Extracts of *Bridelia ovata* and *Croton oblongifolius* induce apoptosis in human MDA-MB-231 breast cancer cells via oxidative stress and mitochondrial pathways

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Abstract. Breast cancer is the most common type of cancer and is also the second leading cause of cancer-associated death in women worldwide. Thus, there is an urgent requirement for the development of effective treatments for this disease. *Bridelia ovata* and *Croton oblongifolius* are herbs used in Thai traditional medicine that have been used to treat various health problems; *B. ovata* has traditionally been used as a purgative, an antipyretic, a leukorrhea treatment and as a birth control herb. *C. oblongifolius* has been used to increase breast milk production, for post-partum care (where it is used as a hot bath herb), and as a treatment for flat worms and dysmenorrhea. However, there is little research investigating the anticancer properties of these herbs. The present study aimed to investigate the anticancer properties of crude ethyl acetate extracts of *B. ovata* (BEA) and *C. oblongifolius* (CEA) in order to explore their underlying mechanisms in breast cancer cell death. The phytoconstituents of the crude extracts of BEA and CEA were studied using gas chromatography-mass spectrometry (GC-MS). GC-MS analysis showed that the primary compound in BEA is friedelan-3-one, and kaur-16-en-18-oic acid in CEA. Cytotoxicity was investigated using an MTT assay, both BEA and CEA showed greater toxicity against MDA-MB-231 breast cancer cells compared with their effect on MCF10A normal epithelial mammary cells. BEA and CEA exerted various effects, including inducing apoptotic cell death, reducing mitochondrial transmembrane potential, increasing the levels of intracellular ROS, activating caspases, upregulating pro-apoptotic and downregulating anti-apoptotic genes and proteins. BEA and CEA were shown to have anticancer activity against breast cancer cells and induce apoptosis in these cells via a mitochondrial pathway and oxidative stress.

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Abbreviations: BEA, Bridelia ovata ethyl acetate extract; CEA, *Croton oblongifolius* ethyl acetate extract; ΔΨm, mitochondrial transmembrane potential; NOXA, phorbol-12-myristate-13-acetate-induced protein 1 or PMAIP1; BAX, Bcl2-associated X protein; Bcl-xL, B-cell lymphoma-extra large

Key words: Bridelia ovata, *Croton oblongifolius*, phytochemicals, breast cancer, apoptosis, extract

Introduction

Breast cancer is the most common type of cancer and the leading cause of cancer-associated mortality and morbidity in women worldwide (1,2). The mortality rate for this disease has decreased due to early diagnosis, but treatment outcomes of patients with advanced breast cancer remain poor (3). There are currently several options for the treatment of breast cancer such as surgery, radiotherapy and chemotherapy, but their use is limited by adverse effects, drug resistance and rapid cancer cell metastasis (4). Therefore, an effective treatment for certain aggressive forms of breast cancer, such as inflammatory breast cancer and triple-negative breast cancer (TNBC), is still required. TNBC cannot be treated with hormonal therapy, as TNBC cells do not express receptors for estrogen, progesterone and epidermal growth factor (5). Despite some considerable limitations, the leading cancer treatments have generally improved over the years; though the financial cost remains high (6). Hence, cancer research should move towards developing and applying herbal treatment strategies with lower costs and fewer side effects to patients.

Bridelia ovata and *Croton oblongifolius*, from the Euphorbiaceae family, are traditional herbs found in Thailand; Thai traditional medicine uses the dried leaf of *B. ovata* as an expectorant and a laxative, whereas the bark is used as a medicinal astringent (7). Few previous phytochemical studies have demonstrated the presence of triterpenes and phytosterols in *B. ovata* (8,9); its crude ethanolic extract has been shown to inhibit the invasive and migratory abilities of HepG2 cells (10), thus the present study focused on the identification of these compounds in *B. ovata*.

C. oblongifolius has been used as a medicinal plant for the treatment of several diseases and ailments; for example, the leaf is used as a blood tonic for general weakness and poor appetite, and the flower is used to treat flatworm infections. Furthermore, the fruit is used to alleviate dysmenorrhea, the seed acts as a purgative (11) and the root is used in the treatment of dysentery (12). In addition, *C. oblongifolius* bark has been used for the treatment of dyspepsia, chronic liver enlargement and remittent fever (13). Moreover, in Thai traditional medicine, *C. oblongifolius* is utilized in combination with *C. sublyratus* to treat gastric ulcers and gastric cancers (14,15). Several types of phytochemical have been isolated from *C. oblongifolius*, including megastigmane glycosides (16), labdane diterpenoids (11,17), clerodanes (18-21), halimanes (22), cembranes (21,23,24), pimarane-types (acantoic acid) (25) and (-)-*ent*-kaur-16-en-19-oic acid (12). A study investigating clerodanes from *C. oblongifolius* showed that this phytochemical has cytotoxic effect against human cancer cell lines, including HepG2, SW620, CHAGO, KATO3 and BT474 (17). However, there are currently no reports of the activity of *B. ovata* and *C. oblongifolius* ethyl acetate extracts against TNBC cells, and to the best of our knowledge, the underlying mechanisms mediating the induction of cancer cell death remain unknown.

In the present study, apoptosis (programmed cell death) was investigated. Apoptosis is an important process for normal tissue homeostasis (26), and any disruption of the underlying mechanisms may cause the onset of a variety of diseases, including autoimmunity, neurodegenerative diseases and cancer (27). Therefore, therapeutic strategies targeting apoptosis have been applied to treat these diseases (28). The two primarily routes of apoptosis are the extrinsic and intrinsic pathways; the extrinsic, or death receptor pathway is induced by the binding of death ligands (such as FasL, TRAIL and TNF) to specific receptors (such as FAS, TRAILR1-2 and TNFR1) at the cell membrane (29). Subsequently, the intracellular death domain of the receptor recruits the adaptor FAS-associated death domain protein and the initiator caspase 8. These form the death-inducing signaling complex (DISC). DISC proteolytically auto-activates caspase 8, which then cleaves and activates the effector caspase 3 to induce apoptosis (30,31).

The intrinsic, or mitochondrial pathway, is triggered by diverse stimuli, including DNA damage, chemotherapeutic drugs and oxidative stress. These stimuli induce the loss of mitochondrial transmembrane potential, permitting cytochrome *c* release from the mitochondria intermembranous space into the cytosol, resulting in apoptosome formation. The apoptosome is a protein complex comprising cytochrome *c*, apoptosis-activating factor 1, adenosine triphosphate (ATP), and procaspase-9 (32). The apoptosome autoactivates caspase 9, which in turn cleaves and activates caspase 3 (33), resulting in apoptosis. Mitochondrial apoptosis is controlled by the Bcl-2 protein family. This comprises anti-apoptotic proteins, including Bcl-2, Bcl-xL and Mcl-1, and two other groups of pro-apoptotic proteins, including the multi-domain proteins BAX and Bcl-2 homologous antagonist/killer (BAK), and BH3-only proteins Bcl-2-like protein 11 (BIM), and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA). During apoptosis, the expression of anti-apoptotic molecules is downregulated, and regulatory BH3-only proteins induce the formation of transport channels on the outer mitochondrial membrane, comprised of dimers of BAX and/or BAK (34). These channels promote cytochrome *c* release and the sequential activation of caspase 9 and 3 (35). Therefore, the aim of the present study was to investigate the pro-apoptotic potential of BEA and CEA against MDA-MB-231 triple-negative breast cancer cells. This cell line has the highest potential for invasiveness (36) and is commonly used as a model for late-stage breast cancer (37).

Materials and methods

Plant materials. The trunks of *B. ovata* and *C. oblongifolius* were collected from northeastern Thailand by the plant genetic conservation project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG). The plants were extracted using ethyl acetate as a solvent, and authenticated by Professor Dr. Bungorn Sripanidkulchai (Center for Research and Development of Herbal Health Products, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand); the voucher numbers of *B. ovata* and *C. oblongifolius* are TT-OC-SK-1253 and TT-OC-SK-1215, respectively. The collected plants were washed, cut and dried, and then crushed into powders. The extracts were obtained using ethyl acetate at a plant-to-solvent ratio of 1:5 at room temperature for 72 h, filtered using Whatman filter paper no. 1 and then centrifuged at 500 x g for 10 min to obtain the supernatant. The extracts were then concentrated under a rotary evaporator and the final products were weighed prior to determination of the percentage yield. For the cell treatment, the crude ethyl acetate extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain an appropriate concentration of the extract stock solutions (to avoid DMSO-associated cellular toxicity). The final concentrations of DMSO under the treatment conditions were less than 0.2%, at which they were not cytotoxic (38).

Reagents and assay kits. The chemicals and solvents used were of analytical grade, which included chloroform, ethyl acetate, ferric chloride, hydrochloric acid, sulfuric acid, acetic acid anhydride, sodium hydroxide, copper acetate, potassium iodide and mercuric chloride (all Merck KGaA). Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's F-12, fetal bovine serum (FBS), horse serum (HS), phosphate-buffered saline (PBS), trypsin-EDTA solution, penicillin and streptomycin were purchased from Gibco; Thermo Fisher Scientific, Inc. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the Annexin V- fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit, 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$), dichlorodihydrofluorescein diacetate (DCFH-DA), dihydroethidium (DHE), DMSO, epidermal growth factor (EGF), cholera toxin, hydrocortisone and insulin were purchased from Sigma-Aldrich; Merck KGaA. Caspase 9 (LEHD-para-nitroaniline; LEHD-*p*-NA), caspase 8 (IETD-*p*-NA) and caspase 3 (DEVD-*p*-NA) activity colorimetric assay kits were obtained from Invitrogen; Thermo Fisher Scientific, Inc. The primers and RevertAid First Strand cDNA Synthesis kit were obtained from Thermo Fisher Scientific, Inc. The RNA Prep Pure kit was purchased from Tiangen Biotech Co., Ltd and the SensiFAST SYBR Lo-ROX kit was purchased from Bioline; Meridian Biosciences. The mitochondria/cytosol fractionation kit, primary antibodies against NOXA (ab13654), BAX (ab32503), Bcl-xL (ab2568), cytochrome *c* (ab6400),

β-actin (ab8227), COX-4 (ab16056) and Rip3 (ab56164), rabbit HRP-conjugated secondary antibody (ab97051), and mouse HRP-conjugated secondary antibody (ab97046) were purchased from Abcam. The ECL Prime Western Blotting System was purchased from GE Healthcare.

Phytochemical screening. The ethyl acetate crude extracts were screened for the presence of bioactive chemical constituents, including alkaloids, flavonoids, phytosterols, terpenoids, saponins, phenols and tannins using the standard methods outlined below (39-41). The results were reported as -, absent; +, present; ++ abundant.

Detection of alkaloids. Dry extracts (50 mg each) were dissolved in 1 ml of 2% hydrochloric acid and filtered using Whatman filter paper no. 1. The filtrates were treated with 100 µl potassium mercuric iodide, and a yellow-colored precipitate indicated the presence of alkaloids.

Detection of flavonoids. Dry extracts (100 mg each) were dissolved in 4 ml ethyl acetate and filtered using Whatman filter paper no. 1. The filtrates were treated with 200 µl sodium hydroxide solution followed by 1% sulfuric acid. The formation of an intense yellow color became colorless when 1% sulfuric acid was added, which indicated the presence of flavonoids.

Detection of phytosterols. Dry extracts (50 mg each) were dissolved in 1 ml chloroform and filtered using Whatman filter paper no. 1. The filtrates were treated with 100 µl acetic anhydride, boiled at 90-100˚C and then subsequently cooled at room temperature. Then, 1 ml 95% sulfuric acid was added and the formation of a brown ring at the junction indicated the presence of phytosterols.

Detection of triterpenoids. Dry extracts (50 mg each) were dissolved in 1 ml chloroform and filtered using Whatman filter paper no. 1. The filtrates were treated with 100 µl 95% sulfuric acid, gently shaken and then allowed to stand for 5 min. Formation of a golden-yellow color indicated the presence of triterpenoids.

Detection of diterpenes. Dry extracts (50 mg each) were dissolved in 1 ml distilled water and treated with 200 µl copper acetate solution. The Appearance of an emerald green color indicated the presence of diterpenes.

Detection of saponins. Dry extracts (50 mg each) were dissolved in 20 ml distilled water and shaken in a graduated cylinder for 15 min. The formation of a 1-cm layer of foam indicated the presence of saponins.

Detection of phenols. Dry extracts (50 mg each) were dissolved in 1 ml distilled water and treated with 100 µl 2% ferric chloride. The formation of a bluish-green or black coloration confirmed the presence of phenols.

Detection of tannins. Dry extracts (50 mg each) were boiled at 90-100˚C in 1 ml distilled water for 5 min and then filtered using Whatman filter paper no. 1; 100 µl 0.1% ferric chloride was added to the filtrates, and the appearance of a brownish-green or a blue-black color indicated the presence of tannins.

Gas chromatography-mass spectrometry (GC-MS) analysis. BEA and CEA were subjected to GC-MS detection, which was performed using a GC 7890A attached to a MSD 5975C (Agilent Technologies, Inc.). The chromatograph was fitted with a HP5-MS capillary column (30 m x 0.25 mm, 0.25-µm film thickness) using a temperature program initially set to 100-150˚C, at a rate of 3˚C/min for 16.67 min. Then, the temperature was raised to 300˚C at a rate of 5˚C/min, for 30 min, and then held at 300˚C for 10 min; the total run time was 56.67 min. The injector was set at 280˚C and the injection volume was at 1.0 µl in the split mode (2:1). The flow speed of helium, the carrier gas, was 1 ml/min. The mass spectra were scanned from m/z 50-550, and the electron impact ionization energy was set at 70 eV. Identification of the components was based on the comparison of their mass spectra and retention times with those of the standard authentic compounds. This was accomplished using Agilent ChemStation Integrator (Agilent Technologies, Inc.) software computer matching using the National Institute Standard and Technology Library (nist.gov/nist-research-library).

Cell culture. The human MDA-MB-231 breast cancer cell line was obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM and supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The human MCF10A mammary epithelial cell line was obtained from the ATCC and cultured in DMEM/Ham's F-12 supplemented with 5% HS, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 50 U/ml penicillin and 50 µg/ml streptomycin. Both cell lines were cultured at 37˚C in a 5% CO_2 atmosphere.

Cytotoxicity determination by MTT assay. MDA-MB-231 and MCF10A cells were seeded into 96-well plates at $5x10^4$ cells/ml and treated with BEA, CEA or Doxorubicin at various concentrations. The cells were then incubated for 24 h at 37˚C before detecting cell viability using the MTT method, as previously described (42). Briefly, MTT reagent was added to the cells, which were then incubated for 4 h at 37˚C. The formazan crystals were dissolved using DMSO and the optical density was measured at 540 nm (with a reference wavelength of 630 nm). The results were expressed as a percentage of cell viability and were compared with untreated control cells. The inhibitory concentration $(IC)_{10}$, IC_{20} and IC_{50} values were calculated in the presence of each of the extracts.

Apoptosis investigation using annexin V-FITC and PI staining. MDA-MB-231 cells were treated with BEA and CEA at IC_{10}, IC_{20} and IC_{50} for 24 h, at 37˚C, and then stained with annexin V-FITC and PI for 15 min. The percentages of apoptotic cells were quantified by annexin V-FITC positive staining (43), detected using the BD FACSAria™ III Cell Sorter flow cytometer (BD Biosciences) and then analyzed with BD FACSDiva software version 7.0 (BD Biosciences). The treated cell results were compared with untreated control cells.

Assessment of mitochondrial transmembrane depolarization. To detect changes in mitochondrial transmembrane potential (ΔΨm), MDA-MB-231 cells were treated with BEA and CEA for 24 h at 37˚C. BEA and CEA-treated and untreated control

cells were stained using $DiOC_6$ at a final concentration of 40 nM for 15 min at 37˚C. Then, the cells were washed and analyzed using a FACSCAtia™ III Cell Sorter flow cytometer (BD Biosciences) (44).

Measurement of reactive oxygen species (ROS) generation. The levels of intracellular ROS were measured, and included those of superoxide anion, hydrogen peroxide and peroxide radicals; two intracellular redox-sensitive fluorescent dyes, DHE and DCFH-DA, were used according to previously described methods (45). Briefly, after the MDA-MB-231 cells were treated with BEA and CEA at IC_{10}, IC_{20} and IC_{50} for 12 h, the cells were stained with DHE and DCFH-DA at a final concentration of 2 μM for 30 min at 37˚C. Fluorescence intensity was measured using a fluorescence microplate reader (BioTek Instruments, Inc.) at 535 nm excitation and 635 nm emission wavelengths for DHE, and 485 nm excitation and 525 nm emission wavelengths for DCFH-DA (46,47). The results were compared between treated and untreated cells. 3% H_2O_2 was used as the positive control for DCFH-DA staining, whereas $FeSO_4$ was used as the positive control for DHE staining.

Determination of caspase activities. Caspase 3, 8 and 9 activities were measured in cellular protein extracts using colorimetric assay kits. Following treatment with BEA and CEA at IC_{10}, IC_{20} and IC_{50}, the MDA-MB-231 cells were lysed and the total protein was quantified using a Bradford protein assay. Caspase activity was detected using the specific substrates DEVD-*p*-NA, IETD-*p*-NA and LEHD-*p*-NA for caspase 3, 8 and 9, respectively. The absorbance was then determined at 405 nm using a microplate reader (BioTek Instruments, Inc.) (44). The results were compared between treated and untreated cells.

Analysis of gene expression levels by reverse transcription-quantitative (RT-qPCR). After treatment of the MDA-MB-231 cells with BEA and CEA at IC_{10}, IC_{20} and IC_{50} (for 24 h at 37˚C), the total RNA was extracted using the Tiangen RNA Prep Pure kit, and then reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit. mRNA expression levels of NOXA, BAX and Bcl-xL were quantified using the SensiFAST SYBR Lo-ROX kit (Bioline; Meridian Biosciences) and the 7500 Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative gene expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (48) with GAPDH as the housekeeping gene. The primers for NOXA, BAX, Bcl-xL and GAPDH were as follows: NOXA, forward 5'GCTGGAAGTCGAGTGTGCTA3' and reverse 5'CCTGAGCAGAAGAGTTTGGA3'; BAX, forward 5'AGCAGATCATGAAGACAGGGG3' and reverse 5'ACACTCGCTCAGCTTCTTGG3'; Bcl-xL, forward 5'GCTGGGACACTTTTGTGGAT3' and reverse 5'TGTCTGGTCACTTCCGACTG3'; and GAPDH, forward 5'TGCACCACCAACTGCTTAGC3' and reverse 5'GGCATGGACTGTGGTCATGAG3'. The primers were designed and selected using the Primer-BLAST tool (ncbi.nlm.nih.gov/tools/primer-blast/) (48,49).

Western blotting. To determine the protein expression levels in MDA-MB-231 cells, the cells were seeded into culture dishes and treated with BEA or CEA for 24 h at IC_{10}, IC_{20} and IC_{50}; untreated MDA-MB-231 cells were used as the control. Following treatment, the cells were harvested and lysed using RIPA lysis buffer supplemented with a protease inhibitor. The mitochondria/cytosol fractionation kit was used to extract cytosolic and mitochondrial proteins per the manufacturer's protocol. Protein concentrations were measured using a Bradford protein assay (50), and protein expression levels were assessed using western blot analysis (51). A total of 20 μg (for NOXA, BAX, Bcl-xL, Rip3, COX-IV and β-actin) and 80 μg (for cytochrome *c*) total protein per lane was loaded onto a 12% gel, resolved using SDS-PAGE and subsequently transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk at room temperature for 1 h, and then incubated with primary antibodies against NOXA, BAX, Bcl-xL, cytochrome *c*, Rip3 and COX-IV (1:1,000) at 4˚C overnight (β-actin was used at 1:20,000). Next, the membranes were washed and incubated with HRP-conjugated secondary antibodies (1:5,000) for 2 h at room temperature. For visualization, an ECL Prime Western Blotting System was used and antibody-specific chemiluminescent bands were detected using an Omega Lum W Imager (Gel Company, Inc.). Quantification of the band density was conducted using ImageJ 1.52p (National Institutes of Health). β-actin and COX-IV were used as the loading controls for whole cell/cytosolic and mitochondrial proteins, respectively.

Statistical analysis. The data are presented as the mean ± standard deviation (SD), calculated from three independent experiments (unless otherwise stated). The statistical differences between multiple-group comparisons were determined using the Kruskal-Wallis test and Dunn's multiple comparisons post-hoc test, and $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 22.0. (IBM Corp.).

Table I. Phytochemical screening test of BEA and CEA.

Phytoconstituent	BEA	CEA
Alkaloids	+	+
Flavonoids	++	++
Phytosterols	++	+
Triterpenoids	++	+
Diterpenoids	-	++
Saponins	+	-
Phenols	++	-
Tannins	++	+

BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; -, absent; +, present; ++ abundant.

Results

Percentage yield and phytochemical screening of BEA and CEA. The percentage yields of BEA and CEA were 0.31 and 0.95%, respectively. For phytochemical screening of the extracts, standard methods were applied (41). BEA contained alkaloids, flavonoids, phytosterols, triterpenoids, saponins, phenols and tannins with an absence of diterpenoids. The

Table II. Phytochemical analyses and identified compound profiles with retention time, name and percentage of total in BEA and CEA treatment.

A, BEA

Retention time (min)	Compounds	% of total
8.948	2-methoxy-4-vinylphenol	1.62
10.960	1,2,3-benzenetriol	7.70
11.618	Benzaldehyde, 4-hydroxy-3-methoxy-	1.15
16.653	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	0.43
20.070	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	0.43
23.150	Tetradecanoic acid	0.88
28.006	Palmitic acid	10.28
30.986	Linoleic acid	0.25
31.429	Oleic acid	7.92
31.733	Stearic acid	0.63
37.568	Di-n-octylphthalate	0.72
42.957	1-Nonadecene	0.69
45.674	1-Hexacosanol	2.59
45.862	α-Tocopherol	1.03
46.593	Cirsilineol	0.83
47.059	Campesterol	4.18
47.477	Stigmasterol	7.74
48.060	24-n-propylidenecholesterol	1.06
48.433	Stigmast-5-en-3-ol	19.55
48.558	Fucosterol	1.09
49.689	Stigmasta-3,5-dien-7-one	1.18
50.367	Stigmast-4-en-3-one	0.92
50.522	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	0.80
52.247	Friedelan-3-one	20.09

B, CEA

Retention time (min)	Compounds	% of total
5.589	Benzoic acid	4.54
27.688	Palmitic acid	2.38
29.082	Biformen	1.15
29.210	(-)-Kaur-16-ene	2.00
33.331	4-Hydroxy-3,5,4'-trimethoxystilbene	3.25
36.634	Kaur-16-en-18-oic acid	36.06
36.794	9-Isopropylidene-5,6,7,8-tetramethyl-1,4-dihydro-1,4-methano-naphthalene	2.66
43.416	Aflatoxin G1	2.54
47.055	Campesterol	1.25
47.413	Stigmasterol	2.52
48.286	Stigmast-5-en-3-ol	4.70
49.673	Stigmasta-3,5-dien-7-one	3.06

BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*.

CEA results revealed the presence of alkaloids, flavonoids, phytosterols, triterpenoids, diterpenoids and tannins with an absence of saponins and phenols. The results of these qualitative phytochemical analyses are presented in Table I.

Identification of BEA and CEA compounds using GC-MS. The characterization of BEA and CEA extracts are presented in Table II. A total of 24 BEA compounds and 12 CEA compounds were identified. Their respective retention times and percentages

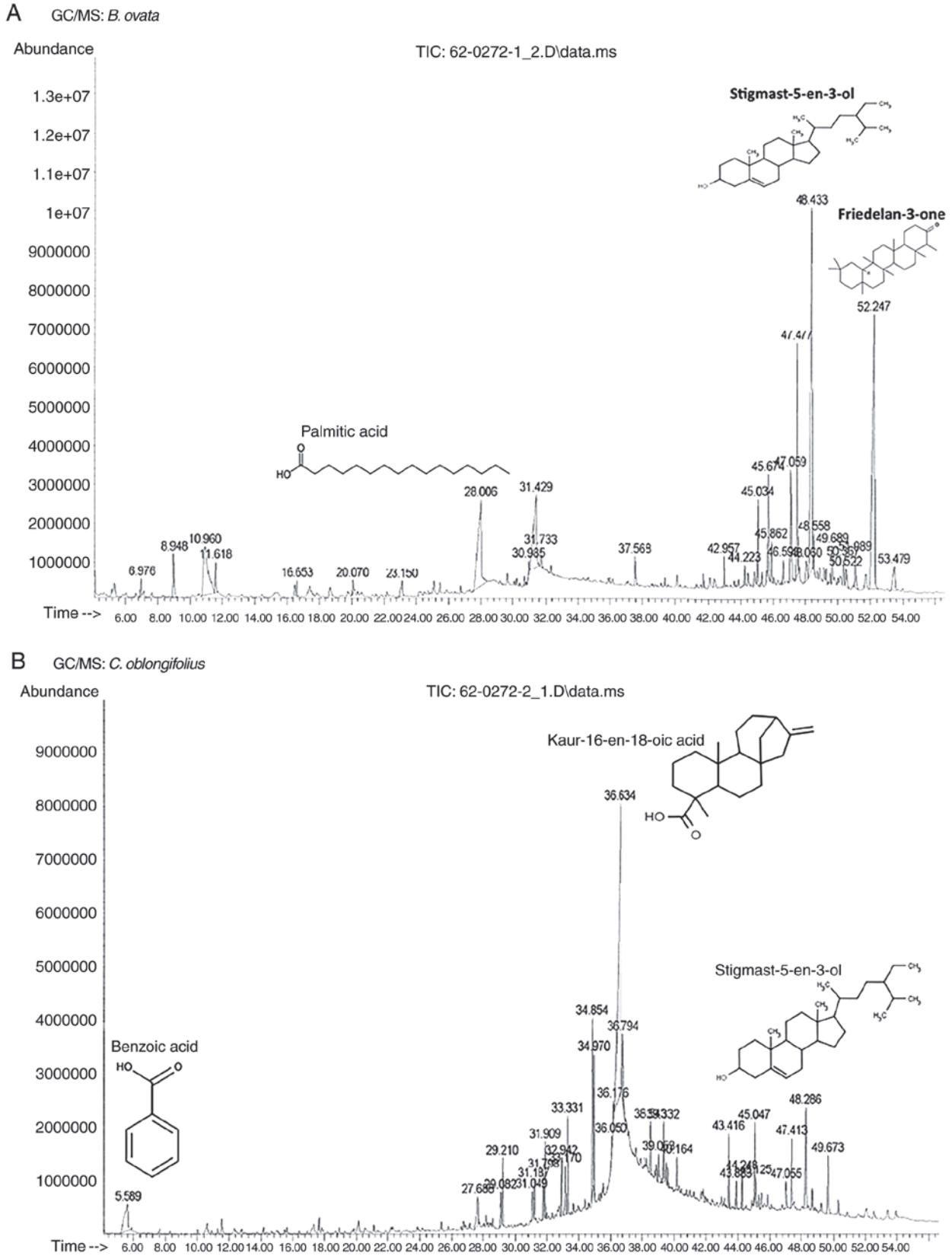

Figure 1. GC-MS chromatographs of BEA and CEA. Chromatograms of (A) BEA and (B) CEA show the retention time on the x-axis and the intensity (absorbance) of the signal at the y-axis. The chemical structures of the three most abundant compounds identified in each extract are presented. GC-MS, gas chromatography-mass spectrometry BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*.

of the total are stated. The GC-MS chromatogram and structures of the 3 major identified compounds of each extract are presented in Fig. 1. The GC-MS results show that the primary phytoconstituent of BEA is friedelan-3-one (20.1%) and that the primary active compound of CEA is kaur-16-en-18-oic acid (36.1%).

Table III. IC_{10}, IC_{20} and IC_{50} values in MDA-MB-231 cells and IC_{50} in MCF10A cells treated with BEA, CEA and doxorubicin.

A, MDA-MA-231

Concentration	BEA ± SD (μg/ml)	CEA ± SD (μg/ml)	Doxorubicin ± SD (μM)
IC_{10}	7.6±1.9	3.9±0.7	-
IC_{20}	14.4±2.4	8.5±1.3	-
IC_{50}	40.0±3.6	29.7±3.4	4.1±0.8

B, MCF10A

Concentration	BEA ± SD, μg/ml	CEA ± SD, μg/ml	Doxorubicin ± SD (μM)
IC_{50}	393.8±4.3	82.0±4.3	8.7±0.8
SI[a]	9.8	2.8	2.1

[a]SI values of each agent, which are ratio of IC_{50} of MCF10A/MDA-MB-231 cells. IC, inhibitory concentration; SI, selectivity index; BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; SD, standard deviation.

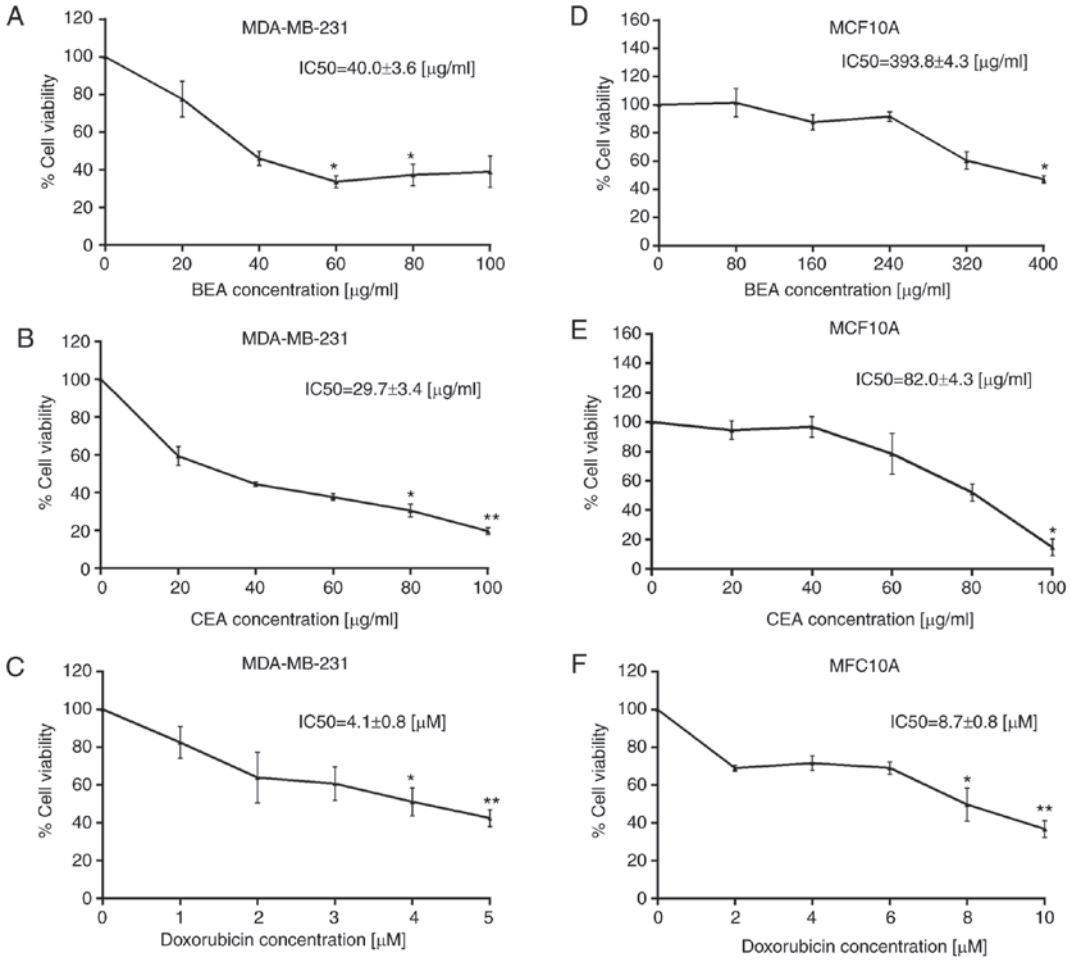

Figure 2. Cytotoxic effects of BEA, CEA and doxorubicin in MDA-MB-231 and MCF10A cells. MDA-MB-231 cells treated with (A) BEA, (B) CEA and (C) doxorubicin at different concentrations. MCF10A cells treated with (D) BEA, (E) CEA and (F) doxorubicin at different concentrations. *P<0.05 and **P<0.01 vs. control. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; IC, inhibitory concentration.

Cytotoxicity of BEA and CEA in MDA-MB-231 and MCF10A cells. BEA and CEA exhibited a cytotoxic effect on MDA-MB-231 and MCF10A cells. The percentage of cell viability was calculated compared with the untreated control

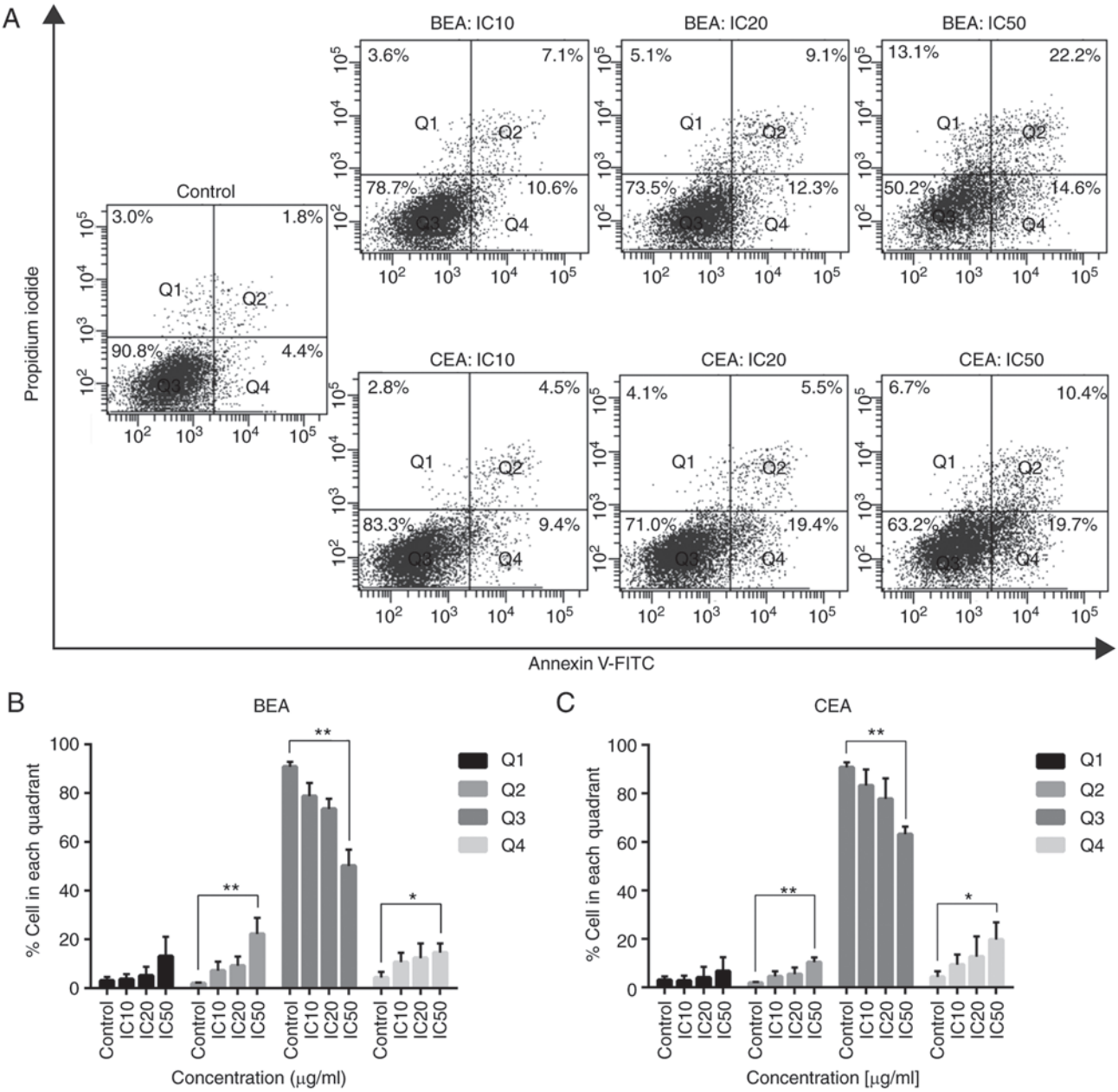

Figure 3. Induction of apoptosis in MDA-MB-231 cells by BEA and CEA. (A) Dot plots represent the cell population in each quadrant, which shows positive and/or negative Annexin V-FITC (x-axis) and PI (y-axis) staining. (B and C) Bar graphs showing the percentages of cells in each quadrant, including Q1 = necrosis/necroptosis, Q2 = late apoptosis/necroptosis, Q3 = living cells and Q4 = early apoptosis. *P<0.05 and **P<0.01 vs. control. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; IC, inhibitory concentration.

cells (100% cell viability). The IC_{10}, IC_{20}, IC_{50} and selectivity index (SI) of BEA, CEA and doxorubicin for both cell types were calculated (Table III). BEA and CEA significantly decreased MDA-MB-231 cell viability in a dose-dependent manner (Fig. 2A and B). The IC_{50} of BEA and CEA were 40.0±3.6 and 29.7±3.4 μg/ml, respectively. However, BEA and CEA also decreased the viability of MCF10A cells (Fig. 2D and E) but at higher concentrations compared with their effect on MDA-MB-231 cells. The results are in accordance with the selectivity index criteria: SI≥3=high selectivity and SI<3=less selectivity (52,53). Therefore, BEA possessed a high SI (9.84), whereas CEA had less selectivity (2.76). Doxorubicin, the conventional chemotherapeutic drug used for breast cancer (54), was used to treat both cell types (Fig. 2C and F). The IC_{50} of doxorubicin in MCF10A cells was higher than that in MDA-MB-231 cells and the SI of both BEA and CEA were higher than doxorubicin (2.13). This suggests that these two extracts have more selectivity towards breast cancer cells than noncancerous cells, which may relate to a reduction in adverse effects.

Mode of cell death induced by BEA and CEA in MDA-MB-231 cells. Annexin V-FITC/PI staining was employed to determine the mode of cell death in MDA-MB-231 cells after treatment with both extracts. Dot plot analyses show the percentage of cells in each quadrant (Fig. 3A). The percentages of cells in early (Q4) and late apoptosis (Q2) were significantly increased compared with the control, whereas

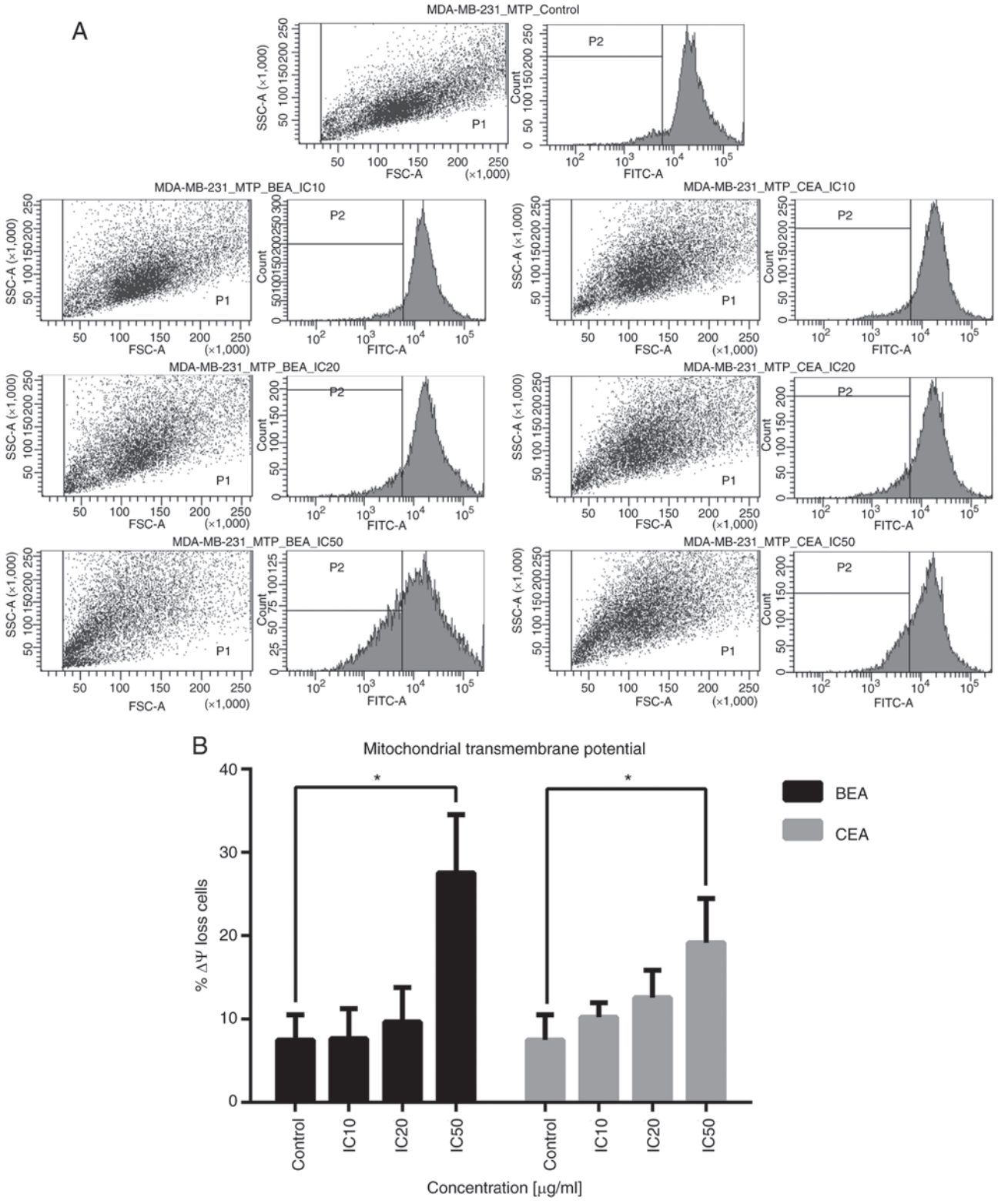

Figure 4. Reduction of mitochondrial transmembrane potential (ΔΨm) in MDA-MB-231 cells induced by BEA and CEA. MDA-MB-231 cells were incubated with BEA and CEA at the indicated IC. A decrease of the $DiOC_6$ indicates the percentage of cells with ΔΨm loss. (A) Histogram of $DiOC_6$ shows the number of cells at each $DiOC_6$ intensity. The x-axis represents the $DiOC_6$ intensity and the y-axis indicates the cell count within the corresponding fluorescence intensity. (B) Bar graph showing the percentage of cells with ΔΨm loss on the y-axis and extract concentrations on the x-axis. *P<0.05 vs. control. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; IC, inhibitory concentration.

the percentage of living cells (Q3) was significantly decreased after BEA treatment at IC_{50} concentration compared with the control; similar results were observed following CEA treatment (Fig. 3B and C). However, the number of necrotic cells in Q1 was increased, but not significantly compared with the control cells; supplementary data demonstrates that necroptosis marker protein RIP3 did not change the expression levels following BEA and BEA treatment (Fig. S1).

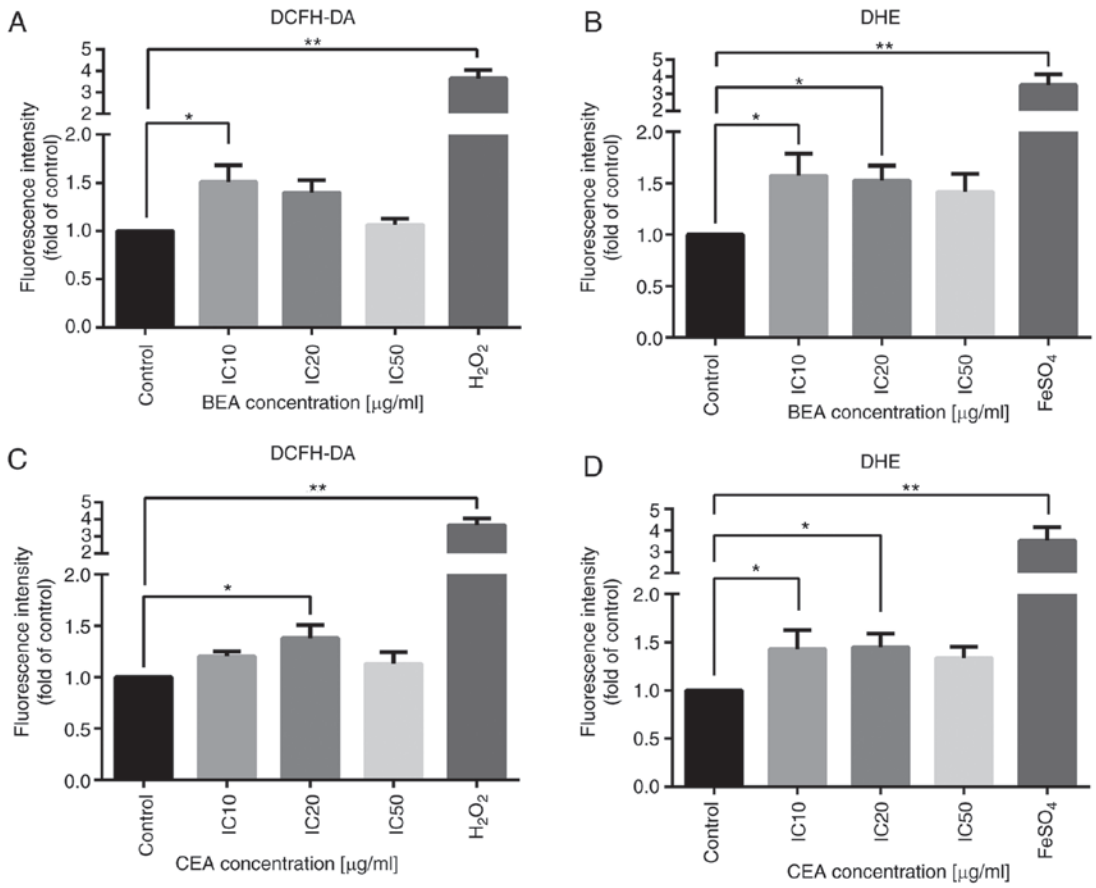

Figure 5. Induction of intracellular ROS generation by BEA and CEA. The fluorescence intensities of the DCFH-DA and DHE staining probes are shown on the y-axis as the fold of the control. MDA-MB-231 cells were treated with (A and B) BEA and (C and D) with CEA. The cells were stained with DCFH-DA using 3% H_2O_2 as the positive control, and with DHE using $FeSO_4$ as the positive control. $^{*}P<0.05$ and $^{**}P<0.01$ vs. the respective control. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; IC, inhibitory concentration.

Therefore, BEA and CEA induced MDA-MB-231 cell death via the apoptosis pathway.

Reduction of ΔΨm and intracellular ROS generation. Mitochondrial dysfunction is a key event in the intrinsic apoptosis pathway, and depolarization of ΔΨm, which occurs during apoptosis (55), was measured to determine the level of mitochondrial disruption induced by BEA and CEA. The percentage of MDA-MB-231 cells with ΔΨm loss was significantly increased after treatment with BEA and CEA at IC_{50} (Fig. 4B). The reduction of ΔΨm indicates mitochondrial dysfunction. The intracellular ROS were measured using two fluorescent dyes. DCFH-DA is widely used probe for detecting H_2O_2 and oxidative stress and DHE is another used probe for detecting intracellular $O^{\bullet-2}$ (56). The fluorescent intensity of both DCFH-DA and DHE probes were increased following BEA and CEA treatment, compared with the untreated control (Fig. 5), thus H_2O_2 and $O^{\bullet-2}$ were generated. However, the level of ROS generation did not increase in a dose-dependent manner (though they were significantly increased). This indicates that BEA and CEA induced oxidative stress and mitochondrial dysfunction during MDA-MB-231 cell apoptosis.

Effect of BEA and CEA on caspase activity. In MDA-MB-231 cells, caspase 3 and 9 activities were significantly increased following treatment with BEA and CEA (Fig. 6A and C), while caspase 8 activity was not significantly altered, compared with untreated control cells (Fig. 6B). These results indicate that BEA and CEA induce MDA-MB-231 apoptosis through the intrinsic pathway.

Effects of BEA and CEA treatment on Bcl-2 family mRNA expression levels. Bcl-2 family proteins play an important role in the mitochondrial pathway (57). Expression levels of NOXA and BAX were significantly increased in cells treated with BEA and CEA, whereas Bcl-xL mRNA expression levels were significantly attenuated compared with those of the control cells (Fig. 7). These results indicate that BEA and CEA induce NOXA expression, which subsequently downregulates Bcl-xL and upregulates BAX expression, inducing apoptosis.

Effects of BEA and CEA treatment on apoptotic-related protein expression levels. The expression levels of NOXA, BAX, Bcl-xL and cytochrome *c* proteins were investigated in MDA-MB-231 cells following BEA and CEA treatment. Protein expression levels of NOXA and BAX were significantly increased, whereas Bcl-xL expression levels were significantly decreased following BEA and CEA treatment (Fig. 8). In addition, cytochrome *c* expression levels were measured in the mitochondria and cytosol. Following treatment with BEA and CEA, cytochrome *c* expression levels in the mitochondria decreased in a dose-dependent

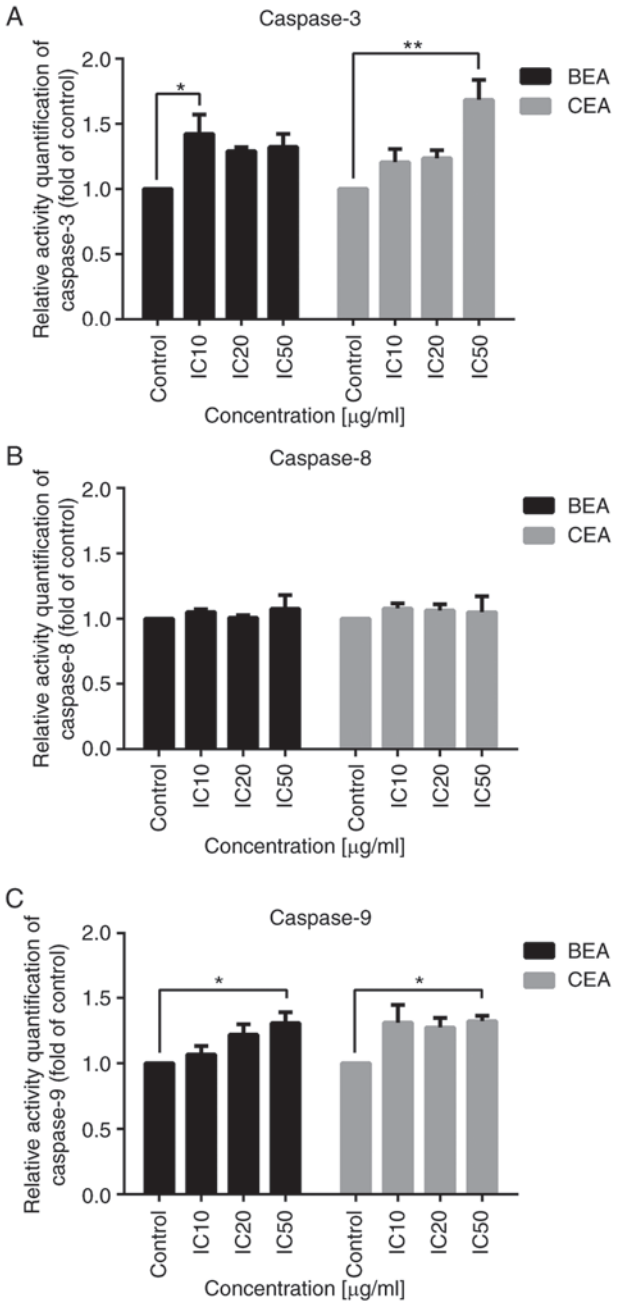

Figure 6. Caspase enzyme activity following BEA and CEA treatment. (A) Activities of caspase 3, (B) caspase 8 and (C) caspase 9 in MDA-MB-231 cells treated with BEA and CEA, and untreated control cells. The y-axis shows the level of caspase activity relative to the no-treatment control. *P<0.05 and **P<0.01 vs. control. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; IC, inhibitory concentration.

manner, while those in the cytosol were increased (Fig. 9). These results suggest that BEA and CEA induce the mitochondrial apoptosis pathway in MDA-MB-231 cells.

Discussion

B. ovata and *C. oblongifolius* are medicinal plants used in Thai traditional medicine. These herbs are used in the treatment of several diseases and symptoms, including as an expectorant (7), and to treat flatworm infections and dysmenorrhea (11). However, previous studies have also reported the cytotoxicity of these herbs against some cancer cell lines (10,17). However, there are no reports investigating the underlying mechanisms of action of these two herbs for potential use in breast cancer treatment. In the present study, BEA and CEA were screened for the presence of phytoconstituent groups using standard methods. The phytochemical screening results showed several groups of phytochemical compounds, such as alkaloids, flavonoids, phytosterols and tannins. However, previous phytochemical reports of *B. ovata* showed the presence of triterpenoids and phytosterols, whereas those of *C. oblongifolius* revealed diterpenoids and megastigmane glycosides. Therefore, GC-MS analysis in the present study primarily aimed to identify triterpenoids, phytosterols and diterpenoids in BEA and CEA. To the best of our knowledge, this is first report of the phytoconstituents of crude ethyl acetate extracts of BEA and CEA by GC-MS analysis.

Friedelan-3-one is a friedelane-type triterpenoid, which was a primary phytoconstituent of BEA in the present study. A previous study reported the cytotoxic effect of friedelan-3-one against mouse 4T1 breast cancer cells (58). Friedelan-3-one also inhibits VEGF-induced Kaposi's sarcoma cell proliferation and induces apoptotic cell death (59). These findings indicate the cytotoxic effect and apoptotic induction of BEA in MDA-MB-231 cells, which may be associated with friedelan-3-one.

Kaur-16-en-18-oic acid (or kaurenoic acid) is a kaurane-type diterpenoid, which is present in some medicinal plants that possess potential anti-cancer properties (60). Kaurane diterpenes have been considered for the development of novel effective anti-cancer chemotherapeutic agents (61). Kaurenoic acid is also reported to possess apoptosis-inducing properties against various cell lines, including the HL-60 human leukemia cell line (62), the PC-3 human prostate cancer cell line and the A-549 human lung cancer cell line (63). Furthermore, the genotoxicity of kaurenoic acid has been demonstrated in gastric cancer cell lines, including ACP02, ACP03, AGP01 and PG100 (64), and cervical cancer cell lines such as HeLa, CaSki and C33A (65). Moreover, the epimer of kaurenoic acid isolated from *Croton antisyphiliticus* exhibits moderate cytotoxic activity against HeLa cervical cancer cells and B-16 murine melanoma cells, by inducing apoptosis (66).

In the present study, the second most abundant compound in both BEA and CEA was the phytosterol stigmast-5-en-3-ol. The anti-proliferative and pro-apoptotic effects of stigmast-5-en-3-ol (from *Dendronephthya gigantean*) have been demonstrated in HL-60 and MCF-7 cells (67). Diterpenes, triterpenes and phytosterols have shown anti-tumor activity in several models (68-71), hence they may have value as novel anti-tumor agents. In the present study, high contents of friedelan-3-one and stigmast-5-en-3-ol in BEA, and kaur-16-en-18-oic acid in CEA, were observed. Therefore, these two herbs show potential as anti-breast cancer agents.

However, in the present study, crude ethyl acetate extracts of *B. ovata* and *C. oblongifolius* were investigated for their anti-cancer activity as an alternative to purified forms of the constituents identified. The present study aimed to investigate all of the constituents in these herbal extracts on the basis of possible synergy required to generate the anti-cancer effects. A previous study demonstrated that the IC_{50} values of crude ethanolic extracts of *B. ovata* and *C. oblongifolius* in the HepG2

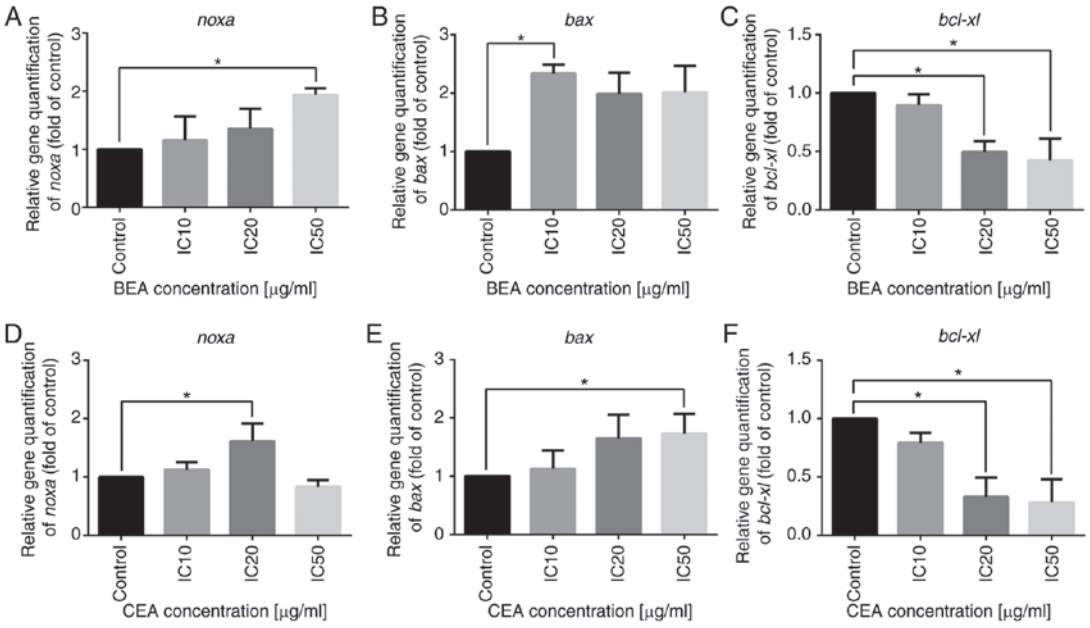

Figure 7. Expression of apoptotic genes after BEA and CEA treatment. MDA-MB-231 cells treated with (A-C) BEA and (D-F) CEA. The mRNA expression of NOXA, BAX and Bcl-xL was determined by reverse transcription-quantitative PCR. *P<0.05 vs. the untreated control. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; BAX, Bcl2-associated X protein; Bcl-xL, B-cell lymphoma-extra large; IC, inhibitory concentration.

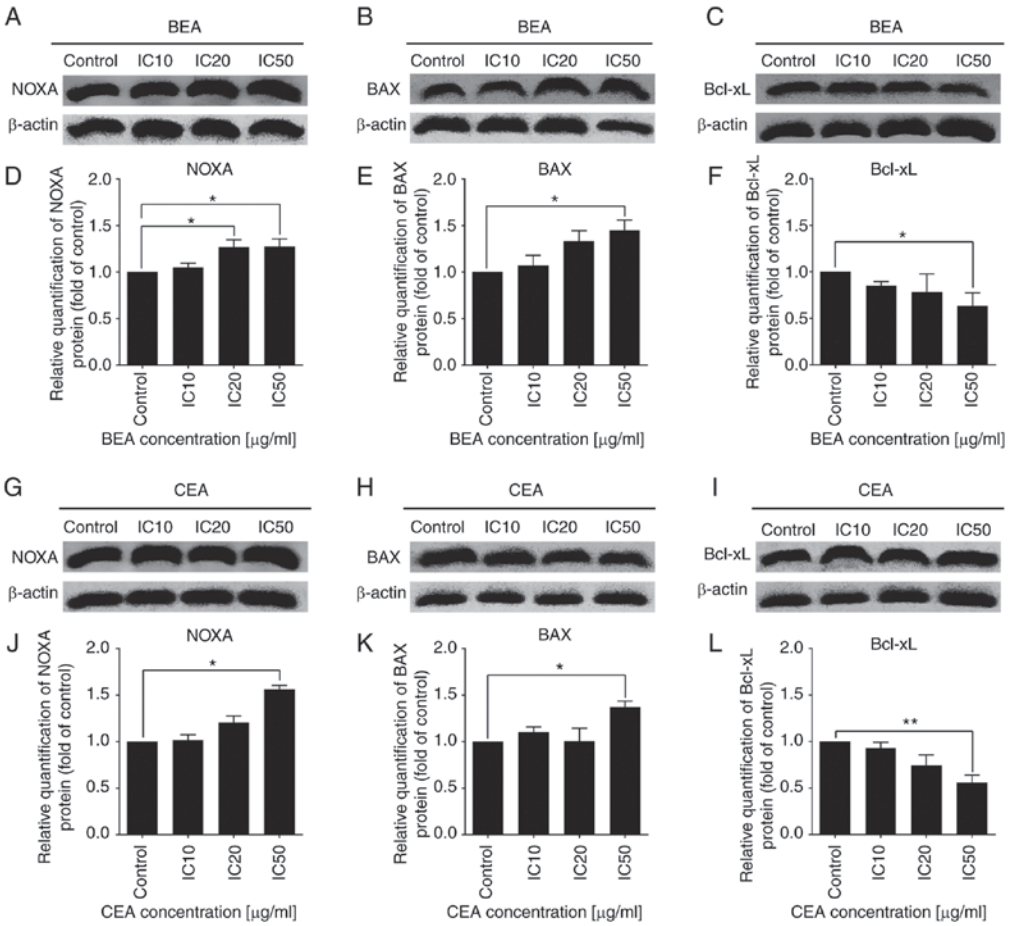

Figure 8. The effect of BEA and CEA on Bcl-2 family protein expression levels in MDA-MB-231 cells. BEA treatment increased the protein expression levels of (A) NOXA and (B) BAX, and deceased the protein expression levels of (C) Bcl-xL. Quantification of the protein band density for (D) NOXA, (E) BAX and (F) Bcl-xL after BEA treatment. CEA treatment increased the protein expression levels of (G) NOXA and (H) BAX, and decreased that of (I) Bcl-xL. Quantification of the protein band density for (J) NOXA, (K) BAX and (L) Bcl-xL after CEA treatment. *P<0.05 and **P<0.01 vs. non-treated control MDA-MB-231 cells. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; BAX, Bcl2-associated X protein; Bcl-xL, B-cell lymphoma-extra large; IC, inhibitory concentration.

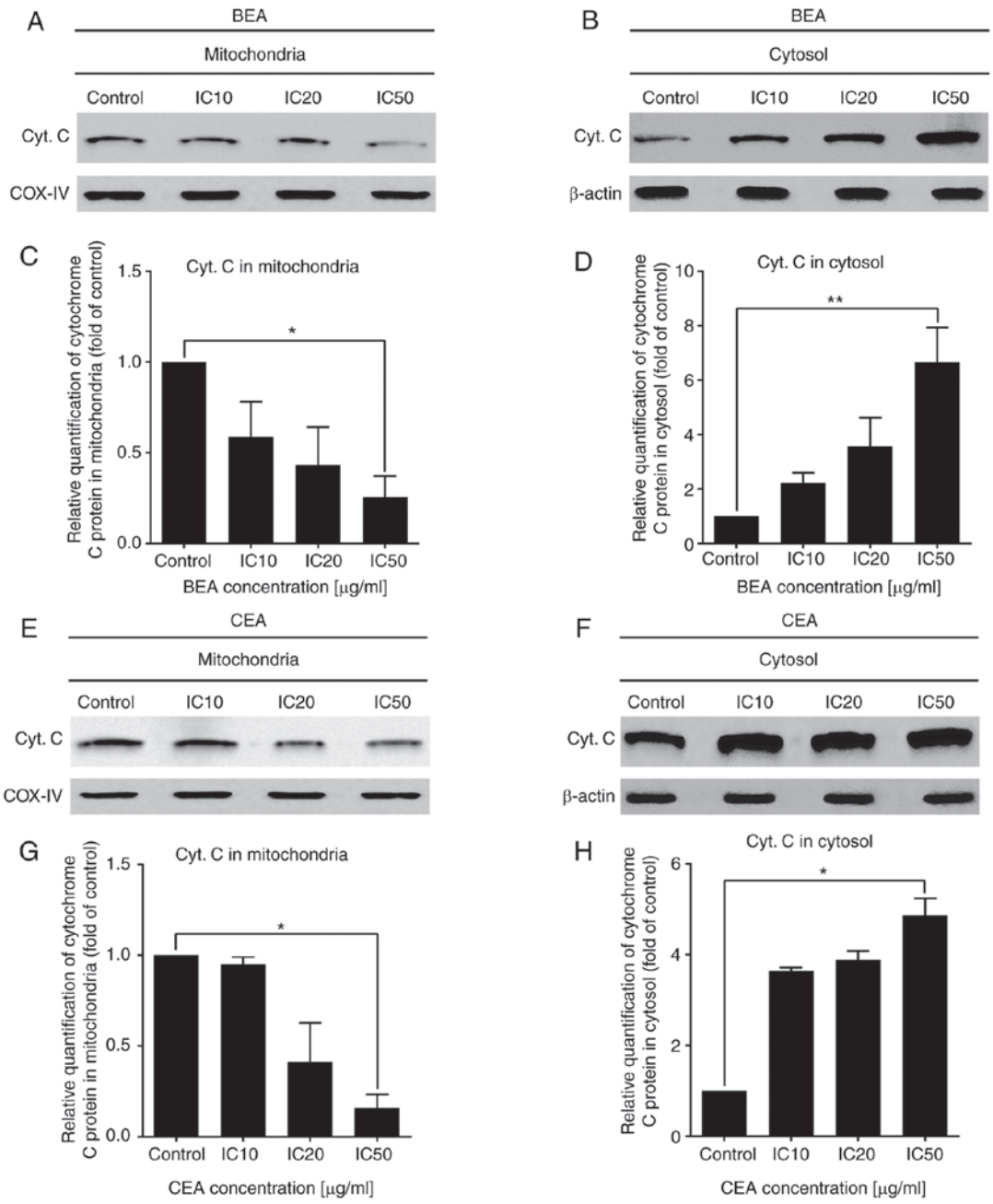

Figure 9. Alteration of Cyt. C protein levels in the mitochondria and cytosol following BEA and CEA treatment in MDA-MB-231 cells. Western blots for (A and B) BEA and (E and F) the CEA treatment. (C and G) BEA and CEA treatment decreased the protein expression levels of cytochrome c in the mitochondria. (D and H) BEA and CEA treatment increased the protein expression levels of cytochrome c in the cytosol. β-actin and COX-IV were used as internal controls for the cytosol and mitochondria, respectively. *P<0.05 and **P<0.01 vs. respective control. Cyt. C, cytochrome C; BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; IC, inhibitory concentration.

cell line (72) were higher than the crude ethyl acetate extracts in the MDA-MB-231 cell line in the present study. These differences suggest that these two herbs have more potential as ethyl acetate extracts against breast cancer cells than ethanolic extracts against liver cancer; however, further study of these extracts against liver cancer is required. Comparing the IC_{50} and SI values of BEA and CEA suggests that CEA exerts greater toxic against MDA-MB-213 cells than BEA, whereas BEA acts more selectively towards breast cancer cells than CEA.

Annexin V-FITC and PI staining showed that MDA-MB-231 cells had undergone apoptosis and necrosis. However, the number of necrotic cells was not significantly different compared with that of the control cells, and Rip3 protein expression levels did not change following BEA and CEA treatment. The percentage of apoptotic cells was significantly increased following treatment with both extracts in a dose-dependent manner, compared with that of the control cells.

The present study further investigated the mechanism of apoptosis, which revealed that the percentage of cells with mitochondrial disruption increased following treatment with both extracts. The mitochondria are the primary source of intracellular ROS production. High generation of ROS induces

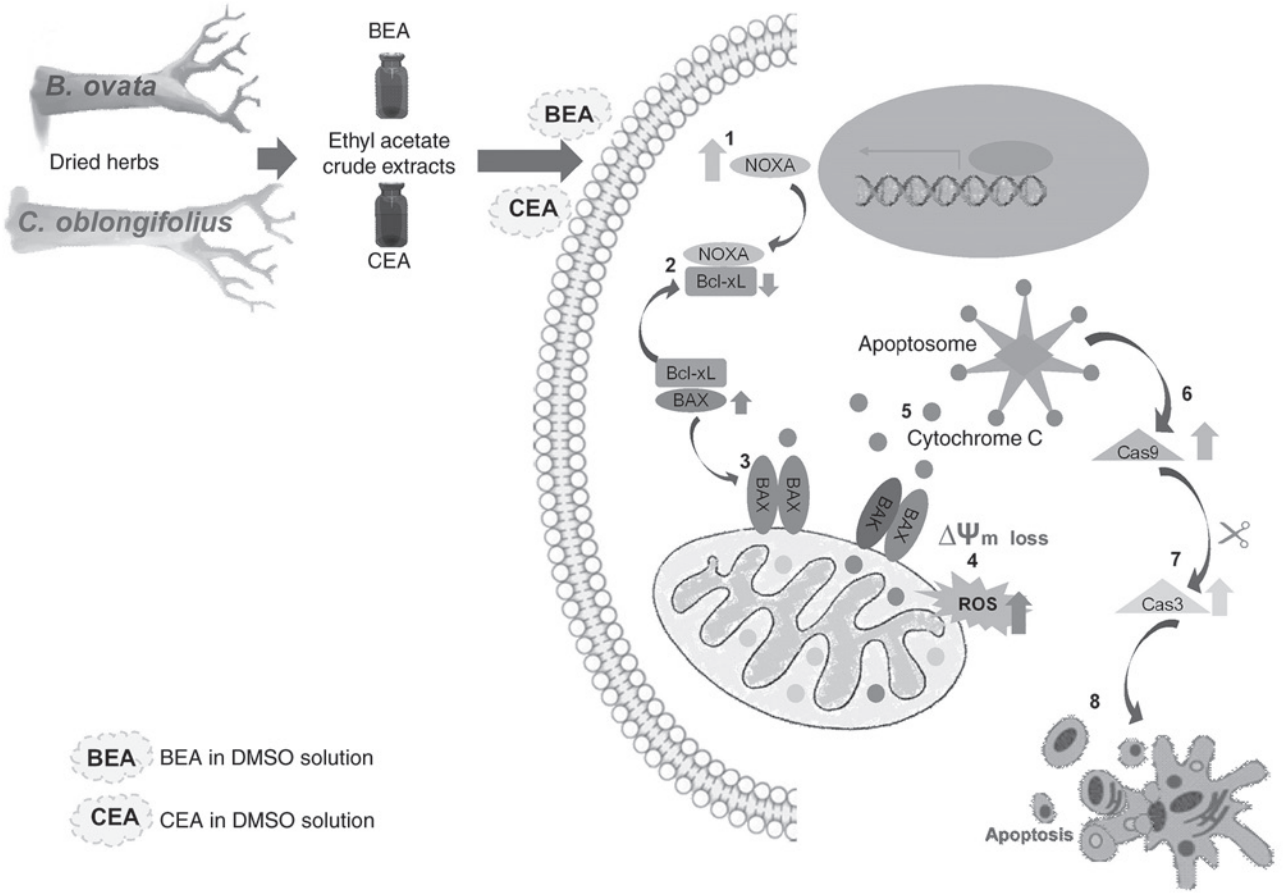

Figure 10. The apoptosis pathway induced by BEA and CEA in MDA-MB-231 cells. BEA and CEA induce gene and protein expression of NOXA and BAX pro-apoptotic proteins and reduced those of anti-apoptotic Bcl-xL, leading to the loss of mitochondrial membrane potential and ROS production. Cytochrome c is then released from the mitochondria into the cytosol, consequently inducing activation of the caspase 9 and 3 cascades, and ultimately resulting in apoptosis. BEA, *Bridelia ovata*; CEA, *Croton oblongifolius*; ROS, reactive oxygen species; Cas9, caspase 9; Cas3, caspase 3; ΔΨm, mitochondrial transmembrane potential; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; BAX, Bcl2-associated X protein; Bcl-xL, B-cell lymphoma-extra large; DMSO, dimethyl sulfoxide.

mitochondrial dysfunction, cell damage and apoptosis (73). This ΔΨm loss was associated with ROS generation, as the high energy source equates to the mitochondrial electron transport chain (74). Intracellular ROS induce cellular stress and consequently induce apoptosis (75). During apoptosis, the loss of mitochondrial membrane potential leads to the inhibition of ATP synthase, subsequently resulting in ROS production (76). However, a number of drugs and natural compounds can directly induce ROS generation; this includes doxorubicin, which also induces mitochondrial dysfunction and apoptosis (77,78). In the present study, the level of ROS increased ~1.5-fold in MDA-MB-213 cells treated with BEA and CEA, compared with the control group, demonstrating that BEA and CEA induce oxidative stress, and subsequent apoptotic cell death. ROS level increased in a dose-independent manner, which may be due to the presence of antioxidants in the cell or the cellular microenvironment (79). Alternatively, this may be due to the oxidant content of the microenvironment, which contains superoxide dismutase (80), reduced glutathione (81), catalase, bilirubin, NADPH oxidase and lipoxygenase and toxins (82,83).

Cellular stress can lead to DNA damage that activates the p53 tumor suppressor gene; this can induce apoptotic cell death and cell cycle arrest. However, the downstream effects of p53 activation depend on cell type, differentiation state and the local microenvironment (84). It has been reported that p53-mediated apoptosis is favorably selected in cells expressing oncoproteins (85); for example, mouse embryonic fibroblasts (MEFs) normally undergo p53-mediated cell cycle arrest, whereas MEFs expressing E1A oncoprotein undergo p53-mediated apoptosis (86). p53-mediated apoptosis involves the transcriptional targets PUMA and NOXA, and the expression of other BH-3 only proteins (85,87). Several studies have reported that NOXA functions as an integral mediator of p53-dependent apoptosis (88). Moreover, PUMA and NOXA indirectly induce mitochondrial outer membrane permeabilization (MOMP) by direct binding to members of the anti-apoptotic Bcl-2 family, such as Bcl-2 and Bcl-xL, which interferes with pro-apoptotic BAX and BAK and anti-apoptotic interactions. Consequently, BAX serves a role in MOMP by homo- or heterodimerizing with BAK, forming pores in the mitochondrial outer membrane and permitting the release of cytochrome *c* from the mitochondrial intermembranous space into the cytosol (89). This subsequently leads to the activation of initiator caspase 9, resulting in downstream activation of executioner caspase 3, which proteolyzes key proteins, including those of cytoskeletal proteins. Also, caspase 3 inactivates other enzymes such as poly(ADP) ribose polymerase (PARP), a DNA repair enzyme (90). Finally, the cells undergo apoptotic cell death and exhibit unique morphological

changes, including DNA fragmentation and membrane blebbing (91).

The present study investigated the effects of BEA and CEA on MDA-MB-231 human breast cancer cells, showing that treatment with these herbs increased the mRNA and protein expression levels of the pro-apoptotic proteins NOXA and BAX. By contrast, BEA and CEA treatment led to the downregulation of the anti-apoptotic protein Bcl-xL. Additionally, cytochrome *c*, which is essential for apoptosome formation and the progression of apoptosis (92), was released from the mitochondria into the cytosol. These data show that BEA and CEA induced breast cancer cell death via the mitochondria-mediated pathway. Caspases are a group of cysteine proteases which play a pivotal role in the apoptosis pathway (93). The mitochondrial dysfunction observed following BEA and CEA treatment demonstrated that only the intrinsic apoptotic pathway was activated by these herbs, and that there was no evidence of a significant increase in caspase 8 activity, which would indicate the activation of the extrinsic pathway. By contrast, significantly enhanced caspase 9 activity was observed. Caspase 3 activity was activated by active caspase 9, and this resulted in MDA-MB-231 cell apoptosis. Therefore, BEA and CEA induced apoptotic cell death via the mitochondrial apoptosis pathway.

To conclude, crude BEA and CEA extracts induced MDA-MB-231 human breast cancer cell apoptosis via oxidative stress and mitochondria-mediated pathways. The altered expression levels of NOXA, BAX and Bcl-xL resulted in disruption of the mitochondrial membrane potential and the release of cytochrome *c* into the cytosol. This led to caspase 9 and 3 activation and induced apoptotic cell death. The pathway by which BEA and CEA induce MDA-MB-231 cell apoptosis is illustrated in Fig. 10. The lack of *in vivo* experiments using animal models and patient studies are the primary limitations of the present study. Therefore, further investigation into the associated active compounds in these extracts is required, and *in vivo* experiments need to be performed before these herbs can be developed as therapeutic agents for human breast cancer treatment.

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Availability of data and materials

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

Authors' contributions

JP and RB designed the study. BS collected the herbs and took responsibility for the extraction. JP performed the biochemical experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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