Background

Centratherum anthelminticum (L.) seeds (CA) is a well known medicinal herb in Indian sub-continent. We recently reported anti-oxidant property of chloroform fraction of Centratherum anthelminticum (L.) seeds (CACF) by inhibiting tumor necrosis factor-α (TNF-α)-induced growth of human breast cancer cells. However, the active compounds in CACF have not been investigated previously.

Methodology/Principal Findings

In this study, we showed that CACF inhibited growth of MCF-7 human breast cancer cells. CACF induced apoptosis in MCF-7 cells as marked by cell size shrinkage, deformed cytoskeletal structure and DNA fragmentation. To identify the cytotoxic compound, CACF was subjected to bioassay-guided fractionation which yielded 6 fractions. CACF fraction A and B (CACF-A, -B) demonstrated highest activity among all the fractions. Further HPLC isolation, NMR and LCMS analysis of CACF-A led to identification of vernodalin as the cytotoxic agent in CACF-A, and -B. 12,13-dihydroxyoxalic acid, another major compound in CACF-C fraction was isolated for the first time from Centratherum anthelminticum (L.) seeds but showed no cytotoxic effect against MCF-7 cells. Vernodalin inhibited cell growth of human breast cancer cells MCF-7 and MDA-MB-231 by induction of cell cycle arrest and apoptosis. Increased of reactive oxygen species (ROS) production, coupled with downregulation of anti-apoptotic molecules (Bcl-2, Bcl-xl) led to reduction of mitochondrial membrane potential (MMP) and release of cytochrome c in both human breast cancer cells treated with vernodalin. Release of cytochrome c from mitochondria to cytosol triggered activation of caspase cascade, PARP cleavage, DNA damage and eventually cell death.

Conclusions/Significance

To the best of our knowledge, this is the first comprehensive study on cytotoxic and apoptotic mechanism of vernodalin isolated from the Centratherum anthelminticum (L.) seeds in human breast cancer cells. Overall, our data suggest a potential therapeutic value of vernodalin to be further developed as new anti-cancer drug.


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Competing interests: The authors declare that all individuals named as authors in this manuscript have no competing interests, including Nitika Rai from Amritum Bio-Botanica Herbs Research Laboratory Pvt Ltd, Jogli, India. Nitika Rai is the CEO of Amritum Bio-Botanica Herbs Research Laboratory Pvt Ltd, Jogli, India. The isolated compound, vernodalin is not patented, in development or marketed by the company. Dr. Aditya Arya was a former employee of Amritum Bio-Botanica Herbs Research Laboratory Pvt Ltd until 2007. At present, he does not hold any position or shares in this company. Thus, this does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

Introduction

Breast cancer is one of the most common malignancies in women. Global breast cancer incidence has increased at an annual rate of 3.1% over the last three decades to more than 1.6 million cases in year 2010 [1]. In Malaysia, breast cancer is the most common cancer among females. There were 3,242 female breast cancer cases diagnosed in 2007, accounted for 18.1% of all cancer cases reported and 32.1% of all female cases (National Cancer Registry Report 2007). Different subtypes of breast cancers arise from different gene mutations occurring in luminal or basal progenitor cell population, causing difficulty in breast cancer diagnosis and treatment [2]. Being both genetically and histopathologically heterogeneous, the mechanisms underlying breast cancer development remains uncertain [3]. Owing to this, conventional chemotherapy, surgery or radiation shows very limited effects. On the other hand, specific natural or synthetic chemical compounds have been widely applied for cancer chemoprevention to inhibit or revert carcinogenesis and to suppress the malignancy of cancer [4].

Medicinal plants have been used for centuries to treat a variety of diseases and maintain health before the advent of modern medicine [5], [8]. The accumulation and developing knowledge of the medicinal properties of plants by personal experimentation, local custom, anecdote, and folk tradition leads to the formation of numerous traditional medical systems and therapies, including traditional Chinese medicine (TCM), Ayurvedic medicine, indigenous medicine, naturopathy and aromatherapy [7]. [8], [9]. In modern medicine, plants have been a source for new anti-cancer drugs. For example, vinblastine was traditionally obtained from Catharanthus roseus, taxol was isolated from the bark of the Pacific yew tree Taxus brevifolia, camptothecin was isolated from the bark and stem of Camptotheca acuminate [10], [11], [12]. The advancement of technology such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have speed up the process of drug screening and discovery [13]. LC-MS is a highly sensitivity and selectivity method used in drug development at many different stages including profiling of secondary metabolites in plants, impurities detection, metabolic stability or degradant analysis [14], [15].

Centratherum anthelminticum (L.) Kuntze, commonly known as kalajiri, somraj, black cumin or bitter cumin, is a robust leafy plant belongs to Asteraceae family of the

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The powdered seeds of C. anthelminticum were purchased from the medicinal plant cultivation zone of Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd, Betul Madhya Pradesh India. The seeds were identified by the quality control department of the company itself. Voucher specimen (CA-9) was deposited at the Pharmacology Department of University Malaya, Malaysia.

Cell Culture

The human breast cancer cell line, MCF-7 was purchased from Cell Lines Service (300273; Eppelheim, Germany) and MDA-MB-231 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Human mammary epithelial cells were purchased from ScienCell (7610; Carlsbad, CA) and maintained in mammary epithelial cell medium (ScienCell). MCF-7 and MDA-MB-231 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Inc, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine, 1% penicillin and streptomycin. Cells were cultured in tissue culture flasks (Corning, USA) and were kept in CO2 incubator at 37°C in a humidified atmosphere with 5% CO2. For experimental purposes, cells in exponential growth phase (approximately 70–80% confluency) were used.

Extraction and Isolation

The powdered seeds of C. anthelminticum (100 g) were extracted successively with hexane (3×250 ml) (Merck, Darmstadt, Germany), chloroform (CHCl3) (3×250 ml) (Merck, Darmstadt, Germany) and methanol (MeOH) (3×250 ml) (Merck, Darmstadt, Germany), in a Soxhlet apparatus for 24 hours. The resultant extracts were filtered using Whatman No. 1 filter paper (Whatman, England) and dried under vacuum to yield 20.1, 7.7, 11.6 g, respectively of the extracts. Then the dried fractions were kept at ~20°C until further use. In our previous paper, the chloroform extract (CACF) showed highest activity on MTT assay, therefore CACF was chosen for this study [23].

Bioassay Guided Isolation

The chloroform extract was fractionated using reversed phase C18 (Merck, Germany) flash column chromatography. The column was preconditioned with water, then the extract was added to the column and eluted using a step gradient of water and methanol as follows: MeOH:H2O (1:1, 3×100 ml); MeOH:H2O (6:4, 3×100 ml); MeOH (3×100 ml); MeOH:H2O (7:3, 3×100 ml); MeOH:H2O (8:2, 3×100 ml); MeOH:H2O (9:1, 3×100 ml) and MeOH (10.0, 5×100 ml). The fractions were dried using a rotary evaporator. Similar fractions were pooled according to their liquid chromatography mass spectrometry (LC-MS) profile using Shimadzu UFLC-IT-TOFMS, into six fractions (CACF-A, CACF-B, CACF-C,……CACF-F). Each fraction was tested for their cytotoxic activity using MTT assay on MCF-7 cell line.

The cytotoxic active fractions CACF-A, CACF-B and CACF-C were then further purified using either preparative high performance liquid chromatography (HPLC) (Gilson GX-281/322 system) using a Waters Novapak C18 column (25×100 mm, 6 µm) or by recrystallisation. The major active compound of the fractions CACF-A and CACF-B were obtained by preparative HPLC. The fractions were eluted at a flow rate of 12 ml per min over 75 min. The gradient started at 10% solvent A (acetonitrile with 0.1% formic acid) and 90% solvent B (water with 0.1% formic acid) for 5 min. The gradient then changed from 10% to 60% (A) over 5 minutes, followed by 60–100% (A) for 50 minutes and finally an isocratic elution of 100% (A) from 60 to 75 min. The fractions yielded 10 mg of compound (I) (colorless oil) eluted at about 30 min. Fraction CACF-C was purified by recrystallisation from ethyl acetate (EtOAC) (Merck, Darmstadt, Germany)/diethyl ether (Merck, Darmstadt, Germany) to give a white powder of compound (2) (183.5 mg).

Identification of Compounds

The 1H and 13C Nuclear Magnetic Resonance (NMR) spectroscopy were carried out on a JOEL NMR 400 and 100 MHz with TMS as internal standard. HR-ESI-MS analyses have proven that extracts from seeds of C. anthelminticum possess various pharmacological properties. The methanolic extract from the C. anthelminticum seeds demonstrates antiviral properties [16] whereas acetone and ethyl acetate extracts demonstrate antifilarial activity against Setaria cervi [17]. Besides, petroleum ether and alcohol extracts show analgesic, anti-pyretic and anti-inflammatory effect in rat model [18], [19]. Different extracts from C. anthelminticum seeds also show antimicrobial and antifungal properties when screened on various pathogens in vitro [20]. A recent report also suggests C. anthelminticum seeds phenols inhibit liposomal peroxidation and protect oxidative damage to genomic DNA of Bacillus, therefore can function as an anti-oxidant agent [21].

Figure 1. Photo of Centratherum anthelminticum (L.) seeds.

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In 2004, Lambertini et al. reported the in vitro anti-proliferative effect of extracts from C. anthelminticum on human breast cancer cells [22]. We recently reported that the chloroform, but not hexane or methanol fractions from C. anthelminticum (L.) seeds (CACF) exhibited anti-oxidant property by inhibiting tumor necrosis factor-α (TNF-α)-induced human cancer cell growth by interrupting the activation of nuclear factor-kappa B (NF-κB) [23]. However, the active compounds in CACF were not examined in the previous reports. In this study, we showed that CACF inhibited MCF-7 breast cancer cell growth. Administration of CACF caused morphological changes, disrupted cytoskeletal structures and DNA fragmentation. Bioassay-guided fractionation led us to the identification of eumodal as the cytotoxic agent in CACF. To the best of our knowledge, this is the first report on the cytotoxic and the apoptotic mechanism of eumodalin isolated from C. anthelminticum seeds in human breast cancer cells.

Materials and Methods

Plant Material

The seeds of C. anthelminticum were extracted from the medicinal plant cultivation zone of Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd, Betul Madhya Pradesh India. The seeds were identified by the quality control department of the company itself. Voucher specimen (CA-9) was deposited at the Pharmacology Department of University Malaya, Malaysia.

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Identification of Compounds

The 1H and 13C Nuclear Magnetic Resonance (NMR) spectroscopy were carried out on a JOEL NMR 400 and 100 MHz with TMS as internal standard. HR-ESI-MS
Spectral Data

Vernodalin (1) was isolated as colorless oil. The 1H and 13C NMR spectral data obtained for CDC12 solution of the compound were in agreement to those previously identified as vernodalin [24]. The positive ion HR-ESI-MS spectrum showed a molecular ion peak at m/z 361 [M+H]+ consistent to molecular formula C23H32O2. 1H NMR (CDCl3, 400 MHz): δ 6.74 (1H, s, H-15α), 6.29 (1H, s, H-4′α), 6.20 (1H, d, J = 10.52, H-13α), 5.96 (2H, s, H-4′β, H-15β), 5.71 (1H, dd, J = 11, 17.4, H-11), 5.64 (1H, d, J = 3.82,11.53), 5.32 (2H, dd, J = 6.84,11.53, H-2), 5.13 (1H, td, J = 4.56,10.52, H-6), 4.48 (1H, d, J = 12.36, H-14α), 4.35 (1H, s, H-3′), 4.28 (1H, dd, J = 1.36,12.36, H-14β), 4.06 (1H, L = 11, H-6), 3.03 (2H, m, H-5, H-7), 2.24 (1H, dd, J = 4.6, 14.2, H-9), 2.11 (1H, br, s, OH), 1.71 (1H, dd, J = 10.52, 14.2, H-9). 13C NMR (CDCl3, 100 MHz): δ 168.4 (C-12), 165.1 (C-1), 163.2 (C-3), 136.6 (C-2′), 138.8 (C-1′), 136.0 (C-15), 135.6 (C-11), 129.9 (C-4′), 127.2 (C-4), 121.7 (C-13), 117.2 (C-2′), 78.1 (C-6), 70.6 (C-14), 68.8 (C-8), 62.3 (C-3′), 50.5 (C-7), 46.7 (C-5), 41.1 (C-10), 39.0 (C-9).

13, 12-dihydroxy-acetic acid (2), was obtained as white powder. The LCMS-IT-TOF spectra showed molecular ion peaks, [M+H]+ at m/z 313 consistent to molecular formula C19H18O4. 1H NMR (CDCl3, 400 MHz): δ 5.56 (1H, m, H-10), 5.4 (1H, m, H-9), 3.49 (2H, q, J = 2.4, 4.8, H-11, H-12), 2.36 (4H, m, H-8, H-11), 2.07 (2H, q, J = 6.4, 14, H-2), 1.65 (2H, m, H-3), 1.51-1.32 (16H, m, (CH2)16), 0.91 (3H, t, J = 6.8, H-18). 13C NMR (CDCl3, 100 MHz): δ 178.3, 133.6, 124.7, 73.9 (C-34), 34.0, 30.3, 23.5, 21.8, 21.6, 21.5, 21.1, 20.9, 20.8, 20.7, 20.6, 20.4, 20.2, 13.0, 13.0, 13.0, 13.0.

MTT Cell Viability Assay

1×10^4 cells per well were seeded into 96-well plate overnight. Cells were treated with various concentrations of compound or extract (dissolved in dimethyl sulfoxide, DMSO) for 24 hours. As negative control, cells were treated with vehicle (DMSO) only. Next, cells were incubated with 50 µl of 4,5-dimethylthiazolyl-2-yl-2,5-diphenyltetrazoliumbromide (MTT) (2 mg/ml) at 37°C for 2 hours. After dissolving the formazan crystals in DMSO, plates were read in Chameleon™ multitechnology microplate reader (Hidex, Turku, Finland) at 570 nm against 620 nm. This experiment was performed in triplicates and repeated for 3 times. Mean values ± SD for each concentration was determined. Calculation of cell viability was described previously with slight modification [25]. Cell viability (in percentages, %) was showed as ratio of absorbance (A570 nm) in treated cells relative to absorbance in control cells (DMSO) (A570 nm). The IC50 was defined as the concentration of sample needed to reduce 50% of absorbance relative to the vehicle (DMSO)-treated control.

\[
\text{Cell viability} = \left( \frac{A_{570\text{nm}}(\text{Sample})}{A_{570\text{nm}}(\text{Control DMSO})} \right) \times 100
\]

Real Time Cell Growth Assay

Cell proliferation was measured using xCELLigence Real-Time Cellular Analysis (RTCA) system (Roche, Germany), which allows us to monitor the cell viability and cell growth continuously at multiple time point. Briefly, background measurements were taken after adding 50 µl of the culture medium to the wells. Next, cells were seeded at density 1×10^4 on a specialized 16-well plate with electrodes for 18 hours to allow cells to grow to the log phase. Cells were treated with 100 µl of CACF or vernodalin in various concentrations (µM) dissolved in cell culture media and continuously monitored for up to 72 hours. Cell sensor impedance was expressed as an arbitrary unit called the Cell Index. Cell index were recorded every 5-10 minutes by RTCA analyzer. To eliminate variation between wells, cell index values were normalized to the value at the beginning of treatment time-point.

Real Time Cell Invasion Assay

The kinetics of cell invasion was assayed using the xCELLigence Real-Time Cell Analyzer (RTCA DP; Roche). CIM-plates (Roche) were pre-coated with 30 µl of matrigel (BD Biosciences) diluted 1:10 in DMEM for 1 h at 37°C. The upper chambers contained pre-warmed serum-free DMEM, whereas the lower chambers contained either DMEM with 10% FCS or DMEM medium only (negative control). Indicated concentrations of vernodalin were added into the medium of upper and lower chambers. 1×10^4 MDA-MB-231 cells were seeded into each well of the upper chambers. Cells were allowed to settle for 30 min at room temperature before being placed back to the RTCA DP in a humidified incubator at 37°C with 5% CO2. Readings were taken every 10 min for 16 h and plotted curves represented the averages from two independent wells per measurement.

Apoptosis Assay

For in vitro fluorescent staining, 1×10^5 cells per well were seeded in 96-well plate overnight. Cells were then treated with CACF at various concentrations for 12 hours. Live cells were stained with FITC-annexin V (BD Biosciences, San Jose, CA) for 15 minutes before fixed with 4% paraformaldehyde. Cells were washed 3 times with PBS and the fluorescent images were acquired using Cellomics ArrayScan high content screening (HCS) reader (Thermo Scientific, Pittsburgh, PA). Compartmental analysis bioapplication module was used to quantify the fluorescence intensity of FITC-annexin V.

For apoptosis assay by flow cytometry, cells were seeded at 1×10^5 per ml on 25 cm2 flask overnight before treated with vernodalin at various concentrations for 24 hours. Determination of apoptotic cells by fluorescent staining was done as described previously [25]. Briefly, cells were incubated with FITC-annexin V and propidium iodide (PI) (BD Biosciences) in binding buffer for 15 minutes in dark. Stained cells were immediately subjected to flow cytometry analyses using FACS Carso II flow cytometer (BD Biosciences).

Cytoskeletal Rearrangement Analysis

1×10^4 MCF-7 cells per well seeded overnight in 96-well plate were exposed to DMSO (negative control) or CACF at various concentrations for 24 hours. Cells were fixed, washed with wash buffer before probing using phalloidin conjugated with DyLight™ 554 and Hoechst 33258 according to the manufacturer's instructions. Cells were washed and images were acquired using Cellomics ArrayScan HCS reader (Thermo Scientific). Morphology bioapplication module was used to quantify the fluorescence intensity of phalloidin.

Cell Cycle Analysis

1×10^5 cells per ml seeded overnight in 25 cm² flask were treated with vernodalin for 24 hours. Cells were then fixed with 70% ethanol overnight. Cells were washed twice with PBS and stained with CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences) according to manufacturer's instructions. Cell cycle distribution of nuclear DNA was determined by flow cytometry (BD Biosciences) by analyzing at least 20,000 cells per sample. The percentage of cells in G1, S and G2 phases were analyzed by Diva software (BD Biosciences).

Reactive Oxygen Species (ROS) Analysis

1×10^4 cells per well were seeded onto 96-well plate. Cells were treated with vernodalin or DMSO (negative control) at indicated concentrations for 12 hours. Dihydrorhodamine (DHE) dye contained in Cellomics ROS kit was added into live culture for 30 minutes. Cells were fixed and washed with wash buffer as described by the manufacturer's instruction. Stained cells were visualized and acquired using Cellomics ArrayScan HCS reader (Thermo Scientific). Target activation bioapplication module was used to quantify the fluorescence intensities of DHE dye in the nucleus.
Nuclear Morphology, Membrane Permeability, Mitochondrial Membrane Potential Δψm (MMP) and Cytochrome C Release Analysis

CACF inhibited cell growth of MCF-7 breast cancer cells in dose- and time-dependent manners. A sudden decrease in nCI values about 1–2 hours after treated with 25 or 12.5 µg/ml of CACF, indicating acute toxicity at high dosages. Together, our results showed inhibition as reflected by increased normalized cell index (nCI) values. Whereas MCF-7 treated with doxorubicin at concentration 6.25 µg/ml resulted in cell growth between MTT and Alamar blue staining assays. Showing IC50 value at 2.0±0.8 µg/ml. To verify MTT results, we repeated the experiments using Alamar blue staining for cell viability. We found comparable results between MTT and Alamar blue staining assays (Figure S1A).

Western Blot Analysis

Bioluminescent assays for Caspase-3/7, -8 and -9 activities were performed using GraphPad Prism 5 software. Statistical significance was defined when P<0.05.

Results

CACF inhibits survival of human breast cancer MCF-7 cells

We first determined the cytotoxic effect of CACF on cell survival using a well-characterized human breast cancer cell line, MCF-7. MTT assay was used to determine cell viability. The survival of MCF-7 decreased significantly in a concentration dependent manner with IC50 value at 6.8±1.2 µg/ml (Figure 2A). No significant cell inhibitory effect was observed in DMSO (solvent)-treated samples. As a positive control, we treated MCF-7 cells with doxorubicin, a cancer chemotherapy drug, which showed IC50 value at 2.0±0.8 µg/ml. To verify MTT results, we repeated the experiments using Alamar blue staining for cell viability. We found comparable results between MTT and Alamar blue staining assays (Figure S1A).

Figure 2. CACF inhibits MCF-7 cells proliferation in a time- and dose-dependent manner.

(A) MCF-7 cells were treated with control DMSO, various concentrations (0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 µg/ml) of CACF or anti-cancer drug doxorubicin for 24 hours. Cell viability was determined by MTT assays. (B) Real-time cell proliferation was measured using xCELLigence Real-Time Cellular Analysis (RTCA) system. MCF-7 cells were treated with DMSO (control), indicated concentration of CACF or doxorubicin (DOX) and normalized cell index for 3 consecutive treatment days was shown. Data were mean ± SD. Arrow showing time-point of CACF administration.

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MTT assays are end point assays which only detect cell viability at certain time-point. Next, we observed the subtle changes or the pattern of cell growth after CACF treatment for 3 consecutive days using real-time cell proliferation assay (RTCA). In control wells (vehicle, DMSO only), we observed an exponential increased of cell growth as reflected by increased normalized cell index (nCI) values. Whereas MCF-7 treated with doxorubicin at concentration 6.25 µg/ml resulted in cell growth inhibition (Figure 2B). A dose-dependent attenuation of cell proliferation was observed in CACF-treated MCF-7 (Figure 2B). As shown in Figure 2B, we observed a sudden decrease in nCI values about 1–2 hours after treated with 25 or 12.5 µg/ml of CACF, indicating acute toxicity at high dosages. Together, our results showed that CACF inhibited cell growth of MCF-7 breast cancer cells in dose- and time-dependent manners.
Morphological Assessment of CACF-treated MCF-7 Cells

Next, we examined if CACF treatment resulted in cell death through apoptotic pathway. We treated MCF-7 cells with control (DMSO solvent) or CACF for 12 hours before staining live cells with apoptosis marker annexin V conjugated to FITC. Exposure of 6.25 and 12.5 µg/ml of CACF led to higher annexin V staining compared to control, suggesting apoptotic activities (Figure 3A).

Figure 3. Morphological assessment of CACF-treated MCF-7 cells.
(A) Representative figures of MCF-7 cells were treated with CACF for 12 hours. Cells were stained with apoptosis marker annexin V (green) and nucleus marker Hoechst 33258 (blue). Histogram shows mean fluorescence intensities of annexin V in MCF-7 cells treated with various concentration of CACF. Data were mean ± SD, *P<0.05. (B) Representative figures of cytoskeletal F-actin formation in control or CACF-treated MCF-7 cells. Cells were fixed, stained with DY544-phalloidin (red) and Hoechst 33258 (blue) after treated with 6.25 µg/ml CACF or solvent DMSO for 12 hours. Histogram shows mean fluorescence intensities of phalloidin in MCF-7 cells treated with various concentration of CACF. Data were mean ± SD, *P<0.05. (C) Representative figures of MCF-7 cells treated with DMSO (control), 6.25 or 12.5 µg/ml of CACF for 24 hours. Cells were also treated with a standard drug doxorubicin (DOX) as positive control of apoptosis induction. Cells were stained with Hoechst 33258 dye (blue). All images were visualized and captured using Cellomic HCS array scan reader (objective 20 ×).

Under light microscope, we observed that MCF-7 cells exposed to CACF resulted in reduction of cell size and cell-cell contact areas (Figure 3B). To further investigate this, we examined cytoskeletal F-actin structure by staining the cells with phalloidin conjugated to DyLight™ 554, which detect polymerized actin (F-actin). Control cells demonstrated well-organized actin filament bundles or stress fibers in the cytoplasm (Figure 3B). On the contrary, CACF treatment (6.25 and 12.5 µg/ml) on MCF-7 cells caused a drastic reduction in phalloidin stain (Figure 3B) and loss of stress fibers in the cytoplasm. Furthermore, F-actin was no longer distributed evenly at the cell periphery, but appeared as punctuate stain at the plasma membrane. This result suggests that CACF treatment led to the disruption of cytoskeletal structure in MCF-7 cells.

Because apoptotic activity is usually associated with DNA cleavage, we examined the effect of CACF on nuclear morphology of MCF-7 cells using Hoechst 33258. After CACF treatment for 24 hours, a population of condensed and fragmented nuclei was observed (Figure 3C). The number of cells with fragmented nuclei increased with higher dosages of CACF administrated while no detectable DNA damage was detected in control cells. Together, these data indicated that CACF treatment triggered apoptotic pathway as evidenced by higher annexin V staining, cell shrinkage, disrupted cytoskeleton and DNA damage in MCF-7 cells.

Cytotoxic Activities of CACF Fractions

To identify the active compound in CACF extracts which possesses cytotoxicity activity against human breast cancer cells, we performed HPLC analysis. The cytotoxic extract CACF was fractionated into six fractions using preparative HPLC (Figure 4A and 4B). Among these fractions, CACF-A and CACF-B showed highest activity on MCF-7 cells with IC_{50} values of 5.8±0.6 µg/ml and 5.5±0.3 µg/ml, respectively. However, CACF-C showed moderate activity on MCF-7 cells with IC_{50} value of 38.2±1.6 µg/ml while the other fractions (CACF-D to F) exhibited low cytotoxic activity (IC_{50}>100 µg/ml). Compounds in fractions CACF-A, -B and -C were further isolated as described in Materials and Methods.
Major compounds in the CACF-A, -B and -C fractions were subjected to LC-MS analysis. LC-MS analysis showed that vernodalin (1) (10 mg), eluted at 3.0 min, was the major compound of CACF-A and CACF-B fractions (Figure 5A and 5C). In addition, 12,13-dihydroxyoleic acid (2), eluted at 4.75 min, (183.5 mg) was largely detected in CACF-C fraction while vernodalin was minor (1 mg) (Figure 5B and 5D). Chemical structures of vernodalin (1) and dihydroxyoleic acid (2) were depicted in Figure 6A and 6B.

Figure 5. Mass spectra of CACF isolated fractions.  
(A, B) LC-MS chromatograms of fraction CACF-A and CACF-C. (A) Single peak detected in the fraction CACF-A was identified as vernodalin (1). (B) Major peak detected in fraction CACF-C was identified as 12,13-dihydroxyoleic acid (2) while vernodalin (1) constituted a small part in the fraction. (C) HR-ESI-MS spectrum (positive mode) of vernodalin (1). (D) HR-ESI-MS spectrum (negative mode) of 12,13-dihydroxyoleic acid (2).

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Figure 6. Chemical structure of vernodalin (1) and 12,13-dihydroxyoleic acid (2).

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Vernodalin Inhibits Cell Growth of MCF-7 and MDA-MB-231 Cells

To examine the in vitro anti-cancer efficacy of vernodalin, we included a highly invasive and metastatic variant of human breast cancer cell-line, MDA-MB-231, apart from MCF-7 (non-metastatic). Both MCF-7 and MDA-MB-231 cells were exposed to various concentrations of vernodalin for 24 hours. Cell viability was determined by MTT assays. The IC<sub>50</sub> values for vernodalin treated MCF-7 and MDA-MB-231 were 2.5±0.3 µg/ml and 3.4±0.6 µg/ml, respectively (Figure 7A). On the other hand, the IC<sub>50</sub> of normal mammary epithelial cells was 12.7±0.5 µg/ml, relatively more resistant to cell killing by vernodalin. We further verified the results using Alamar blue proliferation assay (Figure S1B). Meanwhile, 12,13-dihydroxyoleic acid, another compound isolated showed no cytotoxicity effect on both breast cancer cell lines, IC<sub>50</sub>&gt;100 µg/ml (data not shown).
Next, we monitored real-time vernodalin-mediated cell growth inhibition for 3 consecutive days by RTCA. As shown in Figure 7B and 7C, a dose-dependent cell-growth inhibition was observed in vernodalin-treated MCF-7 and MDA-MB-231 cells. Reduced nCI was observed at concentration 3.125 µg/ml, whereas no significant increment in nCI at 6.25 µg/ml or higher after vernodalin treatment compared to control (Figure 7B and 7C). These results showed that vernodalin inhibited cell growth of breast cancer cells, MCF-7 and MDA-MB-231 in a dose- and time-dependent manner.

Vernodalin Inhibits Invasive Potential of Metastatic Breast Cancer Cells

Next, we examined whether vernodalin has an influence on the invasive potential of metastatic breast cancer cells MDA-MB-231 by using modified Boyden chamber (CIM plates, Roche) with matrigel as a substrate. The presence of chemoattractant FCS strongly induced non-treated MDA-MB-231 cell migration to the lower chamber. In contrast, no signal or cell invasion was detected in lower chamber without addition of FCS. On the other hand, dose-dependent inhibition of cell invasion was observed upon vernodalin treatment (Figure 8). Of note, we previously showed that CACF treatment resulted in reduced phalloidin staining (Figure 3B), implying that vernodalin-mediated cell invasion inhibition was probably mediated through disruption of actin polymerization.

Vernodalin causes Apoptosis and Cell Cycle Arrest

To examine whether cells undergo apoptosis, untreated or vernodalin-treated MCF-7 and MDA-MB-231 breast cancer cells were stained with annexin V and PI. Flow cytometry analysis of stained cells can distinguish cells into four groups, namely viable (annexin V- PI-), early apoptosis (annexin V+ PI-), late apoptosis (annexin V+ PI+) and necrotic (annexin V- PI+) cells. As shown in Figure 9A and 9B, vernodalin exposure at different concentrations (3.125, 6.25 and 12.5 µg/ml) resulted in higher population of early apoptotic population (30.0±19.7% to 48±10.8% in MCF-7 cells; 26.1±8.5% to 28.3±6.8% in MDA-MB-231 cells) when treated with 3.125, 6.25 or 12.5 µg/ml of vernodalin. Less than 10% of population showed necrotic signs when treated with high dosage (12.5 µg/ml) of vernodalin.
Vernodalin Induces ROS Generation

ROS is produced especially when cells undergo chemical or environmental stress and could be one of the causative factors leading to cell cycle arrest or apoptosis. Next, we examined the ROS level in control or vernodalin-treated breast cancer cells by staining with DHE dye. ROS convert non-fluorescent DHE to fluorescent ethidium, which then intercalates into DNA. Hoechst 33258, a DNA binding dye is used to identify the nuclei of individual cells then the DHE fluorescence is quantified using the Cellomics HCS machine to evaluate the oxidative stress level. As shown in Figure 11A and 11B, ROS production was at the basal level in control DMSO-treated MCF-7 or MDA-MB-231 cells. In contrast, treatment with vernodalin (12 hours) resulted in dose-dependent increased of ROS production as shown by increased DHE staining in the nucleus (Figure 11A and 11B).
Figure 11. Vernodalin mediates ROS production.
(A) MCF-7 or MDA-MB-231 cells were treated with DMSO (control) or indicated concentration of vernodalin for 12 hours. Live cells were stained with DHE dye (green) before cells were fixed and stained with Hoechst 33258 (blue). Images were acquired using Cellomic HCS array scan reader (objective 20 ×). Representative figures (control or 6.25 µg/ml vernodalin-treated) were shown. (B) Bar chart showing average fluorescence intensities of DHE dye in the nucleus. Data were mean ± SD of fluorescence intensity readings representative of three independent experiments. (*P<0.05).

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Effect of Vernodalin on Nuclear Morphology, Membrane Permeabilization, MMP (Δψm) and Cytochrome c Release

Since high ROS production could lead to plasma membrane, DNA, mitochondrial damage, we further examined the nuclear morphology, membrane permeability, mitochondrial membrane potential (MMP, Δψm) and cytochrome c release and localization. As shown in Figure 12A and 12B, 24 hours exposure to vernodalin revealed a concentration-dependent increment of membrane permeability, attenuation of MMP and increased cytochrome c in the cytosol compared to control. In some vernodalin-treated cells, cytochrome c was localized in the nucleus and we could observe nuclear condensation and fragmentation in these cells (Figure 12A). Whereas in control samples, nucleus remained rounded and uniform in size. Moreover, plasma membrane was intact as shown by the weak staining of permeability dye (green, Figure 12A). Cytochrome c (cyan) was distributed homogenously in the cytosol, which colocalized with MMP dye (red), indicating that cytochrome c was not released from the mitochondria in control cells (Figure 12A).

Figure 12. Effect of vernodalin on nuclear morphology, membrane permeabilization, MMP (Δψm) and cytochrome c release.
MCF-7 or MDA-MB-231 cells were plated in 96-well plates and treated with either vehicle (DMSO) or indicated dosages of vernodalin for 24 hours. Cells were fixed and stained according to the manual. Images were acquired using Cellomic HCS array scan reader (objective 20 ×). (A) Representative figures showing changes in DNA content (blue), cell permeability (green), MMP (red) and cytochrome c (cyan). Arrows showed condensed or fragmented DNA. (B) Bar chart showing dose-dependent increased in cell permeability, reduced MMP and increased cytochrome c release in vernodalin-treated samples. Data were mean ± SD of fluorescence intensity readings of three independent experiments. (*P<0.05).

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Effect of Vernodalin Treatment on Caspase-3/7, -8, -9
Apoptosis is a complex activity that mobilizes a number of molecules and is classified into caspase-dependent or caspase-independent mechanisms. Caspase-dependent pathway can be further divided into extrinsic or intrinsic pathway, as determined by involvement of caspase-8 or caspase-9, respectively. Both intrinsic and extrinsic pathway involve activation of caspase-3/7 which is important for inducing downstream DNA cleavage molecules. To examine the molecular mechanism underlying apoptosis process, we stained cells with aminoluciferin-labeled substrate of caspase and determined the caspase-3/7, -8, -9 activities by measuring the luminescence intensities every three hours. As shown in Figure 13A, we observed a gradual increased of caspase-9 and caspase-3/7 activity, which peaked at 18 hours in both MCF-7 and MDA-MB-231 cells treated with 6.25 µg/ml of vernodalin (Figure 13A and 13B). The activity of caspase-3/7 increased significantly from 6 to 12 hours, but remained high even after 30 hours of treatment, indicating a more latent effect of vernodalin in MDA-MB-231 cells (Figure 13B). In contrast, there were no significant changes in the activity of caspase-8 for the time span of 30 hours in vernodalin treated-MCF-7 or MDA-MB-231 cells. Our data suggested that vernodalin induced activation of intrinsic caspase pathway in both breast cancer cell lines.

To examine whether caspases and the downstream PARP molecule were involved in vernodalin-induced apoptosis, we performed Western blot analysis using cell lysates of untreated/vernodalin-treated MCF-7 or MDA-MB-231 cells. Results indicated that vernodalin dose-dependently caused cleavage of caspase-7 and -9 in MCF-7, whereas caspase-3, -7 and -9 were activated in MDA-MB-231 cells (Figure 13C). On the other hand, PARP cleavage was also detected in both cells, suggesting involvement of caspase cascade and PARP inactivation in vernodalin-mediated apoptosis (Figure 13C).

Vernodalin Downregulates Anti-apoptotic Molecules

Cell survival is maintained by pro-survival (anti-apoptotic) molecules such as Bcl-2 and Bcl-xL. To examine if the vernodalin initiated apoptosis by affecting the cellular level of these molecules, we performed Western blot analysis using control or vernodalin-treated breast cancer cells. Cells were also treated with a standard drug doxorubicin as a positive control of apoptosis induction. Our data showed that vernodalin dose-dependently reduced the expression level of Bcl-2 and Bcl-xL (Figure 14).
Discussion

In this study, chloroform extract of the seed of C. anthelmintica (CACF) was tested for its cytotoxicity effect on MCF-7 human breast cancer cells. We showed that CACF effectively inhibited cell growth of MCF-7. CACF-treated cells exhibited morphological hallmarks of apoptosis including cell shrinkage, loss of cytokeratin- F-actin structure, higher stain with apoptotic marker annexin V and DNA damage. In fact, defects in apoptotic pathway are thought to contribute to a number of human malignancies [27]. Thus, anti-cancer agents that induce apoptosis is one of the efficient strategies in cancer chemotherapy [28].

Bioassay-guided isolation is a procedure whereby extract is chromatographically fractionated until a pure and active compound is isolated. Each fraction produce during the process is evaluated in bioassay system (e.g. in vitro cell culture, in vivo rat or zebrafish model etc) and the active fractions are chosen for further purification [29], [30], [31]. In this study, bioassay guided isolation of CACF extract led us to the identification of a potent compound vernodalin. Vernodalin is a sesquiterpene lactone isolated from various plant species including the seeds of C. anthelmintica [32]. Vernodalin exhibits anti-marial and anti-bacterial activities and is a constituent of Vernonia amygdalina (Composite), a plant ingested by wild chimpanzees sometimes suffering from parasite-related diseases in the Mahale Mountains National Park, Tanzania [33], [34], [35]. So far, only two studies have been done on the anti-cancer effect of vernodalin, which demonstrated cytotoxic activity on melanoma and ovarian cancer cell lines [36] and human carcinoma of the nasopharynx (KB) [37]. These two studies mainly examined cell viability of vernodalin treated cancer cells by MTT based assays and less information was available on anti-cancer mechanism by vernodalin. To the best of our knowledge, this is the first report on cytotoxicity and mechanism of vernodalin on human breast cancer cells.

12,13-dihydroxyoyleic acid (2), a compound isolated from CACF-C (a moderate active fraction) did not show inhibitory activity on breast cancer cells. The moderate activity of CACF-C could be due to the presence of trace quantity of vernodalin (Figure 5B). The structure of the compound was confirmed by comparing its spectral data (MS, 1H NMR, 13C NMR) with those reported for a synthetic 12,13-dihydroxyoyleic acid [38]. The MS spectrum (Figure S2A) showed ions at m/z 202 and 181 corresponding to the allylic cleavage and indicated the double bond at C9 and C10 (Figure S2B). Fragmentation ions at m/z 213 and 157 corresponding to the alpha cleavage on the either side of the OH groups which confirmed their positions at C12 and C11 [38], [39]. A characteristic peak at m/z 25 (100%) as a result of ions formed through C9 and C10 cleavage and losing two protons form [M+H+2](+). This is the first time for the compound (2) to be reported as secondary metabolite in plant. 12,13-hydroxyoyleic acid (2) is normally synthesized by acetylation of vernolic acid, an epoxy fatty acid obtained from C. anthelmintica oil [40]. Hydroxy fatty acids are important in industry for the production of oleochemicals [38], [39].

We showed that vernodalin induced cell cycle arrest in breast cancer cells. Cell cycle progression is a hallmark for cell proliferation. Deregulation of cell cycle has been linked with cancer initiation and progression [41]. Thus, cell cycle has emerged as one of the attractive therapeutic target in the treatment of cancer. Nevertheless, siRNA or small molecule inhibitors that target cell cycle have been developed, for example flavopiridol is the first cell cycle inhibitor to be tested in clinical trials [42], [43]. To date, most of the chemotherapeutic agents caused cell cycle arrest either at G0/G1 or the G2/M stage, whereas cell cycle arrest at the S-phase is rare. For instance, synthetically methylated analogue of resveratrol induces G1 cell cycle arrest of human breast cancer MCF-7 cells [44] whereas curcumin induced G2/M cell cycle arrest in cisplatin resistant ovarian cancer cells [45]. The cell cycle is controlled by a group of cyclin family proteins called cyclin dependent kinase (CDK) enzymes [46]. The regulation of CDKs activities is achieved by their association with cyclin partners and kinases, phosphatases and specific inhibitors [46]. Future works are needed to examine the detail mechanism of cell cycle arrest in vernodalin-treated breast cancer cells.

ROS are either free radicals or reactive anions containing oxygen atoms, such as oxygen ions and peroxides. ROS could be a by-product of aerobic respiration, tissue-specific enzyme or microsomal cytochrome P450 metabolism of xenobiotic compounds [47]. High level of ROS can destroy the integrity of plasma membrane, affects dynamic of actin cytoskeleton and causes DNA damage, cumulatively known as oxidative stress [48], [49], [50]. Interestingly, we observed that ROS production in vernodalin-treated human breast cancer cells were 2-4 fold higher compared to control. Although harmful to cells, the anti-cancer effect of several conventional treatments such as ionizing radiation, etoposide or arsenates rely on their ability to stimulate ROS production, which modulate cellular redox balance leading to oxidative stress, destabilization of mitochondria and subsequently induction of apoptosis [51]. For example, etoposide caused severe ROS accumulation preferentially in the human glioblastoma-astrocytoma cells and elevated ROS rendered these cells highly sensitive to cell death [52].

Studies have shown that mitochondria have played a key role in the apoptotic process [53], [54], [55]. Changes in the MMP (Δψm) increase the release of apoptogenic factors such as cytochrome c from the outer mitochondria membrane space into the cytosol. Released cytochrome c then form apoptosome with pro-caspase-9, apoptotic protease activating factor-1 (Apaf-1) and ATP, which in turn activates downstream apoptotic signal such as caspase-3/7 [56], [57]. However, malignant tumor cells predominantly produce ATP through glycolysis rather than oxidation of pyruvate in mitochondria like most normal cells, a phenomena known as Warburg effect [53]. Hence, tumor mitochondria are less susceptible to mitochondrial membrane permeabilization rendering them more resistant to mitochondrial pathway of apoptosis [53]. In this report, we showed that vernodalin induced attenuation of MMP (Δψm) possibility through ROS production, which promote mitochondria membrane permeabilization and subsequent induction of apoptosis.

Caspases is a family of cysteine proteases that is divided into executioner caspases such as caspase-3 or -7, and initiator caspases, such as caspase-8 and -9. Initiator caspase-8 is known to be activated through extrinsic pathway whereas caspase-9 is activated in the event of mitochondrial cytochrome c leakage [57]. Both initiators commit caspase-3/7 pathway. Incubation with vernodalin caused a time-dependent activation of caspase-9, while caspase-8 activities remained at basal level. The increase in caspase-9 activity was concomitant with the increase in caspase-3/7 activity. These results suggest that vernodalin induced apoptosis via mitochondrial-dependent intrinsic pathway. Of note, caspase-3/7 cleaves several target proteins, one of which is DNA repair enzyme, PARP. Interestingly, DNA fragmentation was detected in vernodalin-treated MCF-7 and MDA-MB-231 cells. Since MCF-7 is deficient in caspase-3 expression, it is possible that DNA fragmentation could be mediated by activation of caspase-3 and PARP cleavage, as shown previously by other studies [59], [60].

Members of the Bcl-2 family are major regulators of cell death or cell survival. Bcl-2 and Bcl-xL act as apoptosis inhibitors in the cells. Our data showed that vernodalin treatment reduced expression of pro-survival/anti-apoptotic proteins Bcl-2 and Bcl-xL, implying the relevance of Bcl-2 family proteins for breast cancer cell survival. Another study by Shimizu et al. highlighted the importance of Bcl-2 and Bcl-xL in protecting mitochondria against loss of function during apoptosis and some forms of necrotic cell death [61]. Presence of Bcl-2 in mitochondria blocks cell death by inhibiting apoptosis-associated release of cytochrome c from the mitochondria [52], or by regulating ion flux [63]. Whereas Bcl-xL interacts with Apaf1 to prevent apoptosis by inhibiting Apaf1 dependent activation of caspase 9 [64]. Therefore, downregulation of Bcl-2 and Bcl-xL upon vernodalin treatment could lead to loss of MMP which facilitated cytochrome c release and activation of caspase cascade.

Estrogen stimulates proliferation of various breast cancer cells via estrogen receptors (ER). Studies show that compounds such as phytoestrogens, alkylphenols, organochlorine pesticides and phthalates could bind to estrogen receptors and mediate estrogen responses [41]. Estrogen stimulates proliferation of various breast cancer cells via estrogen receptors (ER). Studies show that compounds such as phytoestrogens, alkylphenols, organochlorine pesticides and phthalates could bind to estrogen receptors and mediate estrogen responses [41]. Estrogen stimulates proliferation of various breast cancer cells via estrogen receptors (ER). Studies show that compounds such as phytoestrogens, alkylphenols, organochlorine pesticides and phthalates could bind to estrogen receptors and mediate estrogen responses [41]. Estrogen stimulates proliferation of various breast cancer cells via estrogen receptors (ER). Studies show that compounds such as phytoestrogens, alkylphenols, organochlorine pesticides and phthalates could bind to estrogen receptors and mediate estrogen responses [41]. Estrogen stimulates proliferation of various breast cancer cells via estrogen receptors (ER). Studies show that compounds such as phytoestrogens, alkylphenols, organochlorine pesticides and phthalates could bind to estrogen receptors and mediate estrogen responses [41].

In conclusion, this report showed that CACF has profound activity against MCF-7 human breast cancer cell line. Through bioassay guided isolation, we identified vernodalin as the active compound responsible for the anti-cancer property in CACF. Our collective data suggest that vernodalin inhibits cell growth of MCF-7 and MDA-MB-231 breast cancer cells through induction of cell cycle arrest and apoptosis. Vernodalin induces apoptosis by generating ROS and downregulating pro-survival molecules Bcl-2 and Bcl-xL. These processes subsequently lead to attenuation of MMP and cytochrome c release. Release of cytochrome c activates caspase cascade and PARP cleavage to execute apoptotic program through fragmentation of chromatin DNA. The findings in this report indicate potential therapeutic value of vernodalin and further research in animal tumor models is necessary to confirm its anti-cancer activity in vivo.

Supporting Information

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Figure S1.

Proliferation assay by Alamar blue assay. (A) MCF-7 cells were treated with vehicle (DMSO) or various concentrations (0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 µg/ml) of CACF for 24 hours. After treatment, Alamar blue stain was added into culture medium for 2 hours (10% of total volume). Cell viability was determined by Alamar blue staining assay (AbD Serotec, Oxford, UK). The fluorescent intensity was measured with Bio-Tek Synergy H4 hybrid microplate reader (Bio-Tek, US) at 590 nm emission (560 nm excitation). Cell viability was calculated according to manufacturer's manual. IC50 value for CACF-treated MCF-7 was 7.6±0.5 µg/ml, IC50 value for doxorubicin-treated MCF-7 was 3.4±0.5 µg/ml. (B) MCF-7, MDA-MB-231 and primary mammary epithelial cells were treated with various concentrations of vernodalin for 24 hours. Cell viability was determined as described above. MCF-7 (IC50 = 2.1±0.8 µg/ml), MDA-MB-231 (IC50 = 3.8±0.4 µg/ml), and primary mammary epithelial cells (IC50 = 14.2±1.3 µg/ml).

Figure S2.

Mass spectra of 12,13-dihydroxyoleic acid. (A) MS spectrum of 12,13-dihydroxyoleic acid (2). (B) MS fragmentation pattern of 12,13-dihydroxyoleic acid (2). The MS spectrum showed ions at m/z 182 and 131 corresponding to the allylic cleavage and indicated the double bond at C9 and C10.

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Author Contributions

Conceived and designed the experiments: CYL MRM. Performed the experiments: CYL FKC BM KHL. Analyzed the data: CYL BM WFW. Contributed reagents/materials/analysis tools: AA KM NR. Wrote the paper: CYL WFW.

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