinese herbal medicine for general health management and may additionally represent a testable therapeutic alternative for TNBC. The present study examined the growth inhibitory efficacy of the nutritional herb *Cornus officinalis* (CO) in MDA-MB-231 cells, which represent a cell culture model for TNBC, and identified potential mechanistic leads. In MDA-MB-231 cells, CO induced dose-dependent cytostatic growth arrest [inhibitory concentration (IC)₀, 0.1% and IC₉₀, 0.5%], and inhibited anchorage independent colony formation. Mechanistically, CO inhibited G₁ to S phase transition leading to G₂ arrest and decreased the expression of cyclin D1 and phosphorylated-retinoblastoma proteins. CO additionally altered apoptosis specific BCL-2 associated X protein/B-cell lymphoma-2 expression and upregulated pro-apoptotic caspase-3/7 activity. Collectively, these data provided mechanistic evidence for the efficacy of CO, and validated a mechanism-based approach to prioritize efficacious nutritional herbs as testable alternatives for secondary prevention/treatment of TNBC.

**Introduction**

The American Cancer Society projections for the incidence of invasive breast cancer and mortality estimate 246,660 newly diagnosed invasive breast cancer cases and 40,450 invasive breast cancer related deaths in women in 2018 (1). Therapy resistant invasive breast cancer continues to remain the leading cause of mortality in women in the USA. The triple negative breast cancer (TNBC) represents an aggressive molecular subtype that lacks the expressions of estrogen receptor-α (ER-α), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2). TNBC is notable for its resistance to conventional endocrine or HER-2 targeted therapy (2-4). Current treatment options for the TNBC subtype are restricted to anthracyclin, taxol and platinum based conventional chemotherapy or to poly(ADP-ribose) polymerase (PARP), phosphadityl-inositol-3 kinase (PI3K) and mammalian target of rapamycin (m-TOR) selective inhibitor based targeted therapy (4,5). These conventional and targeted treatment options are frequently associated with long-term dose limiting systemic toxicity, *de novo* or acquired tumor resistance, and emergence of drug resistant cancer stem cells that impact therapeutic efficacy, and thereby, promote drug resistant disease progression (6). These limitations emphasize a need to identify novel, non-toxic treatment options as testable alternatives to existing treatment strategies.

Complementary and alternative approaches utilizing herbal medicines are being extensively used for general health issues and for management of breast cancer. Nutritional herbs represent widely used non-toxic natural substances in traditional Chinese herbal medicine (7-9). Non-toxic natural substances offer a testable alternative as potentially novel approach for secondary prevention/therapy of breast cancer, reduction of therapy associated toxicity and/or for enhanced therapeutic efficacy (8,9). Extracts from mechanistically distinct Chinese nutritional herbs have displayed growth inhibitory effects on cell culture models for Luminal A and the triple-negative molecular subtypes for clinical breast cancer (10-17). *Cornus officinalis* (CO) is a nutritional herb in the form of a cherry fruit. It is used as a major ingredient in some Chinese herbal formulations for general health management purposes. Several *Cornus* species have documented anti-proliferative,

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anti-oxidant and anti-inflammatory properties. Anthocyanins are among the major bio-active agents (18,19). Additionally, CO extract exhibits growth inhibitory effects on the estrogen receptor positive Luminal A model predominantly via inhibiting estrogen stimulated growth and altering the cellular metabolism of estradiol to favor the generation of anti-proliferative metabolites (12). In an effort to evaluate the utility of CO in the TNBC model, the experiments in the present study were designed to examine the growth inhibitory effects of CO in a cell culture model for TNBC, and to identify potential mechanistic leads and molecular targets for its efficacy.

Materials and methods

Experimental model. The human mammary carcinoma derived ER-α, PR and HER-2 MDA-MB-231 cells (20,21) represented the model for TNBC. The MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in RPMI medium with L-glutamine and 5% fetal bovine serum (Life Technologies, Grand Island, NY, USA) following the protocol recommended by the vendor.

Dose response. For the dose response experiments the non-fractionated aqueous extract of CO was prepared following previously published protocol (12). Briefly, the herb was sequentially boiled in water and centrifuged to concentrate their water soluble components in the final volume of 20 ml. This 100% stock solution of CO was serially diluted in the culture medium to obtain the final concentration range of 1.0, 0.5, 0.1 and 0.05% CO. The cells were treated with the CO extract within this concentration range. The dose response of CO extract was determined by the cell viability assay using the trypan blue dye exclusion test kit (Sigma-Adrich; Merck KGaA, Darmstadt, Germany). The cell viability measurements were conducted for CO treated and control cultures at day 7 post-seeding of 10^5 cells. The cell viability was calculated as % viable cells=[1.00-(Number of blue cells ÷ Number of total cells)] x100. These data were used to identify minimum effective, half maximum, maximum cytostatic and toxic concentrations. The maximum cytostatic concentration was defined by the cell viability equal to or higher than the initial seeding density. The toxic concentration was defined by the cell viability lower than the initial seeding density. The data were expressed as viable cell number (x10^5), relative to the initial seeding density.

Anchorage independent (AI) growth assay. This assay was performed following the optimized protocol (13,16). The stock solution of agar was prepared by mixing DNA grade agar (Sigma-Adrich; Merck KGaA) with an appropriate volume of 2X RPMI medium (Sigma-Adrich; Merck KGaA) to obtain a 6% agar stock solution. To prepare the basement layer, this stock solution was diluted to 0.6% using the culture medium, dispersed in a 6-well plate and allowed to solidify overnight at 37°C. MDA-MB-231 cell suspension, at a density of 5x10^5 cells per ml, was prepared in RPMI medium containing 0.33% agar, and this cell suspension was overlaid on the basement layer in the presence or absence of CO. The cultures were incubated at 37°C in a CO₂ incubator for 21 days. The AI colonies were stained with 0.005% crystal violet and colony counts were determined at 10X magnification. The data were expressed as AI colony number.

Cell cycle progression. For the analysis of cell cycle progression, 5x10^5 cells were seeded in T-25 flasks and treated at 24 h. post-seeding with different concentrations of CO for 48 h. The cells were harvested by trypsinization, pelleted at 500 x g, and washed twice with cold phosphate buffered saline pH 7.4 (PBS; Sigma-Adrich; Merck KGaA). The cells were then fixed with cold 70% ethanol, washed with cold PBS, and stained with 50 µg/ml propidium iodide (PI; Sigma-Adrich; Merck KGaA) in PBS, followed by the addition of 10 µg/ml ribonuclease (Sigma-Adrich; Merck KGaA) and incubation for 4 h. in the dark. DNA content was analyzed by flow cytometry using Becton Dickinson FACSCAN Flow Cytometer (BD Biosciences, Research Triangle Park, NC, USA) and analyzed with FACS Express software (De Novo Software, Glendale, CA, USA). The data were expressed as G₁:S+G₂/M ratio.

Western blot analysis. For the western blot assay, cells were seeded in 10-cm dishes at 70% confluence 1 day before the treatment. Different concentrations of CO were added and incubated for 48 h. in a CO₂ incubator at 37°C. Cells were harvested and immediately lysed with radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitors (Sigma-Adrich; Merck KGaA), and centrifuged for 15 min at 10,000 x g. An equal quantity of protein was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) and were maintained in RPMI medium with L-glutamine and 5% fetal bovine serum (Life Technologies, Grand Island, NY, USA) following the protocol recommended by the vendor. The chemo-luminescent signal was developed with ECL-plus reagent (Bio-Rad Laboratories, Hercules, CA, USA) and detected by autoradiography. The data were expressed as arbitrary scanning unit (ASU).

Reverse transcription-polymerase chain reaction assay for gene expression. The effect of CO on the status of select apoptosis specific gene expression was evaluated using the semi-quantitative Reverse Transcription PCR assay. Gene amplification was monitored for apoptosis specific BAX and BCL-2 genes using gene specific promoters (Cepheid, Inc. Sunnyvale, CA, USA). Briefly, a total of 25 µl of reaction mix was prepared that contained MgCl₂ (2 mmol/l), 12.5 µl of 2X Taq PCR Master Mix (Qiagen, Inc., Valencia, CA, USA), 0.25X SYBR dye (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and gene specific primer sets (0.3 µmol/l, provided by the core facility, University of Texas Health Sciences Center, San Antonio, TX, USA). The PCR reaction was set for 40 cycles and the data were compared after normalization with β-actin RNA levels. The data were expressed as ∆∆Ct values for relative gene expression.

Caspase assay. Caspase-3/7 activity in the MDA-MB-231 cells was measured using Caspase-Glo assay kit (Promega Life Sciences, Inc., Madison, WI, USA). Briefly, the cells treated with
CO were homogenized by sonication in homogenization buffer (25 mmol/l HEPES, pH 7.5, 5 mmol/l MgCl₂, and 1 mmol/l EGTA) and protease inhibitors (all from Sigma-Adrich; Merck KGaA). The homogenate was centrifuged at 6,500 x g at 4°C for 15 min. To 10 µl of the supernatant containing the cellular protein, equal volume of the assay reagent was added and incubated at room temperature for 2 h. The luminescence was measured using Luminometer (Thermo Fisher Scientific, Inc.). The data were expressed as relative luminescence units (RLU).

### Statistical analysis

The experiments for dose response, anchorage independent growth, caspase-3/7 activity and relative gene expression profile were conducted in triplicate. The data are expressed as the mean ± standard deviation. Statistically significant differences between the control and multiple treatment groups were assessed by one-way analysis of variance and Dunnett’s test as a post hoc test with a threshold of α=0.05, using Microsoft Excel 2013 XLSTAT-Base software.

### Results

#### Growth characteristics of MDA-MB-231 cells

The data presented in Table I summarizes the growth pattern of the experimental model. The MDA-MB-231 cells exhibited a short population doubling time of 15 h and high saturation density, exhibiting a ~33-fold increase relative to the initial seeding density. Additionally, these cells exhibited accelerated cell cycle progression as evidenced by a substantially low G₁:S+G₂/M ratio of 0.6, relative to a ratio of 2.3 exhibited by non-tumorigenic triple-negative 184-B5 cells (data not shown). Furthermore, unlike the non-tumorigenic 184-B5 cells, these breast carcinoma derived MDA-MB-231 cells exhibited a high number of AI colonies.

### Dose response of CO

The data presented in Fig. 1A demonstrate a dose-dependent growth inhibition of MDA-MB-231 cells in response to a 7-day treatment with CO. In response to treatment with CO concentrations of 0.05, 0.1 and 1.0%, the viable cell number was reduced to 17.8±1.6x10⁵, 12.5±1.1x10⁵ and 2.5±0.2x10⁵, respectively, relative to 24.8±2.3 x10⁵ in the control. This dose response experiment identified IC₅₀ as 0.05%, IC₉₀ as 0.1% and IC₉₀ as 0.5% for CO respectively, relative to control. Statistical analysis using ANOVA and Dunnett’s test confirmed that control >IC₅₀ and control >IC₉₀ (α=0.05). The concentration of 1% CO exhibited >98% inhibition in viable cell number. In this treatment group the viable cell number was lower than the initial seeding density of 1.0 x10⁶. The concentration of 1% was therefore considered toxic.

### Effect of CO on anchorage independent (AI) growth

The data presented in Fig. 1B summarizes the growth inhibitory effect of CO on AI colony formation of 21 day duration in MDA-MB-231 cells. In response to treatment with CO at the pre-determined IC₅₀, IC₉₀ and IC₉₀ concentrations, the number of AI colonies was reduced to 222±49, 120±27 and 40±9, respectively, relative to 255±57 AI colonies in the control. Statistical analysis using ANOVA and Dunnett’s test confirmed that control >IC₅₀ and control >IC₉₀ (α=0.05).

### Effect of CO on cell cycle progression

The experiment presented in Fig. 2A examined the effect of CO on the cell cycle progression of MDA-MB-231 cells. The data presented as the G₁:S+G₂/M ratio demonstrate that a 48 h. treatment with CO was associated with a dose-dependent increase in this ratio. Thus, treatment with 0.1% CO exhibited the G₁:S+G₂/M ratio of 4.8±0.4, and treatment with 1.0% CO exhibited a ratio of 7.8±0.2, relative to the ratio of 2.5±0.2 exhibited by control. Statistical analysis using ANOVA and Dunnett’s test confirmed that control <0.1% CO and control <1.0% CO (α=0.05). These

### Table I. Status of proliferation end points in a cellular model for triple-negative breast cancer.

<table>
<thead>
<tr>
<th>Proliferation end point</th>
<th>Model MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling time (h)</td>
<td>15.0±2.2</td>
</tr>
<tr>
<td>Saturation density (x10⁵)</td>
<td>32.9±2.3</td>
</tr>
<tr>
<td>G₁:S+G₂/M ratio</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>AI colony numberd</td>
<td>257±57</td>
</tr>
</tbody>
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data indicate that CO treatment induced a 92% and a 2.2-fold progressive increase due to \(G_1\) arrest and inhibition of the \(S\) and \(G_2/M\) phases of the cell cycle.

The experiment presented in Fig. 2B examines the status of select regulatory proteins in the RB signaling pathway in response to treatment with CO. These data demonstrate that a 48 h CO treatment resulted in ∼60-66% decrease in cyclin D1 expression, and ∼40-52% decrease in p-RB expression depending on CO concentration, relative to that in the untreated control cells.

**Effect of CO on cellular apoptosis.** The experiment presented in Fig. 3A examines the status of apoptosis specific BAX and BCL-2 gene expressions. These data presented as \(\Delta \Delta C_t\) values demonstrate that in response to a 48 h treatment with 0.5% CO and 1.0% CO the expression of pro-apoptotic BAX gene was 2.5±0.2 and 4.9±0.5, while that of anti-apoptotic BCL-2 gene was 0.9±0.1 and 0.2±0.1, relative to untreated control. Statistical analysis using ANOVA and Dunnett’s test confirmed that control <0.5% CO and control <1.0% CO (\(\alpha=0.05\)). Thus, these data demonstrate that in response to treatment with CO the expression of pro-apoptotic BAX gene exhibited a ∼2.5- and ∼2.9-fold increase, while that of anti-apoptotic BCL-2 gene exhibited a ∼10 and ∼80% decrease, relative to the untreated control.

The experiment presented in Fig. 3B examined the effect of a 48 h treatment with CO on pro-apoptotic caspase-3/7 activity. The data presented as RLU demonstrate that treatment with 0.1% CO resulted in RLU value of 5.7±0.3, and treatment with 1.0% CO resulted in RLU value of 7.9±0.5, relative to RLU value of 1.9±0.1 for the control. Statistical analysis using ANOVA and Dunnett’s test confirmed that control <0.1% CO and control <1.0% CO (\(\alpha=0.05\)). Thus, treatment with CO induced a 2.0- and 3.1-fold increase in caspase-3/7 activity.

**Discussion**

Approximately 10-20% of clinical breast cancers lack the expressions of ER-\(\alpha\), PR and HER-2 and are classified as TNBC (2,4,22,23). The treatment option for the TNBC molecular subtype is predominantly restricted to conventional chemotherapy or to pathway selective small molecule based targeted therapy (3-5). Long-term therapeutic efficacy of these treatment options is compromised predominantly due to acquired tumor resistance and emergence of drug resistant cancer stem cell population (6,22-24). These limitations emphasize a need for identification of non-toxic effective alternatives as testable therapeutic options.

Human tissue derived cell culture models offer valuable mechanistic approaches to identify clinically translatable leads to the inhibitory efficacy of a number of nutritional herbs against the Luminal A and TNBC models (10-17). Compared to the non-tumorigenic triple-negative 184-B5 cells, the present TNBC model exhibits loss of homeostatic growth control and...
persistent cancer risk as evidenced by hyper-proliferation, accelerated cell cycle progression and increased AI colony formation. In the present TNBC model several nutritional herbs induce cytostatic growth arrest and/or cellular apoptosis and inhibit anchorage independent colony formation, thereby re-establishing cellular homeostasis and reducing cancer risk. At the mechanistic levels, growth inhibitory efficacy of these herbs involve RB, BAX/BCL-2, caspase, and RAS-RAF-MEK-ERK mediated signaling pathways (15-17).

In the present study, MDA-MB-231 cells in response to treatment with CO exhibited dose-dependent growth inhibitory effects as evidenced by cytostatic growth arrest, and reduction in AI colony formation. The data generated from dose response experiments identified the effective range of 0.05-0.5% for CO in the present model, suggesting potent growth inhibitory efficacy at relatively low doses of CO. Additionally, the cytostatic growth arrest induced by CO was associated with a progressive increase in the G$_1$S+G$_2$/M ratio, indicating an effect of CO on cell cycle progression.

The data from the experiment designed to examine the effect of CO on the RB pathway clearly demonstrate that CO inhibited the expression of cyclin D1 and of phosphorylated RB (p-RB) in a dose-dependent manner. The tumor suppressor retinoblastoma (RB) gene has a well-documented function in the regulation of cell cycle progression. The tumor suppressive function of RB via the cyclin D-CDK4/6-pRB pathway has been documented to be frequently compromised in therapy resistant basal-like and triple-negative molecular subtypes of clinical breast cancer and therefore, may represent a therapeutic target (25-27). Additionally, small molecule inhibitors of CDK4/CDK6 in combination with aromatase inhibitors or with selective estrogen receptor modulators have documented clinical efficacy in hormone receptor positive metastatic breast cancer (28,29). Specific small molecule inhibitors of CDK4/CDK6 may also represent viable treatment options for TNBC (30,31). In the present study, the dose-dependent decrease in cyclin D1 and pRB expression suggests involvement of the RB pathway in the efficacy of CO. In this context it is noteworthy that kinase-dependent site specific phosphorylation of RB represents one of the major post-translational modification that is critical for its tumor suppressive function via inactivation of cell cycle progression and promotion of cellular apoptosis (25,26). It is also noteworthy that another nutritional herb *Dipsacus asperoides* exhibits inhibition of CDK4 and CDK6 expression in the MDA-MB-231 model for TNBC (17). Additionally, recent evidence on human TNBC samples and on patient derived TNBC xenograft models suggests that expression of EGFR, it’s recently identified partner MT4-MMP and RB is strongly associated with the efficacy of a combination of EGFR and CDK4/6 based targeted therapy (32). Thus, the present data on inhibition of cyclin D1 and pRB provide mechanistic leads that the RB pathway might represent a molecular target for the efficacy of CO in the present model system.

The experiment designed to examine the effect of CO on cellular apoptosis demonstrated that treatment with CO produced a progressive increase in pro-apoptotic BAX expression, while that of anti-apoptotic BCL-2 was decreased. Additionally, this CO-mediated modulation in the BAX/BCL-2 pathway positively correlated with a dose-dependent increase in the pro-apoptotic caspase-3/7 activity. It is well established that the intrinsic mitochondrial apoptotic pathway involves altered membrane permeability, cytochrome-c release, and apoptosis mediated activation of caspase-9 and subsequently of caspase-3/7 (33,34). In the present TNBC model, treatment with CO has identified pro-apoptotic mechanistic leads for the efficacy of CO (16). Thus collectively, these data on cellular apoptosis support the evidence that induction of cellular apoptosis by CO may be via a caspase-dependent mechanism. This suggestive mechanistic lead indicates involvement of the intrinsic apoptotic cascade where expression of caspase-3/7 activity represents a late-occurring event. Investigating additional molecular pathways relevant to intrinsic apoptosis cascade represents future research directions that are focused to examine the specific effect of CO on cellular apoptosis and to investigate mechanisms and molecular targets responsible for pro-apoptotic efficacy of CO.

Traditional Chinese herbal medicine utilizes combination of several herbs for herbal formulations that are boiled in water to prepare herbal tea for consumption by the patients. Thus, non-fractionated aqueous extract of CO used in the present study simulates clinical administration of herbal formulations. In this context it is conceivable that individual constitutive herbs in the formulations may contain multiple bio-active agents with distinct growth modulatory roles. Although in the present study, non-fractionated aqueous extract from CO has exhibited potent growth inhibitory effects via potential mechanistic leads for its efficacy, little evidence is available regarding the identity of active agent(s) responsible for these effects. However, it is noteworthy that anthocyanins, representing major bio-active agents, may in part be responsible for the cellular and biochemical effects of CO (18,19).

In conclusion, the present study outcome validates a relevant cell culture model for the triple-negative molecular subtype of clinical breast cancer, and offers a facile experimental approach to prioritize efficacious natural substances as testable alternatives for endocrine therapy resistant breast cancer.

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Availability of data and materials

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

Authors’ contributions

NTT conceived the study design, formulated the experimental protocols and prepared the manuscript. HBN performed the
experiments, and organized and analyzed the data. GYCW selected the nutritional herb for the present study, and contributed to the data interpretation and preparation of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References