Vitex rotundifolia fractions induce apoptosis in human breast cancer cell line, MCF-7, via extrinsic and intrinsic pathways

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Abstract

Breast cancer is amongst frequently diagnosed cancer type throughout the world. Due to reduced efficacy of current chemotherapeutics, several natural products have been screened for better alternatives. The cytotoxic activity of fractions prepared from leaves extract of Vitex rotundifolia (V. rotundifolia) on human breast cancer cell line, MCF-7 was studied. The fractions F1, F2, F3, and F5 of V. rotundifolia produced concentration-dependent cytotoxic effects on MCF-7 cell line. The relative potential of cytotoxicity of the fractions on MCF-7 cell line was found to be F3 > F2 > F5 > F1. The active fractions induce apoptosis in MCF-7 cell line determined by annexin V base assay. The phosphatidylserine externalization and the presence of DNA fragmentation in treated cells confirms the early and late apoptosis in treated cells. The V. rotundifolia fractions induced apoptosis by both pathways; extrinsic pathways via activation of caspase-8 and intrinsic pathways through enhanced bax/bcl-2 ratio and activation of caspase-3/7 and caspase-9 proapoptotic proteins. Furthermore, chemical profiling indicates various phenolic, flavonoids, and terpenoids compounds in the active fractions. Thus, V. rotundifolia might be a suitable candidate to investigate further and develop molecular targeted cancer therapeutics by understanding the fundamental mechanisms involved in the regulation of cell death in cancer cells.

Keywords: Apoptosis; Breast cancer; Caspases; DNA fragmentation, Vitex rotundifolia.

INTRODUCTION

Cancer is the second leading cause of mortality after heart disease. It accounts for more than 6 million deaths each year worldwide (1-2). Lung cancer, breast cancer, prostate cancer, and colorectal cancer are primary diagnosed cancers. Breast cancer is the most commonly diagnosed cancer type in women. It accounts for 23% of all newly occurring cancer cases and 13.7% of all cancer deaths in men and women (2-3). The estimated numbers of 1,762,450 cancer cases diagnosed in the United States in 2019, which is the equivalent of more than 4,800 new cases each day. Also, there will be approximately 62,930 new cases of female breast carcinoma in situ (4). However, it remains the most frequently diagnosed cancer and a prevalent cause of death in women (5).

Although extensive preclinical and clinical studies have been carried out in finding anticancer drugs, the majority of potent agents are still not useable due to the harmful effects these candidates may possess. Currently, the development of plant-derived anticancer drugs has gained much interest since naturally-derived compounds are considered to have less toxic side effects compared to synthetic-based drug treatment (6). Plants are rich sources of biologically active and chemically diverse compounds. The secondary metabolites have found considerable use in the treatment of numerous diseases and have been considered lead molecules itself and also as templates for medicinal chemistry (7-8). Over 3000 plant species have been reported to have anticancer properties, and so far about 30 compounds have been isolated from plants and tested in cancer clinical trials (9).

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The plants of the *Vitex* genus widely known for their bioactivities possess anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, antihistamine, and anti-asthmatic properties. Thus, the evaluation of *Vitex rotundifolia* (*V. rotundifolia*) focusing on its bioactive components as potent biological activities in the development of new drugs that may provide an effective way of treating different human ailments such as heart problems and cancer (10).

*V. rotundifolia* has been used as a traditional medicine for the treatment of headaches, colds, migraine, eye-pain, asthma, chronic bronchitis, gastrointestinal infections, bacterial dysentery, and diarrhea (11-12). Furthermore, the fruit of *V. rotundifolia* has been used for the treatment of night blindness and neuralgia (13). Major compounds isolated from *V. rotundifolia* in the previous studies include phenols, flavonoids, iridoid compounds, C-glycoside flavones, and terpenoids (14-15).

Few compounds have been isolated from the fruit extract of *V. rotundifolia* and studied for the anticancerous effect, decidedly less scientific data and publications regarding the potential use of constituents of leaves of *V. rotundifolia* on cancer specifically breast cancer. In this study, the cytotoxic activity of the extract and fractions prepared from *V. rotundifolia* (leaves) on MCF-7 cell line investigated. The mode of cell death discussed focusing on early and late apoptosis and anti and pro-apoptotic proteins, Bcl-2, Bax, and caspases of intrinsic and extrinsic pathways of apoptosis.

**MATERIALS AND METHODS**

**Materials**

The breast cancer cell line MCF-7 purchased from American Type Cell Culture, Manassas, Virginia, USA. The materials used for cell culture were from Sigma Aldrich, St. Louis, Missouri, USA. CellTiter® 96 AQeuous non-radioactive cell proliferation assay (MTS assay) kit, annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and DeadEnd™ fluorometric apoptosis detection kit purchased from Promega, Madison, Wisconsin, USA. NE-PER nuclear and cytoplasmic extraction reagents purchased from Thermo Scientific, USA. Mouse monoclonal IgG1 primary antibodies (Bax, Bcl-2, caspase-8, caspase-9, and caspase-3) and anti-mouse m-IgGk BP-HRP secondary antibody purchased from Santa Cruz Biotechnology, Inc., Heidelberg, Germany. All the standard compounds used purchased from Permsula, USA. All chemicals used were of analytical grade.

**Preparation of the fractions from the *V. rotundifolia***

Leaves of *V. rotundifolia* were collected from Teluk Ketapang, GPS coordinates (5°22’54 N,103°06’59 E) Kuala Terengganu, Malaysia. The *V. rotundifolia* fractions encoded as COAO115001, was deposited in the Institute of Marine Biotechnology, UMT, Malaysia. The sample was cleaned, chopped, and lyophilized (Labconco, UK). Ten g of dried sample was soaked in 100 mL of methanol for 24 h and repeated three times. After this, the filtrate was evaporated to dryness using rotavapor (BUCHI, Switzerland) at 40 °C.

Subsequently, the extract was fractionated using solid phase extraction using a column. For fractionation, crude methanol extracts was impregnated with silica gel until homogeneous column was prepared. Further seven different fractions were obtained by using seven different solvent systems; F1, hexane; F2, hexane:dichloromethane (DCM) (1:1); F3, DCM; F4, DCM:ethyl acetate (1:1); F5, ethyl acetate; F6, ethyl acetate:methanol (1:1); and F7, methanol. Afterwards, the fractions were evaporated to dryness using rotavapor.

**Cell viability assay**

Cytotoxic activity of extract and fractions of *V. rotundifolia* (leaves) against breast cancer cell line, MCF-7, was determined using MTS assay (16). Cells were counted using Countess™ automated cell counter and cultured onto 96-well plates at a cell density of $6 \times 10^4$ cells/well and incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO$_2$ for 24 h. When the cell growth reached 80% confluency, the media was discarded and replaced with fresh media containing different concentrations of each sample and incubated
for 24, 48, and 72 h individually. All dried fractions diluted in dimethyl sulfoxide (DMSO) (Sigma, USA) and in all cases the concentration of DMSO in every well was 1% (v/v). Vincristine sulfate (Sigma, USA) used as positive control in the cytotoxicity screening assay. After treatment, 20 µL of MTS reagent was added at each incubation period for 3 h at 37 ºC. Finally, the absorbance was recorded at 490 nm using ELISA 96-well plate reader (Multiskan Thermofisher, USA).

Annexin V-fluorescein isothiocyanate apoptosis detection

Annexin V-FITC apoptosis detection kit used to determine the mode of cell death exerted by the active fractions on the MCF-7 cells (17). Briefly, MCF-7 cells were treated with active fractions at an inhibitory concentration (IC50) and incubated for 12, 24, and 36 h. Untreated control cells incubated in the presence of 1% DMSO (v/v) and designated as the negative control. Cells treated with vincristine sulphate (0.625 µg/mL) used as positive control. After treatment, cells were washed with 1× binding buffer and subsequently incubated with binding buffer (5 µL of annexin V-FITC and 10 µL of propidium iodide (PI)) at room temperature for 5-15 min. Finally, cells observed under ImageXpress Micro XLS Widefield High Content Screening (Sunnyvale, San Jose, California, USA).

Detection of DNA fragmentation by TUNEL assay

DeadEnd™ apoptosis detection system was used to determine the DNA fragmentation in cancer cells. The MCF-7 cell line was treated against active fractions at a concentration of an IC50 and then incubated for 36 h. The assay performed according to the manufacturer protocol. Briefly, the cells were washed with phosphate buffered saline (PBS) and fixed by immersing the slides in 4% (v/v) methanol-free formaldehyde solution (prepared in PBS), for 25 min at 4 ºC. The slides were then washed with PBS for 5 min at room temperature, and subsequently, the cells were permeabilized by immersing the slides in 0.2% (v/v) Triton™ X-100 solution (prepared in PBS) for 5 min. After permeabilization, the cells washed with PBS for 5 min, and 50 µL of recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer was added into the cells and incubated for 60 min at 37 ºC. The rTdT incubation buffer prepared by mixing equilibration buffer, nucleotide mix, and rTdT enzyme in the ratio (45:5:1) respectively. Finally, the slides were immersed in diluted 20× saline-sodium citrate solutions in deionized water (1:10) for 15 min at room temperature. The cells were then washed with PBS for 5 min and stained by immersing the slides in 50 µL of PI solution (diluted to 1 µg/mL in PBS) for 15 min at room temperature in the dark. The slides were then observed under ImageXpress Micro XLS Widefield High Content Screening to detect the green fluorescence of FITC-labelled apoptotic cells.

Western blot analysis

Western blot analysis was used to evaluate the regulation of protein expression. The protein was separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After the completion of electrotransfer, PVDF membrane was blocked for 3 h and incubated in primary antibodies against Bax, Bcl-2, caspase-8, caspase-9, and caspase-3 overnight at 4 ºC. Subsequently, the membrane was incubated with secondary antibody and incubated for 2 h. The density of the detected proteins was used to observe and calculate using Gel doc 2000 and densitometer (BioRad, California, USA).

Caspases-8, -9, and -3/7 activities

Caspase Glo™ assay (Promega, USA) was performed to examine the association of caspases in triggering or initiating apoptosis induced by the fractions of V. rotundifolia following manufacturer’s protocol. MCF-7 cell line was treated with the fractions F2 and F3 at a concentration of their respective IC50 (72 h) throughout 48 h at 37 ºC in the presence of 5% (v/v) CO2. Moreover, caspase-8, -9 and -3/7 activity was determined. Successively, 100 µL of Caspase-Glo™ reagent added to each well-containing 100 µL of the sample and
negative control. Subsequently, the contents were mixed by shaking plate at 300 rpm for 30 sec and incubated for 2 h. After that, the luminescence was measured using luminometer.

**Thin layer chromatography**

The active fractions of *V. rotundifolia* (F1-F3, and F5) were diluted in a suitable amount of requisite solvent and spotted on the pre-coated silica gel 60 F 254 thin layer chromatography (TLC) plate (Merck, Massachusetts, USA, 1.05735.0001). TLC plate placed in a developing chamber containing a mixture of solvent. The best solvent system selected was a mixture of hexane and ethyl acetate (1:1 v/v). After a while, the plate was taken out from a chamber and left to dry. The plate observed under UV light and spot marked accordingly. The plate was also derivatized with anisaldehyde sulphuric acid reagent.

**High performance thin layer chromatography**

The chemical profiling of active fractions studied using high-performance TLC (HPTLC). The HPTLC plate pre-coated with silica gel 60 F254 glass plates was used. Active fractions of *V. rotundifolia*, F1, F2, F3, F5, standard compounds S1 (ferulic acid), S2 (4-hydroxybenzaldehyde), S3 (4-hydroxybenzoic acid), and S4 (luteolin) were diluted in a suitable amount of requisite solvent. Sonication of sample and standards was done to ensure complete solubility and then filtered to remove undissolved particles. The fraction sample (4 µL) and standard compound sample (2 µL) were spotted (bandwidth of 6.0 mm), 20 mm apart and 8.0 mm from the bottom using automatic sample applicator Camag Linomat V (Camag Muttenz, Switzerland). The chromatograms were developed using hexane and ethyl acetate (1:1) as the mobile phase. Ascending development of chromatogram to a distance of 70 mm was performed at room temperature (28 ± 2 °C) in a glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried and then scanned using Camag TLC Scanner (Wilmington, USA). The HPTLC plate was then visualized under UV light at the wavelength of 254 nm and 365 nm and derivatized with anisaldehyde-sulphuric acid and ferric chloride reagent. The plot was accessed by using WINCATS software.

**Statistical analysis**

Cytotoxicity experiments performed in triplicates and results presented as percentage growth inhibition. IC_{50} values of growth inhibition computed from a nonlinear regression model (curve fit) which was based on the sigmoidal dose-response curve (variable) using GraphPad Prism software (GraphPad, USA). The mean and standard error mean (SEM) were calculated by analysis of variance (ANOVA) (one-way) and Dunnett’s multiple comparison test for post comparison tests, using SPSS software (IBM SPSS Software, US).

**RESULTS**

**Cytotoxicity of Vitex rotundifolia fractions on MCF-7 cell line**

In this study, the cytotoxic effects of the crude methanol extract and fractions prepared from *V. rotundifolia* on human breast cancer cell line MCF-7 investigated by using the MTS assay. The crude methanol extract of *V. rotundifolia* exhibited cytotoxic activity against MCF-7 cell line in a concentration-dependent manner. In this study, IC_{50} was determined. At 72 h incubation, the crude methanol extract was able to inhibit 50% of MCF-7 cells proliferation at the IC_{50} value 63.09 µg/mL. On treatment with F1, the MCF-7 cell line showed an increase in cell death rate with an IC_{50} value 29.51 µg/mL. Similarly, F2 exhibited significant activity with an IC_{50} value 19.95 µg/mL. Interestingly, F3 exhibited considerable cytotoxicity in MCF-7 cell line with a lower IC_{50} value of 6.30 µg/mL than F2 and F1 after 72 h treatment exposure. Furthermore, F5 exhibited the cytotoxic effect with an IC_{50} value of 26.30 µg/mL comparable to F1, and F4 exhibited activity with IC_{50} value above 100 µg/mL. However, F6 and F7 exhibited an IC_{50} value above 100 µg/mL against MCF-7 cell line after 72 h treatment. The fractions F1-F3 and F5 of *V. rotundifolia* showed dose-dependent cytotoxic effects on MCF-7 cell line at all three time points (24, 48, and 72 h) as shown in Fig. 1A-1D.
Vitex rotundifolia fractions induce apoptosis in MCF-7 cells. Fig. 1. Cytotoxic effects of fractions (A) F1, (B) F2, (C) F3, and (D) F5 of Vitex rotundifolia on MCF-7 cell line at 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 µg/mL after 24, 48, and 72 h incubation. Each value represented mean ± SD of six replicates. * And ** indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) in comparison with negative control (0 µg/mL).
Table 1. IC\textsubscript{50} values of \textit{Vitex rotundfolia} methanol extract and fractions, n= 03.

| Samples | IC\textsubscript{50} (µg/mL) |
|---------|-----------------------------|
|         | 24 h | 48 h | 72 h |
| Extract | -    | -    | 63.09 ± 1.98 |
| F1      | > 100 | 44.66 ± 1.73 | 29.51 ± 2.0 |
| F2      | > 100 | 30.19 ± 3.73 | 19.95 ± 2.68 |
| F3      | > 100 | 15.84 ± 2.58 | 6.30 ± 0.52 |
| F4      | > 100 | 99.82 ± 0.14 | 33.11 ± 3.60 |
| F5      | > 100 | > 100 | > 100 |
| F6      | > 100 | > 100 | > 100 |
| F7      | > 100 | > 100 | > 100 |
| Vincristine | -    | -    | 0.625 ± 0.08 |

Fig. 2. The early apoptosis study, green stain (annexin-FITC), early apoptosis; red stain (PI), late apoptosis after 36 h.
F, Fraction; FITC, fluorescein isothiocyanate; PI, propidium iodide.

The relative potential of cytotoxicity of the fractions on MCF-7 cell line was found to be F3 > F2 > F5 > F1. Vincristine sulfate was used as a positive control in the cytotoxicity screening assay with an IC\textsubscript{50} value of 0.625µg/mL at 72h. The fractions F1-F4 were cytotoxic (judged by the criterion set by American National Cancer Institute (NCI) which describes cytotoxicity as an IC\textsubscript{50} value < 30 µg/mL after 72 h) (18). Table 1 shows the IC\textsubscript{50} values of \textit{V. rotundfolia} methanol extract and fractions.

**Phosphatidylserine externalization**

The apoptotic study was carried out using annexin V to monitor the exposure of phosphatidylserine (early apoptosis) and TUNEL assay for DNA fragmentation (late apoptosis). The results obtained by annexin V-FITC staining method demonstrate that fractions F1, F2, F3 and F5 at the concentrations of IC\textsubscript{50} (72 h) successfully induce the translocation of phosphatidylserine (annexin V\textsuperscript{+}) labeled by green stain of FITC, which shows the presence of early apoptosis in MCF-7 cells at 12 h and 24 h (results not shown). The exposure of phosphatidylserine to the outer membrane of the cell is one of the characteristic features of apoptosis (19). However, after 36 h of incubation, most of the cells responded positively to both annexin V and PI (annexin V\textsuperscript{+}/PI\textsuperscript{+}) which signify the presence of late apoptotic cells (Fig. 2). Vincristine sulfate used as a positive control was positive for annexin-V binding. Untreated control (vincristine control) MCF-7 cells were not stained by both annexin V and PI (annexin V\textsuperscript{-}/PI\textsuperscript{-}), which shows that cells were viable and lack of apoptosis. Thus, the results strongly indicated that all four fractions F1, F2, F3, and F5 exhibited
Vitex rotundifolia fractions induce apoptosis in MCF-7 cell line via early apoptosis.

**DNA fragmentation (late apoptosis)**

To further study of late apoptosis via DNA fragmentation, TUNEL assay performed. The results demonstrated that the nuclei of MCF-7 cells were stained green when incubated with fractions F1, F2, F3, F5, and vincristine sulfate at the concentration of their respective IC$_{50}$ (72 h) for 36 h indicating the presence of DNA fragmentation. DNA fragmentation, the hallmark feature of apoptosis, plays a key role in cell death (20). Thus, the results strongly indicated that all four fractions induced apoptosis via DNA fragmentation in MCF-7 cell line as shown in Fig. 3.

**Mechanism of apoptotic pathway activation in MCF-7 cell line**

To find out the potential mechanism of fractions induced apoptosis caspase Glo™ assay and western blot analysis performed, and activity of caspase and Bcl-2 family proteins examined. Figure 4A and 4B showed western blot analysis of the expression profile of Bax, Bcl-2, caspases-8, -9, and -3 at 24 h, ratio of Bax/Bcl-2 expression in MCF-7 cells. Figure 4C indicated the effect of the fractions F2 and F3 of *V. rotundifolia* leaves on the expressions of apoptotic pathway proteins in MCF-7 cells. Figure 4D and 4E represents the results of Caspase Glo™ assay which showed activation of caspases (-8, -9 and -3/7) in MCF-7 cells treated with fraction F2 and F3.

**Fig. 3.** TUNEL assay, the presence of green stain (FITC), which indicates DNA fragmentation. F, Fraction; FITC, fluorescein isothiocyanate; PI, propidium iodide.
Fig. 4. (A) Western blot analysis of ratio of Bax/Bcl-2 expression in MCF-7 cells at 24 h, (B) the expression profile of Bax, Bcl-2, caspases-8, -9, and -3 at 24 h, (C) effect of fractions F2 and F3 of *Vitex rotundifolia* leaf on the expressions of apoptotic pathway proteins in MCF-7 cells. Caspase GloTM assay, activation of caspases-8, -9, and -3/7 in MCF-7 cells treated with fractions (D) F2 and (E) F3 at 0, 1, 3, 6, 9, 12, 16, 20, 2, 36, and 48 h time intervals respectively. Each value represented mean ± SD of six replicates. * And ** indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) in comparison with negative control (0 h).
The findings obtained by western blot analysis demonstrate that fractions F2 and F3 induced the activation of Bax in MCF-7 cells as compared to control cells throughout 24 h. While the reduction in activation of Bcl-2 observed in MCF-7 cells treated with fractions F2 and F3 at 24 h. The ratio of Bax/Bcl-2 increased in MCF-7 cells treated with fractions F2 and F3 as compared to control cells. Thus, the results showed the simultaneous up-regulation of Bax and down-regulation of Bcl-2 in MCF-7 cells. The caspase-8 was inactivated at 24 h in MCF-7 cells treated with active fractions F2 and F3 of *V. rotundifolia*. The results showed the activation of caspase-9 in MCF-7 cells when treated with F2 and F3 compared to control throughout 24 h. The caspase-3 enzyme was not detected by western blot in control as well as treated MCF-7 cells. Furthermore, the results obtained by Caspase Glo™ assay indicated that the treatment of MCF-7 cells with fractions significantly (*P* < 0.05) increased the activation of caspases-8, -9, and -3/7 at different time points. As shown in Fig. 4D and 4E, F2 and F3 induced significant activation of caspase-8 in MCF-7 cells at 36 h. The level of active caspase-9 significantly increased in MCF-7 at 20, 24, 36, and 48 h when the cells were treated with fraction F3 while fraction F2 significantly increased the activation of caspase-9 at 24, 36, and 48 h. The level of active caspase-3/7 significantly (*P* < 0.05) increased in MCF-7 cells treated with fractions F2 and F3 at 48 h, reaching its peak at 36 h and decreased again at 48 h. Thus, the results suggest that both intrinsic and extrinsic pathway may involve in apoptosis induced by fractions F2 and F3 of *Vitex rotundifolia* in MCF-7 cells. Fig 5 shows the schematic representation of apoptotic pathways activated in our study.

**Chemical profiling**

The chemical profiling of fractions F1-F3 and F5 prepared from the methanol extract of *V. rotundifolia* leaves was analyzed using TLC and HPTLC. The solvent system of hexane:ethyl-acetate (1:1 v/v) was used to separate the sample constituent. TLC plate viewed under short wavelength (UV 254 nm) and long wavelength (UV 365 nm).

![Fig. 5. An overview of apoptotic cell death induced by fractions F2 and F3 of *Vitex rotundifolia* in MCF-7 cell line.](image-url)
The results showed that phenolic, flavonoid, and terpenoid compounds are present in the active fractions (Fig. 6A). HPTLC showed the presence of selected standard bioactive compounds in V. rotundifolia fractions. HPTLC silica gel 60 F254 plates used for HPTLC analysis was more sensitive than conventional TLC plates and had high separation ability. The solvent system of hexane:ethyl acetate (1:1 v/v) was used to separate the sample constituents and standard compounds. Phenolic acids namely S1, S2, S3, and S4 used as standard compounds for the HPTLC analysis. The identification of standard compounds in fractions F1, F2, F3, and F5 detected by comparing the retention factor (Rf) value of the compounds fractions with the Rf value of standard compounds. The standard compounds S4, S1, S3, and S2 were separated as four bands at Rf 0.12, 0.18, 0.23, and 0.52, respectively. The results obtained by 2D and 3D HPTLC densitogram at 254 nm demonstrated that F1 showed phenolic acid 4-hydroxybenzaldehyde (Fig. 6B-6D). The F2 represent standard compounds S2 and S3 acid. The HPTLC analysis also confirmed the presence of standard compounds S1 and S2 in F3. The F5 may contain S1, S2, and S4. The peak intensity of S1 and S2 in active fractions was determined in the order S1, F3 > F5 > F2 and for S2, F3 > F2 > F1 > F5. Moreover, S3 was showed in F2 and S4 was detected in F3 and F5 with intensity F5 > F3.

DISCUSSION

The present study was carried out to determine the cytotoxic effects and mechanism of apoptosis induced by fractions of V. rotundifolia in MCF-7 cell line. The results obtained demonstrate that among seven fractions, F1, F2, F3, and F5 presented cytotoxic effects in MCF-7 cell line with IC_{50} < 30 µg/mL after 72 h of treatment. The relative potential of cytotoxicity of the active fractions on MCF-7 cell line was found to be F3 > F2 > F5 > F1. The results obtained by annexin V-FITC staining method and TUNEL assay indicated that all four active fractions of V. rotundifolia induced early and late (DNA fragmentation) apoptosis in MCF-7 cell line at 36 h. Apoptosis is a fundamental regulatory mechanism of normal cells, any dysregulation in apoptosis could trigger the uncontrol multiplication of cells (21). The V. rotundifolia fruit extract suppress the cell viability and induce apoptotic cell death in human colorectal cell lines, HCT116 and SW480 (22). Lignan compounds from V. negundo have also been reported to inhibit the proliferation of breast cancer cell line MDA-MB-435 and liver cancer cell line SMMC-7721 via apoptosis (23). Caspases and Bcl-2 families of proteins play an essential role in the initiation and execution of apoptosis (24). Caspase-9 is associated with the activation of the mitochondrial pathway of apoptosis while caspase-8 plays its role in apoptosis by activating the extrinsic pathway of apoptosis (25). The results obtained suggest that apoptotic cell death induced by fractions F2 and F3 in MCF-7 cells might involve the activation of both caspase-8 and -9. Caspase-8 and -9 mediated cell death has described earlier, V. agnus-castus fruit extract induced apoptosis in human gastric signet ring carcinoma KATO-III cells by the activation of caspases-8 and -9 (26). These results were in parallel with the previously reported study; the MCF-7 cells are deficient in caspases-3 and -7 were involved in the induction of apoptosis by seeds of Centratherum anthelminticum (L.) determined by western blot analysis and Caspase Glo™ assay (27). Similarly, the absence of caspase-3 in MCF-7 cells and involvement of other effector caspases such as caspase-6 or -7 in mediating DNA fragmentation induced apoptotic cell death have been reported previously (28).

Furthermore, western blot analysis suggests that the expression of Bax significantly increased while the expression of Bcl-2 protein significantly decreased in MCF-7 cell line with the fractions F2 and F3 of V. rotundifolia for 24 h. It was well established that the intrinsic pathway of apoptosis is mediated by proapoptotic member of Bcl-2 family protein (Bax) insertion into the mitochondrial membrane (29). Bax regulates mitochondrial function and releases cytochrome c (30). On the contrary, the anti-apoptotic Bcl-2 protein prevents the release of cytochrome c and restrains apoptosis (31). Therefore, based on the results from the present study,
similar mechanisms of action was responsible for inducing apoptosis in MCF-7 cells.

Similarly, Vitexicarpin isolated from *V. trifolia* induced apoptosis in K562 cells by downregulating the ratio of Bcl-2/Bax (32). Furthermore, the results obtained by TLC and HPTLC profiling suggest that phenolic, flavonoid, and terpenoid compounds might be present in the active fractions. The active fractions possess standard compounds S2, S3, S1, and S4. The standard compounds used in the present study have been previously reported to possess anticancer activities such as ferulic acid and luteolin (33,34). Therefore, the presence of these standard compounds in the fractions might result in their cytotoxicity activity.

Fig. 6. (A) TLC profile of active fractions of *Vitex rotundifolia* visualized under UV light and derivatized with anisaldehyde reagent, (B) HPTLC profiling of active fractions of *Vitex rotundifolia* and standard compounds visualized under UV 254 nm, (C) 3D HPTLC densitogram of active fractions of *Vitex rotundifolia* and standard compounds at UV-254 nm, (D) 2D HPTLC densitogram of active fractions of *Vitex rotundifolia* and standard compounds at UV-254 nm. F1, Hexane; F2, hexane:dichloromethane; F3, dichloromethane; F5, ethyl acetate; S1, ferulic acid; S2, 4-hydroxybenzaldehyde; S3, 4-hydroxybenzoic acid; S4, luteolin; HPTLC, high performance thin layer chromatography.
CONCLUSION

The fractions prepared from *V. rotundifolia* exert cytotoxicity in MCF-7 cell line. The mode of cytotoxicity mainly due to apoptosis, the translocation of phosphatidylserine to the outer surface of cell membrane and DNA fragmentation in treated cells confirms the early and late apoptosis. Fractions induced apoptosis may involve both the extrinsic and intrinsic pathway of apoptosis. Accordingly, *V. rotundifolia* could have potential as an anticancer agent with more study in understanding the role of their active compounds in cell death mechanism.

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REFERENCES

1. Weir HK, Anderson RN, Coleman King SM, Soman A, Thompson TD, Hong Y, et al. Heart disease and cancer deaths-trends and projections in the United States, 1969-2020. Prev Chron Dis. 2016;13:E157.
2. Garbi MI, Osman EE, Kabbashi AS, Saleh MS, Yusof YS, Mahmoud SA, et al. Cytotoxicity of *Vitex trifolia* leaf extracts on MCF-7 and Vero cell lines. J Sci Innov Res. 2015;4(2):89-93.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016;66(1):7-30.
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019;69(1):7-34.
5. Leon Guerrero RT, Novotny R, Wilkens LR, Chong M, White KK, Shvetsov YB, et al. Risk factors for breast cancer in the breast cancer risk model study of Guam and Saipan. Cancer Epidemiol. 2017;50(Pt B):221-233.
6. Desai AG, Qazi GN, Ganju RK, El-Tamer M, Singh J, Saxena AK, et al. Medicinal plants and cancer chemoprevention. Curr Drug Metab. 2008;9(7):581-591.
7. Wachtel-Galor S, Benzie IFF. Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs. In: Benzie IFF, Wachtel-Galor S, editors. Herbal Medicine: Biomolecular and clinical aspects. 2nd ed. Boca Raton (FL): CRC Press/Taylor and Francis; 2011. pp: 1-10.
8. Valli M, Privatto M, Danello A, Castro-Gamboa Ian, Silva DHS, Cavalheiro AJ, et al. Tropical biodiversity: has it been a potential source of secondary metabolites useful for medicinal chemistry? Quim Nova. 2012;35(11):2278-2287.
9. Meena AK, Niranjani US, Rao MM, Padhi MM, Babu R. A review of the important chemical constituents and medicinal uses of *Vitex* genus. Asian J Tradit Med. 2011;6 (2):54-60.
10. Ono M, Yanaka T, Yamamoto M, Ito Y, Nohara T. New diterpenes and nortriterpenes from the fruits of *Vitex rotundifolia*. J Nat Prod. 2002;65(4):537-541.
11. Hu Y, Zhang Q, Xin H, Qin LP, Lu BR, Rahman K, et al. Association between chemical and genetic variation of *Vitex rotundifolia* populations from different locations in China: its implication for quality control of medicinal plants. Biomed Chromatogr. 2007;21(9):967-975.
12. Kim DK. Antioxidative constituents from the twigs of *Vitex rotundifolia*. Biomol Ther. 2009;17(4):412-417.
13. Yoshiokaa T, Inokuchib T, Fujiokac S, Kimurab Y. Phenolic compounds and flavonoids as plant growth regulators from fruit and leaf of *Vitex rotundifolia*. Z Naturforsch C. 2004;59(7-8):509-514.
14. Iwashina T, Setoguchih H, Kitajimaj J. Flavonoids from the leaves of *Vitex rotundifolia* (Verbenaceae), and their qualitative and quantitative comparison between coastal and inland populations. Bull Natl Mus Nat Sci Ser B. 2011;37(2):87-94.
15. Ono M, Yamamoto M, Masuoka C, Ito Y, Yamashita M, Nohara T. Diterpenes from the fruits of *Vitex rotundifolia*. J Nat Prod. 1999;62(11):1532-1537.
16. Hudaya T, Gul-e-Saba, Taib M, Ismail N, Mohammad TST. Methanol extracts of four selected marine sponges induce apoptosis in human breast cancer cell line, MCF-7. Int J Res Pharm Sci. 2017;8(4):667-675.
17. Gul-e-Saba, Islamiah M, Ismail N, Mohammad H, Sung YY, Muhammad TST. Induction of apoptosis by *Aaptos sp.*, fractions in human breast cancer line, MSF-7. Int J Res Pharm Sci. 2017;9(2):328-337.
18. Vijayarathna S, Sasidharan S. Cytoxicity of methanol extracts of *Elaeis guineensis* on MCF-7 and Vero cell lines. Asian Pac J Trop Biomed. 2012;2(10):826-829.
19. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ. 1998;5(7):551-562.
20. Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem. 1997;45(7):923-934.
21. Jan R, Chaudhry GS. Understanding apoptosis and apoptotic pathway targeted cancer therapeutics. Adv Pharm Bull. 2019. In press.
22. Song HM, Park GH, Park SB, Kim HS, Son HJ, Um Y, et al. Vitex rotundifolia fruit suppresses the proliferation of human colorectal cancer cells through down-regulation of cyclin D1 and CDK4 via proteasomal-dependent degradation and transcriptional inhibition. Am J Chin Med. 2018;46(1):191-207.
23. Xin H, Kong Y, Wang Y, Zhou Y, Zhu Y, Li D, et al. Lignans extracted from Vitex negundo possess cytotoxic activity by G2/M phase cell cycle arrest and apoptosis induction. Phytomedicine. 2013;20(7):640-647.
24. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 2013;14:32-40.
25. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell. 2002;9(3):459-470.
26. Ohyama K, Akaike T, Imai M, Toyoda H, Hirobe C, Bessho T. Human gastric signet ring carcinoma (KATO-III) cell apoptosis induced by Vitex agnus-castus fruit extract through intracellular oxidative stress. Int J Biochem Cell Biol. 2005;37(7):1496-1510.
27. Looi CY, Arya A, Cheah FK, Muharram B, Leong KH, Mohamad K, et al. Induction of apoptosis in human breast cancer cells via caspase pathway by vernodalin isolated from Centratherum anthelminticum (L.) seeds. PLoS One. 2013;8(2):e56643.
28. Mc Gee MM, Hyland E, Campiani G, Ramunno A, Nacci V, Zisterer DM. Caspase-3 is not essential for DNA fragmentation in MCF-7 cells during apoptosis induced by the pyrrolo-1,5-benzoxazepine, PBOX-6. FEBS Lett. 2002;515(1-3):66-70.
29. Kim R. Recent advances in understanding the cell death pathways activated by anticancer therapy. Cancer. 2005;103(8):1551-1560.
30. Wang Q, Zhang L, Yuan X, Ou Y, Zhu X, Cheng Z, et al. The relationship between the Bcl-2/Bax proteins and the mitochondria-mediated apoptosis pathway in the differentiation of adipose-derived stromal cells into neurons. PLoS One. 2016;11(10):e0163327.
31. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin. 2005;55(3):178-194.
32. Wang HY, Cai B, Cui CB, Zhang DY, Yang BF. Vitexicarpin, a flavonoid from Vitex trifolia L., induces apoptosis in K562 cells via mitochondria-controlled apoptotic pathway. Yao Xue Xue Bao. 2005;40(1):27-31.
33. Peng CC, Chyau CC, Wang HE, Chang CH, Chen KC, Kuang-Yu Chou, et al. Cytotoxicity of ferulic acid on T24 cell line differentiated by different microenvironments. Biomed Res Int. 2013;2013. Article ID: 579859.
34. Lin Y, Shi R, Wang X, Shen HM. Luteolin, a flavonoid with potentials for cancer prevention and therapy. Curr Cancer Drug Targets. 2008;8(7):634-646.