RESEARCH ARTICLE

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Anti-cancer Effect and Active Phytochemicals of *Houttuynia cordata* Thunb. against Human Breast Cancer Cells

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Abstract

Background: *Houttuynia cordata* Thunb (HCT) is a medicinal herb used in Southeast Asia. **Aim of this work:** This study aimed at investigating the cytotoxicity of this plant extract and fractions towards human breast cancer MDA-MB-231 and MCF-7 cells. HCT's phytoactive compounds are determined. **Materials and methods:** Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The mode of cell death was measured by staining with annexin V-FITC and propidium iodide (PI) employing flow cytometry technique. The oxidative stress was measured by using 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE+) fluorescent probes and using a fluorescence microplate reader. HCT phytochemicals were characterized by high performance liquid chromatography (HPLC). **Results:** The proliferation of MDA-MB-231 and MCF-7 cells was dramatically decreased by the crude extract and individual fraction of HCT. Ethyl acetate was the solvent fraction with the highest toxicity against MCF-7 cells, followed by dichloromethane, crude, and hexane fractions, respectively, whereas in MDA-MB231 cells, dichloromethane, crude, hexane, and ethyl acetate fractions each had the strongest impact, respectively. The methanol fraction had no effect on either cell line up to 200 µg/ml. The extract and fractions were less harmful to the NIH3T3 normal murine fibroblast cell line. The mode of both cell death was apoptosis evidenced by the increase of cell population stained with annexin V-FITC and PI. The fluorescence probes of both DCFH-DA and DHE in MDA-MB-231 cell line were enhanced. Phenolic acids included chlorogenic acid (CA), gallic acid (GA), transcoumaric acid (TCA), vanillic acid (VA), and syringic acid (SA), as well as flavonoids like quercetin and rutin, were identified as the active phytochemicals in the crude and fractions by using HPLC method. **Conclusion:** MDA-MB-231cells underwent apoptosis via oxidative stress when induced with HCT hexane fraction. Phenolic acids and flavonoids were identified in HCT's extract and fractions.

Keywords: *Houttuynia cordata* Thunb- anti-cancer- human breast cancer- active phytochemicals

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Introduction

According to a molecular docking method that determines the binding affinities between the phytochemicals and the growth factor receptors in cancer cells, *Houttuynia cordata* Thunb. (HCT), has anti-cancer ligands. According to Das et al., in 2022, the top-hit phytochemicals from HCT can be employed to dock with the overexpressed growth factor receptors. The ligand-binding pocket of the HER2 and VEGFR2' kinase domains is docked against a total of 100 physiologically active phytochemicals from HCT (Das et al., 2022). Finding high affinity phytochemicals that have a perfect fit for the ATP ligand-binding pocket requires competitive docking. When docked to HER2 and VEGFR2 using both hydrogen and hydrophobic contacts of the kinase domains of HER2 and VEGFR2, β-sitosterol and quercetin have the highest binding affinity among the top-hit phytochemicals from HCT (Das et al., 2022).

We also previously reported the anti-cancer effect of HCT extract against human breast cancer cell proliferation, migration, invasion and apoptosis (Subhawa et al., 2020). In castrate-resistant (PCai1) and androgen-sensitive prostate cancer cells (LNCaP), Subhawa et al., in 2021, showed that HCT extract and its ethyl acetate fraction (EA) induce apoptosis through activation of caspases, down-regulation of androgen receptor, and inactivation of AKT/ERK/MAPK signaling. One of HCT's active ingredients, rutin, exerts effects on LNCaP cells that are comparable to those of HCT/EA. Moreover, in LNCaP cells, HCT/EA

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block the STAT3/Snail/Twist and epithelial-mesenchymal transition phenotypes. Treatment with 1% HCT orally dramatically slows the growth of PCai1 xenograft tumors. HCT inhibits the development of prostate cancer and CRPC both *in vitro* and *in vivo* (Subhawa et al., 2021).

In 2021, Liu et al., conducted research on the effects of heat treatment on various HCT components and the inhibitory effects on gastric cancer cell proliferation. Alamar blue assay is used to evaluate the effect of various extracts (heated aerial stem, heated subterranean stem, heated leaves, and non-heated classified as NAS, NSS, and NL) on cancer cell viability. It's interesting to note that heat treatment intensifies the effects of cellular growth inhibition and triggers apoptosis via the mitochondrial pathway (Liu et al., 2021).

In order to treat cervical cancer cells biologically, Chen et al., in 2021, produced the copper nanocomplex utilizing HCT (Hc-CuONPs) herbal extract. With a maximum peak at 350 nm, the UV-visible spectroscopy research demonstrated the existence of the nanocomplex, whilst the Fourier transform infrared (FT-IR) spectroscopy absorption peaks exhibited the existence of distinct function groups. Studies using X-ray diffraction and transmission electron microscopy (TEM) revealed that the Hc-CuONPs have a face-centered cubic structure with a size of 40–45 nm. Moreover, the Hc-CuONPs is hazardous to cervical HeLa cancer cells (IC_{50}=5 μg/ml). Acridine orange/ethidium bromide (AO/EtBr), propidium iodide (PI), and 4',6-diamidino-2-phenylindole (DAPI) staining, are used to evaluate apoptotic cell death in the nanocomplex-treated HeLa cells. The analysis results showed that the Hc-CuONPs inhibit HeLa cell proliferation and promote apoptosis in an *in vitro* model through PI3K/AKT signaling pathways (Chen et al., 2021a).

New clinical data show that HCT is highly effective in treating radiation-induced lung injury (RILI). A deadly and dose-limiting consequence of radiation for thoracic malignancies was the subject of two studies, one by Liu et al., in 2023, and the other by Lai et al., in 2022. There are two types of the conditions: irreversible advanced-stage fibrosis and early reversible pneumonitis. Reliable biomarkers are still needed for RILI clinical diagnosis and early prediction.

Inflammation plays a major role in the onset and development of RILI, and certain associated biomarkers are identified during this process. 75 differentially expressed genes (DEGs) are filtered out of two expression profiles that were retrieved from the Gene Expression Omnibus (GEO) database. MMP-9, IL-1, CCR1, and S100A9 were found to be four inflammation-related hub genes in the progression of RILI by combining Gene Oncology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein-protein interaction (PPI) network analysis, viz., created using STRING. In RILI mice models, the hub genes' levels of expression are confirmed, with S100A9 showing the greatest level of overexpression. S100A9 expression in lung tissues and its amount in bronchoalveolar lavage fluid (BALF) are positively linked with the level of inflammation in RILI (Liu et al., 2023).

12 potent compounds against RILI were found by Lai et al., in 2022, after they searched for the main active components from HCT that corresponded to the targets in the Traditional Chinese Medicine System Pharmacology (TCMSP) Database. The GeneCards database is used to retrieve the targets of RILI. By Venn diagram analysis, common targets between active chemicals and disorders are discovered. The network interaction between the medications, active chemicals, targets, and disease is built and visualized using Cytoscape. The effectiveness of the primary active compounds' binding affinities to the central targets is confirmed by molecular docking research. Quercetin, kaempferol, hyperoside, and rutin are the main active chemical constituents. The PPI network's hub nodes for the cancer-signaling pathway include TP53, VEGFA, JUN, TNF, and IL-6. The GO categories are divided into three functional areas: 32 cellular components from the active chemicals in the HCT, 112 biological processes, and 9 molecular functions. The target genes are found in multiple important cancer-related signaling pathways, including the TNF-, PI3K-, AKT-, and HIF-1 signaling pathways, according to the KEGG pathway enrichment study (Lai et al., 2022).

The chemopreventive effect of HCT and its bioactive component 2-undecanone against benzo(a)pyrene (B[a]P)-initiated lung carcinogenesis and the underlying mechanism were reported by Lou et al., in 2019, using a B[a]P-stimulated lung cancer animal model in A/J mice and a normal lung cell model (BEAS.2B) *in vitro.* Without causing systemic toxicity *in vivo*, HCT and 2-undecanone effectively reduce B[a]P-induced lung carcinogenesis. Furthermore, BEAS.2B cells are further noticeably protected by HCT and 2-undecanone, which successfully reduce B[a]P-induced intracellular ROS overproduction, DNA damage, and inflammation by preventing interleukin-1 (IL-1) and phosphorylated H2A.X overexpression. By strikingly activating the Nrf2 pathway, HCT and 2-undecanone, as confirmed by an Nrf2 siRNA molecule, cause the production of the antioxidant enzymes: heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO-1). The Nrf2-HO-1/NQO-1 signaling pathway is thus effectively activated by HCT and 2-undecanone to reduce intracellular ROS production, thereby reducing DNA damage and inflammation brought on by B[a]P activation (Lou et al., 2019).

There are, however, still few studies in comparing the cytotoxicity of HCT extract and different components. As a result, the objectives of this study were to determine the cytotoxic effect of crude extract in comparison to hexane, ethyl acetate, dichloromethane and methanol fraction on human breast cancer MDA-MB-231 and MCF-7 cells, as well as normal murine fibroblast NIT3T3 cell line, mode of cell death, and its preliminary molecular mechanism, including whether or not ROS were generated. The phytochemical components were identified by using HPLC.

Materials and Methods

Chemicals

Rutin, quercetin, 2',7'dihydro dichlorofluorescein diacetate (DCFH-DA), and dihydroethidium (DHE+) were purchased from Sigma-Aldrich in addition to MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide, gallic acid, chlorogenic acid, vanillic acid, syringic acid, and transcoumaric acid (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was bought from Invitrogen (USA). A Roche Annexin V-FITC kit was purchased (Indianapolis, Indiana, USA). The fluorescence probes Fluo3-AM and Rhod2-AM were purchased from Molecular Probes (Eugene, Oregon, USA). Analytical quality hydrogen peroxide, ferrous sulfate, methanol, ethyl acetate, hexane, dichloromethane, and ethanol were purchased from RCI Labscan Limited (Bangkok, Thailand).

Plant materials and extraction

The complete *Houttuynia cordata* Thunb. plants were procured in Thailand's Lop Buri province in May 2018. Plant species was identified and authenticated by Dr. Narin Printarakul, a lecturer and senior plant taxonomist, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The voucher specimen number is CMUB003997001, deposited in CMU Herbarium, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

After the plant's authenticity was confirmed, HCT that had been air dried and finely ground was percolated five times with ten liters of ethanol over the course of five days at room temperature. To create a crude ethanolic extract, the extracts were mixed and dried under decreased pressure. The ethanolic extract was redissolved in a 3:7 mixture of methanol and water, and then partitioned three times with different solvents in a ratio of 1:1: hexane (H), dichloromethane (CH_2Cl_2), and ethyl acetate (EtOAc). The solvent was eliminated from each fraction using a rotary evaporator. Every fraction was kept at 4°C until used.

Cell lines and cell culture

The American Type Culture Collection provided the human breast cancer MDA-MB-231, MCF-7 and murine embryonic fibroblast NIH3T3 cells (ATCC, Manassas, VA, USA). Cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 25 mM $NaHCO_3$, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37°C in an environment with 5% CO_2.

Cytotoxicity test

The crude extract, various fractions, including hexane, dichloromethane, ethyl acetate, and methanol fractions, were applied to MCF-7, MDA-MB-231, and NIH3T3 cells for 24 hours at various concentrations, i.e., two-fold dilution, such as 50, 100, 150, 200, 300, and 400 µg/ml. Cell viability was assessed using a mitochondrial enzyme reaction that changes MTT into formazan crystals. MTT dye solution was added and incubated at 37°C for four hours after the extract or each fraction had been treated for 24 hours. A microplate reader was used to measure the absorbance at 540 nm and the reference wavelength at 630 nm after the formazan crystal was dissolved in dimethyl sulfoxide (DMSO) (BioTek, Winooski, VT, USA) (Banjerdpongchai et al., 2010).

As in the prior study, the percentage of cell viability was calculated. Using the formula below. To further investigate the dosage response effect, the inhibitory concentrations at 20 percent (IC_{20}) and 50 percent (IC_{50}) were computed. Calculating the percentage of cell viability was performed using the following formula (Banjerdpongchai et al., 2010).

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in treated wells}}{\text{Mean absorbance in control wells}} \times 100$$

Mode of cell death determination by flow cytometry

In a 24-well culture plate, $5x10^5$ MDA-MB-231 and MCF-7 cell lines were incubated at IC_0, IC_{20}, and IC_{50} doses. The cells were centrifuged after being washed twice with phosphate-buffered saline (PBS) and incubated at 37°C in a 5% CO_2 environment for 24 hours. The cells were then reconstituted and stained for 15 minutes in the dark with 100 µl of binding buffer containing 5 µl of annexin V-fluorescein isothiocyanate (FITC) and PI (Roche Diagnostics, Mannheim, Germany). The cells' fluorescence intensity was assessed. Using the flow cytometer's Cell Quest software, the cells' dot plot character was examined (Beckman, Dickinson and Company, NJ, USA). Annexin V-FITC and Annexin V-FITC combined with PI staining cells were identified as early and late apoptotic cells. A bar graph was created and the proportion of total apoptotic cells was computed (Banjerdpongchai et al., 2016).

Determination of reactive oxygen species generation

At IC_0, IC_{20}, and IC_{50} concentrations, particular hexane fractions were applied to cells ($1x10^5$ cells/ml in a 96-well culture plate). Each unique set of treated cells received a final concentration of either DHE+ (25 µM) or DCFH-DA (20 mM) before an hour of incubation. The fraction was applied to the cells for 4 hours at 37°C and 5% CO_2. After that, the DCF fluorescence was recorded. As a positive control, hydrogen peroxide (0.3% for 30 minutes; H_2O_2) was used. A fluorescence microplate reader was employed with wavelengths of 485 nm and 530 nm as the excitation and emission wavelengths, respectively (Prommaban et al., 2012). When employing $FeSO_4$ as a positive control, the excitation and emission wavelengths for ethidium (E+) were 535 nm and 635 nm, respectively (Rachakhom et al., 2019).

Characterization of phytochemicals by high performance liquid chromatography (HPLC) analysis

All fractions and the ethanolic extract were dissolved in methanol at a concentration of 1 mg/ml. In methanol at a concentration of 100 µg/ml, all typical chemicals, including gallic acid, chlorogenic acid, vanillic acid, syringic acid, transcoumaric acid, rutin, and quercetin, were dissolved. Agilent 1260 Infinity DAD detector

(Santa Clara, CA, USA) with a binary pumping system was used for HPLC-UV/DAD analysis. Zorbax Eclipse plus C18 column (4.6 mm 100 mm, particle size 3.5 m) was injected with samples (10 ml) (Kumar et al., 2014).

The standard mixture's gradient program designated for 0.5% formic acid in water (solvent A) and methanol (solvent B). For 4 minutes, the solvent gradient was 90:10 (A:B), for 11 minutes, 80:20, for 50 minutes linearly 10:90, and for 5 minutes constantly. Prior to injecting the second sample, an equilibrium phase of 90:10 (A: B) lasted for 5 minutes. The flow rate was 0.6 ml/min, the detecting wavelength was 272 nm, and the injection volume was 5 ml.

The extract was subjected to HPLC under the conditions of 0.5% formic acid in water (solvent A) and methanol (solvent B) as the mobile phase. Before injecting the next sample, the initial A:B ratio was 90:10 for the first 4 min, changing to 70:30 for 16 min, 50:50 for 40 min, 30:70 for 10 min, constant for 10 min, and 90:10 for the final 10 min. The detection wavelength was 272 nm, the injection volume was 5 ml, and the flow rate was 0.6 ml/min.

Formic acid (solvent A) and methanol (solvent B) were the solvents used in the HPLC procedure for the hexane fraction. 50:50, 10:90 for the opening 15 minutes, for A:B. Before injecting the next sample, there was a 5-min 90:10 (A: B) equilibrium phase that followed. Volume of 5 ml was used for the injection, with a flow rate of 0.6 ml/min and a 272 nm detecting wavelength.

Using methanol as the solvent B and 0.5% formic acid in water as the solvent A, HPLC was used to separate the dichloromethane fraction. Initially, the 4:1 ratio of A:B was 90:10, followed linearly by 30 minutes of 80:20 and 20 minutes at 10:90. The following session was a 5-minute 90:10 equilibrium period. The flow rate was 600 µl/ml, the detecting wavelength was 272 nm, and the injection volume was 5 ml.

Statistical analysis

The results are presented as mean + S.D. Three independent experiments carried out in triplicate were used to determine the statistical difference between the control and treated groups using one-way ANOVA (Kruskal Wallis analysis) with a p-value cutoff of 0.05. Data were examined using Student's *t*-test to compare between two groups.

Results

Cytotoxic effect of H. cordata extract and fractions against human breast cancer MDA-MB-231, MCF-7 cells and normal murine fibroblast NIH3T3 cells

In a dose-dependent way, the crude extract decreased the percentage of cell viability in both MCF-7 and MDA-MB-231 cells. However, it had no harmful effects on NIH3T3 cells that were in their normal state (Figure 1A). The crude extract was hazardous to MDA-MB-231 and MCF-7 cells at concentrations below 200 µg/ml, but not to NIH3T3 cells at concentrations over 200 µg/ml. Hexane fraction dramatically decreased the viability of both breast cancer cells in a concentration-dependent manner, but it had no harmful effects on normal NIH3T3 cells (Figure 1B).

At doses under 200 µg/ml for both types of breast cancer MCF-7, MDA-MB-321; and normal healthy fibroblast NIH3T3 cells the dichloromethane fraction treatment showed cytotoxicity. The particular fraction was therefore not included in subsequent research (Figure 1C). The IC_{50} concentration for the ethyl acetate fraction in MDA-MB-231 cells was greater than 200 µg/ml, whereas it was less than 200 µg/ml in the MCF-7 cell line. This demonstrated that MDA-MB-231 cells have lower sensitivity to ethyl acetate than MCF-7 cells. Additionally, NIH3T3 cells that were given the ethyl acetate fraction treatment displayed no harm (Figure 1D).

Methanol fraction did not exhibit cytotoxicity towards MCF-7, MDA-MB-231 and normal NIH3T3 cells at maximal concentration of 200 µg/ml (Figure 1E). Thus, methanol fraction was also excluded in further experiment. We selected hexane fraction for further study since it was intriguing to determine the mode and mechanism of such cell death effect, and the relevant mechanism in breast cancer cells (used as representatives).

When this particular hexane fraction was applied to MCF-7 and MDA-MB-231 cells, the mode of cell death was identified. In order to determine the manner of cell death using the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and flow cytometry technique, the IC_{20} and IC_{50} values of H fraction on MDA-MB-231 cells were calculated to be 189 and 289 µg/ml, respectively. The dosages were 194 and 337 µg/ml, respectively, for the MCF-7 cells. MDA-MB-231 cells appeared to be more sensitive to the particular H fraction than MCF-7 cells based on these inhibitory concentrations (IC) or values.

Apoptosis induction by H. cordata specific hexane fraction in MDA-MB-231 and MCF-7 cell lines

Apoptosis resistance is one of the characteristics of cancer cells (Hanahan and Weinberg, 2011). Therefore, we investigated whether or not a particular hexane fraction may cause the cell lines MDA-MB-231 and MCF-7 to undergo apoptosis. Using data from dot plots and the CellQuest flow cytometer software (Becton, Dickinson and Company, NY, USA), it was discovered that both breast cancer cell lines died in an apoptotic-regulated manner (Figure 2A and 2B).

Higher concentrations of a particular hexane fraction

Table 1. The Standard Components in the Mixture together with Their Retention Time (RT) Determined by HPLC technique

Standard compounds	Retention times (minutes)
Gallic acid (GA)	10.83
Chlorogenic acid (CA)	23.17
Vanillic acid (VA)	25.77
Syringic acid (SA)	26.81
Transcoumaric acid (TCA)	30.49
Rutin (RU)	33.87
Quercetin (QC)	40.07

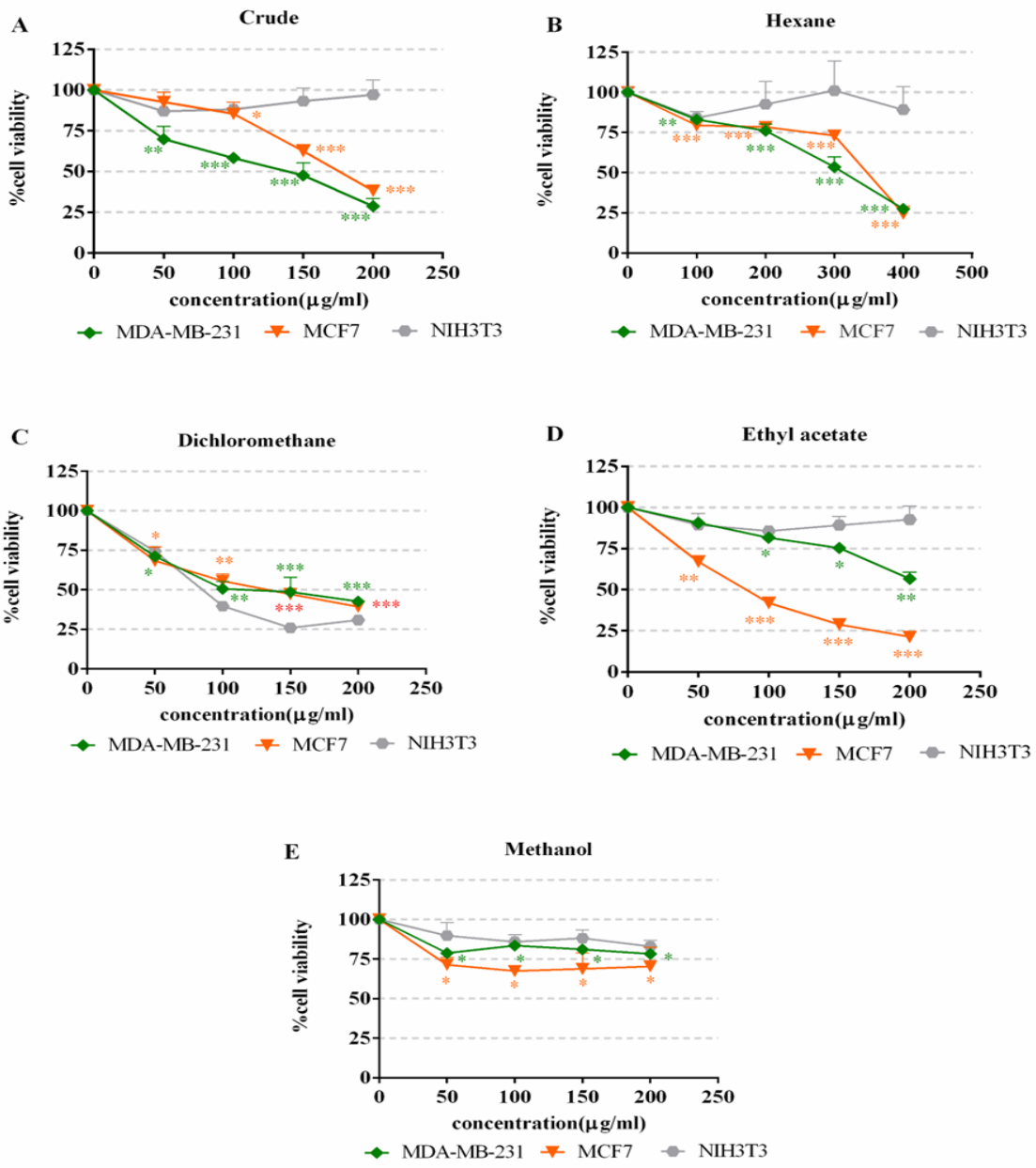

Figure 1. *H. cordata* Crude Extract and Different Fractions' Cytotoxic Effects on the Normal NIH3T3 Murine Embryonic Fibroblast Cell Line as Well as the Breast Cancer MDA-MB-231 and MCF-7 Cell Lines. After 24 hours of exposure to crude extract (A), hexane (B), dichloromethane (C), ethyl acetate (D), and methanol (E) fractions at varied concentrations, the percentage of cell viability was calculated. The data are shown as the mean ± SD of three independent trials done in triplicate. The statistical significance of values is indicated by the symbols *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

significantly increased the proportion of the entire population of apoptotic cells in MDA-MB-231 cells ($p < 0.01$). Additionally, there was a significant difference between the effects caused by IC_{20} and IC_{50} ($p < 0.05$). (Figure 2C). Although not dose dependent, the percentage of MCF-7 apoptotic cells significantly increased at both IC_{20} and IC_{50} ($p < 0.01$) (Figure 2C).

Specific Human breast cancer MDA-MB-231 cells generated ROS as a result of the H. cordata fraction treatment

The fluorescent intensity of DCFH-DA in MDA-MB-231 cells was considerably raised by a specific percentage of HCT dosage at IC_{50} ($p < 0.05$). The formation of ROS, also known as hydrogen peroxide or peroxide radicals, was much higher between the IC_{20} and IC_{50} ranges. ($p < 0.01$) (Figure 3A). At IC_{50}, there was a substantial increase in the fluorescence intensity of dihydroethidium fluorescence ($p < 0.01$) (Figure 3B). Between IC_{20} and IC_{50}, there were also noticeably variable dihydroethidium intensities ($p < 0.05$). Radical superoxide anion is represented by dihydroethidium.

Active phytochemicals in the extract and fractions of H. cordata determined by HPLC approach

A medicinal herb, *H. cordata* Thunb., is grown across Asia (Chun et al., 2014). The following common compounds are among the active phytochemicals found

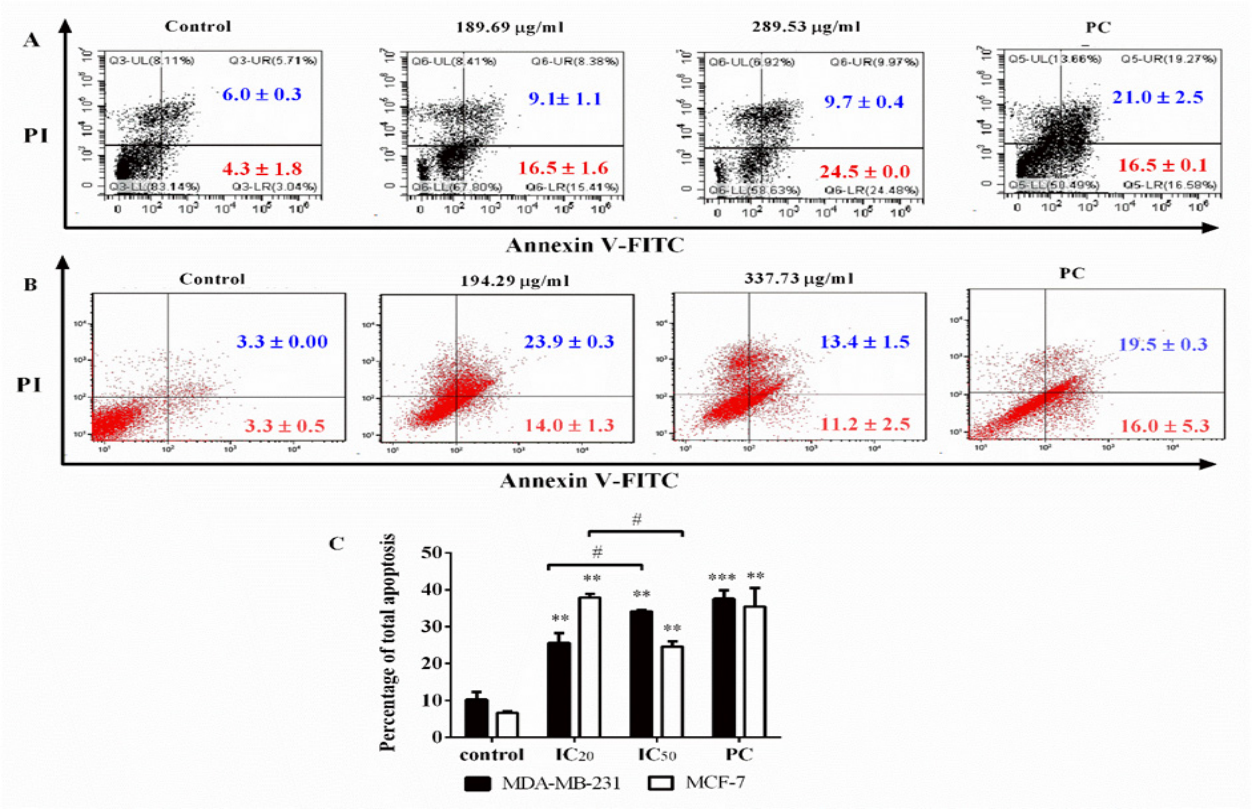

Figure 2. Dot plots showing the induction of apoptosis by the *H. cordata* hexane fraction in MDA-MB-231 (A) and MCF-7 (B) cells after 24 hours of treatment at IC_0, IC_{20}, and IC_{50}, respectively. The positive control utilized was Navelbine (5 µM). Total apoptotic cell bar graphs (C) from three separate studies are displayed as mean ± SD (black bar represents MDA-MB-231 and white bar represents MCF-7 cells). The statistical significance of values is indicated by the symbols *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

in the plant. Several phenolic acids, including gallic acid (GA), chlorogenic acid (CA), vanillic acid (VA), syringic acid (SA), transcoumaric acid (TCA), and sinapinic acids, were shown to be present in commercial fermented *H. cordata* products (Senawong et al., 2014). The phenolic acids and flavonoids in the *H. cordata* extract and fractions were detected in our investigation using the unique HPLC standard system. We employed the standard chemicals displayed in Figure 4A and their retention times are reported in Table 1 for our HPLC analytical apparatus. Figures 4B–4E show the HPLC chromatograms for the crude extract of natural *H. cordata* and each distinct fraction. In the HPLC analysis of crude extract, flavonoids like rutin and quercetin and phenolic acids, such as syringic

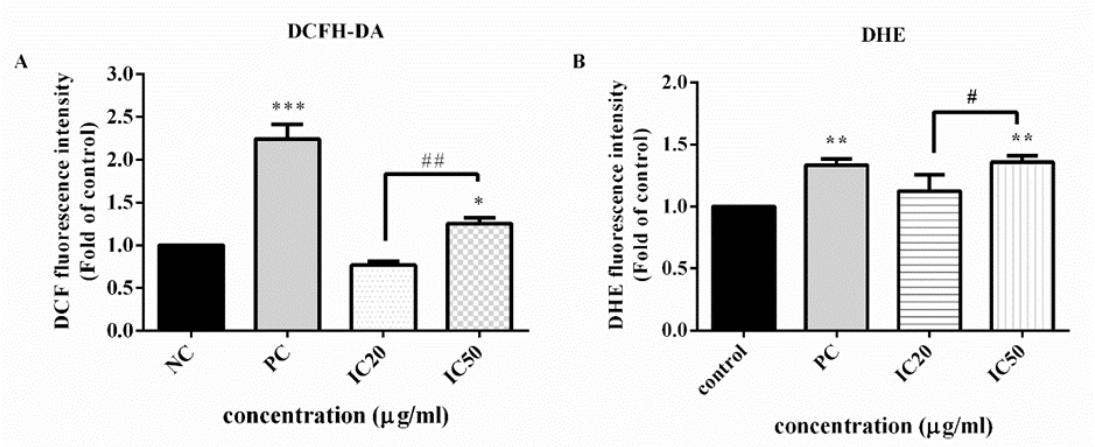

Figure 3. MDA-MB-231 Cells Treated with the Hexane Fraction of *H. cordata* Produce Reactive Oxygen Species (ROS). Fluorescence probes for ROS generation include 2',7'-dichlorodihydrofluoresceine diacetate (DCFH-DA) for measuring peroxide radicals or hydrogen peroxide (H_2O_2) and dihydroethidium (DHE+) for determining superoxide anion radicals (O_2•−). The fluorescence intensity was determined using a fluorescence microplate reader. The results of three separate tests are shown as mean ± SD. The statistical significance of values is indicated by the symbols *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control. The comparison between groups are marked with, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. NC, negative control; PC, positive control, H_2O_2 for DCFH-DA and $FeSO_4$ for DHE.

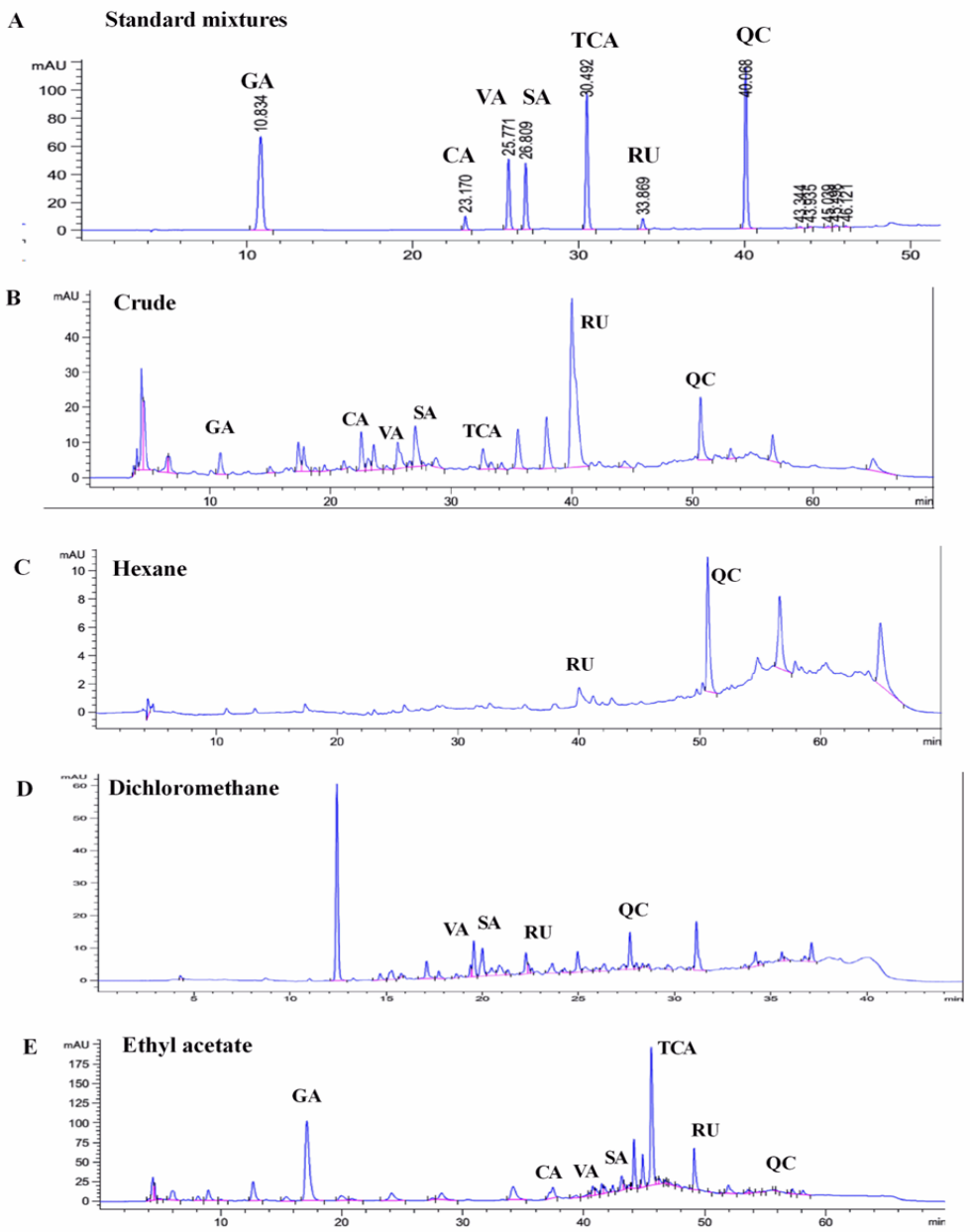

Figure 4. The Following HPLC Chromatograms are Shown: standard chemicals (A) in standard mixes (retention periods 0–50 min), crude extract (B), hexane fraction (C), dichloromethane fraction (D), and ethyl acetate fraction (E). Gallic acid (GA), cholrogenic acid (CA), vanillic acid (VA), syringic acid (SA), transcoumaric acid (TCA), rutin (RU) and quercetin (QC) are the conventional benchmarks. Each tiny panel was not in the same scale.

acid, vanillic acid, chlorogenic acid, transcoumaric acid and gallic acid, were detected, respectively, ranging in quantity from high to low (Figure 4B).

Flavonoids like quercetin were the most prevalent flavonoid component in the hexane fraction (Figure 4C). The primary substances in the dichloromethane fraction were flavonoids, specifically quercetin and rutin, as well as phenolic acids like syringic acid and vanillic acid (Figure 4D). The phenolic acids, such as transcoumaric acid, chlorogenic acid, gallic acid, syringic acid and vanillic acid, were the most prevalent substances in the ethyl acetate fraction, followed by flavonoids such as rutin and quercetin, respectively (Figure 4E).

Discussion

H. cordata considerably boosts the mRNA and protein expression of hypoxia-inducible factor (HIF-1A) and Forkhead box (FOXO3) in hepatocellular carcinoma HepG2 cells. It also promotes the expression of MEF2A and induces apoptosis. MEF2A operates as a proapoptotic factor through increased caspase-3 and caspase-7, as well as the production of proteins from the Bcl-2 family (viz., Bax, Bcl-xL, and Bcl-2) (Kim et al., 2017). Additionally, primary colorectal cancer cells are subjected to cytotoxicity by HCT ethanol extract. It causes a reduction in the mitochondrial membrane potential ($\Delta\Psi(m)$) and an increase in the generation of reactive oxygen species (ROS). The elevated amounts of cytochrome c, Apaf-1, and caspase-3 and -9 are the

mitochondrial dependent apoptotic signaling pathway. Caspase-9 and Caspase-3 are activated by a decrease in ΔΨ(m) and a rise in the Bax/Bcl-2 ratio. It showed that a mitochondria-dependent signaling mechanism in human primary colorectal cancer cells causes HCT to trigger apoptotic cell death (Lai et al., 2010). Additionally, the intrinsic pathway is used to trigger apoptosis in the human colon cancer HT-29 cell line (Tang et al., 2009).

The antiproliferative properties and phenolic acid content of water and ethanolic extracts of the powdered formulation of *H. cordata* Thunb. fermented broth and *P. emblica* fruit mixture were also investigated (Kumnerdkhonkaen et al., 2018). Aqueous extract is less cytotoxic than ethanolic extract. According to the susceptibility of cancer cells to the extract, the ethanolic extract is toxic to human breast MCF-7 cells, human colon cancer HCT116 and HT29 cells, human T-cell leukemia Jurkat cell line, and non-cancer Vero cell lines in that order. The Jurkat cells respond to both extracts the greatest. Apoptosis induction, however, is the underlying mechanism for antiproliferative action. The most vulnerable cells to extract-induced apoptosis are HT29 cells. It has been proven that ethanolic extract works better than water extract at apoptosis induction. Additionally, cell cycle arrest is also shown (Kumnerdkhonkaen et al., 2018). The powder mix extracts of ethanol and water contain seven recognized phenolic acids, including gallic, p-hydroxybenzoic, vanillic, syringic, p-coumaric, ferulic, and sinapinic acids, with p-coumaric acid having the greatest concentration followed by ferulic acid, according to HPLC examination (Kumnerdkhonkaen et al., 2018). However, in our study we could further identify two flavonoids in the extract and fraction, which were rutin and quercetin.

There are several conventional methods for treating cancer, such as chemotherapy or radiotherapy, or even targeted therapy. These, however, frequently have a number of negative consequences and several drawbacks in practical practice. Additionally, concerns regarding drug resistance are growing. Natural chemicals originating from plants are of great interest in the ongoing search for safer and more efficient treatments. Secondary metabolites known as plant phenolics have grown in significance as possible anti-cancer agents.

Phenolics have a significant potential as cytotoxic anti-cancer drugs since they target several components of cancer, increase apoptosis, and decrease proliferation (including angiogenesis, growth, differentiation and metastasis). The subclass of plant phenolics known as phenolic acids, which is further classified into benzoic and cinnamic acids, has been linked to powerful anti-cancer properties in a variety of *in vitro* and *in vivo* scenarios. Additionally, the therapeutic effects of phenolic acids are supported by their functions as epigenetic regulators and promoters of unfavorable reactions or resistance to conventional anticancer therapy. It was stated that one difficult component of the effectiveness of natural chemicals employed in cancer treatment is the encapsulation of phyto-substances into nanocarrier systems (Abotaleb et al., 2020).

Investigated in the human lung cancer A549 cell line are the anti-human lung cancer activity and growth suppression mechanisms of ethanol HCT extract. The G0/G1 and Sub-G1 cell (apoptosis) populations are increased by HCT extract, which also promotes apoptosis with characteristic cell shape. According to the Comet assay, the extract enhances DNA fragmentation and DNA condensation. Additionally, it triggers the activation of caspase-3 and -8. Fas/CD95 protein levels are raised in A549 cells treated with HCT extract. The G0/G1 phase and apoptotic related protein levels, cyclin D1, cyclin A, CDK4 and CDK2 are decreased, and p27, caspase-8 and caspase-3 are upregulated in A549 cells after HCT treatment. Hence, A549 cells died as a result of HCT-induced G0/G1 phase arrest and Fas/CD95-dependent apoptosis (Chen et al., 2013).

As previously established, *Houttuynia cordata* Thunb. (HCT) is a plant with multiple biological features and uses as both food and medicinal. Investigated are the impact of food preparation methods or styles on the anticancer activity of HCT. The plant extracts (designated as heated aerial stem, heated subterranean stem, heated leaves, and not heated as NAS, NSS and NL, respectively) were found to have an inhibitory effect on the viability of four cancer cell lines. Four human tumor cell lines that have received heat treatment from HCT demonstrated anticancer activity. It is intriguing to expect that heat treatment might boost anticancer activity. Heat treatment promotes apoptosis through intrinsic signaling pathways and boosts anticancer activity of aerial stem by 2–14 times in the human gastric cancer SCG–7901 cell line. It is intriguing to note that the caspase-9 specific inhibitor prevented the arial stem - treated cells to die or less viable, demonstrating that apoptosis was being induced by the mitochondrial pathway. HCT's anticancer action is increased by heat treatment, which can be used as a dietary strategy to prevent stomach cancer (Liu et al., 2021).

The absence of some specific types of standard chemicals employed in this technique is the restriction of our HPLC analysis. Due to the use of insufficiently representative reference chemicals, several HPLC peaks could not be distinguished. Gallic acid (GA), chlorogenic acid (CA), vanillic acid (VA), syringic acid (SA), and transcoumaric acid (TCA) were our reference components in the mixture and overlapped with the results of the prior studies (Senawong et al., 2014; Kumnerdkhonkaen et al., 2018). But instead, we have employed two flavonoids, quercetin (QC) and rutin (RU), which are also present in some fractions, as the HPLC standard compounds. However, we discovered and proved that HCT extract and its various fractions were made up primarily of phenolic acids and a small number of flavonoids from the results of the thin layer chromatograms (data not shown).

According to research on flavonoids and apoptosis induction, flavonoids like quercetin's with in the flavanol classification group are distinguished by their unsaturated C rings at C2-C3, which are discovered oxidized at C4 and hydroxylated at C3 (Panche et al., 2016). The most prevalent flavonoid, 3,5,7,3′,4′-pentahydroxyflavone, or quercetin, is a phytoestrogen with structural similarities to natural estrogen 17-estradiol (Ranganathan et al., 2015). Numerous research back up quercetin's capacity to fight

cancer. Quercetin is demonstrated as high propensity to enhance Bax and capsase-3, decrease Bcl-2 and necroptosis by overexpression of RIPK1 and RIPK3 in MCF-7 cells (Ranganathan et al., 2015; Duo et al., 2012; Chou et al., 2010). But in the invasive ductal breast cancer BT-474 cell line, quercetin increases extrinsic apoptosis without having any effect on Bcl-2 or Bax, and as a result, does not start any intrinsic apoptotic pathway (Seo et al., 2016). In HepG2 liver cancer cells, the substance lowers ERK1/2 and AKT phosphorylation and blocks the NF-kappaB pathway (Fantini et al., 2015; Granado-Serrano et al., 2010).

Our findings showed that HCT-specific hexane fractions could reduce breast cancer cell viability and trigger cell death by generating superoxide anion and hydroxide radicals. According to the current findings, oxidative stress, or excess ROS generation, caused human invasive breast cancer MDA-MB-231 cells to undergo apoptosis. However, each flavonoid or phenolic acid should be examined separately on breast cancer cells in order to demonstrate that they were the culprits behind the controlled apoptotic cell death. To confirm the effects of the active phytochemicals, the compound should be utilized to test in human primary breast cancer MDA-MB-231 cells *in vitro* and *in vivo* and evaluate the antiproliferative effect and mode / mechanism(s) of cell death at molecular levels.

The current study has shown, in summary, that a specific extract and fraction of *H. cordata* have a strong potential to suppress the growth of human invasive breast cancer cells by inducing apoptosis and oxidative stress through the production of hydroxide and superoxide anion radicals. This herb is a good source of flavonoids, particularly quercetin and phenolic acids. The growth of human breast cancer cells may have been inhibited by phenolic acids and flavonoids. Further investigation into the use of *H. cordata* as a supplementary medicine for cancer treatment and prevention in both animal models and clinical trials is necessary in light of the study's findings.

Author Contribution Statement

All authors have contributed to the current work. PI performed laboratory investigations. RB contributed substantially to the conception and design of the study, and funding acquisition. PI, HP and RB carried out the acquisition, analysis and interpretation of data. PI and RB drafted the manuscript, and RB performed the critical revision and correction of the manuscript. PK supervised the study. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

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Availability of data and materials

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

Competing interests

Neither of the authors discloses any potential or actual conflict of interest. No financial or nonfinancial benefits have been or will be received from any party relate directly or indirectly to the subject of this article.

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