

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

Gul-e-Saba Chaudhry\*, Rehmat Jan, Habsah Mohamad,  
and Tengku Sifzizul Tengku Muhammad\*

Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia.

### 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).

#### Access this article online



Website: <http://rps.mui.ac.ir>

DOI: 10.4103/1735-5362.258496

\*Corresponding authors:

G. Chaudhry, Tel: +609-6683810, Fax: +609-6684944

Email: [sababiochem@gmail.com](mailto:sababiochem@gmail.com)

TST. Muhammad, Tel: +609-6685184, Fax: +609-6684944

Email: [sifzizul@umt.edu.my](mailto:sifzizul@umt.edu.my)

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<sup>®</sup> 96 AQueous non-radioactive cell proliferation assay (MTS assay) kit, annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and DeadEnd<sup>™</sup> 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 Permula, 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<sup>™</sup> automated cell counter and cultured onto 96-well plates at a cell density of  $6 \times 10^3$  cells/well and incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub> 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  $\mu$ L of MTS reagent was added at each incubation period for 3 h at 37 °C. 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 ( $IC_{50}$ ) 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  $\mu$ g/mL) used as positive control. After treatment, cells were washed with 1 $\times$  binding buffer and subsequently incubated with binding buffer (5  $\mu$ L of annexin V-FITC and 10  $\mu$ L 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  $IC_{50}$  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 °C. 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  $\mu$ L of recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer was added into the cells and incubated for 60 min at 37 °C. 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 $\times$  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  $\mu$ L of PI solution (diluted to 1  $\mu$ g/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 °C. 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  $IC_{50}$  (72 h) throughout 48 h at 37 °C in the presence of 5% (v/v) CO<sub>2</sub>. Moreover, caspase-8, -9 and -3/7 activity was determined. Successively, 100  $\mu$ L of Caspase-Glo™ reagent added to each well-containing 100  $\mu$ L 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<sub>254</sub> 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 F<sub>254</sub> 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 (CamagMuttentz, 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.

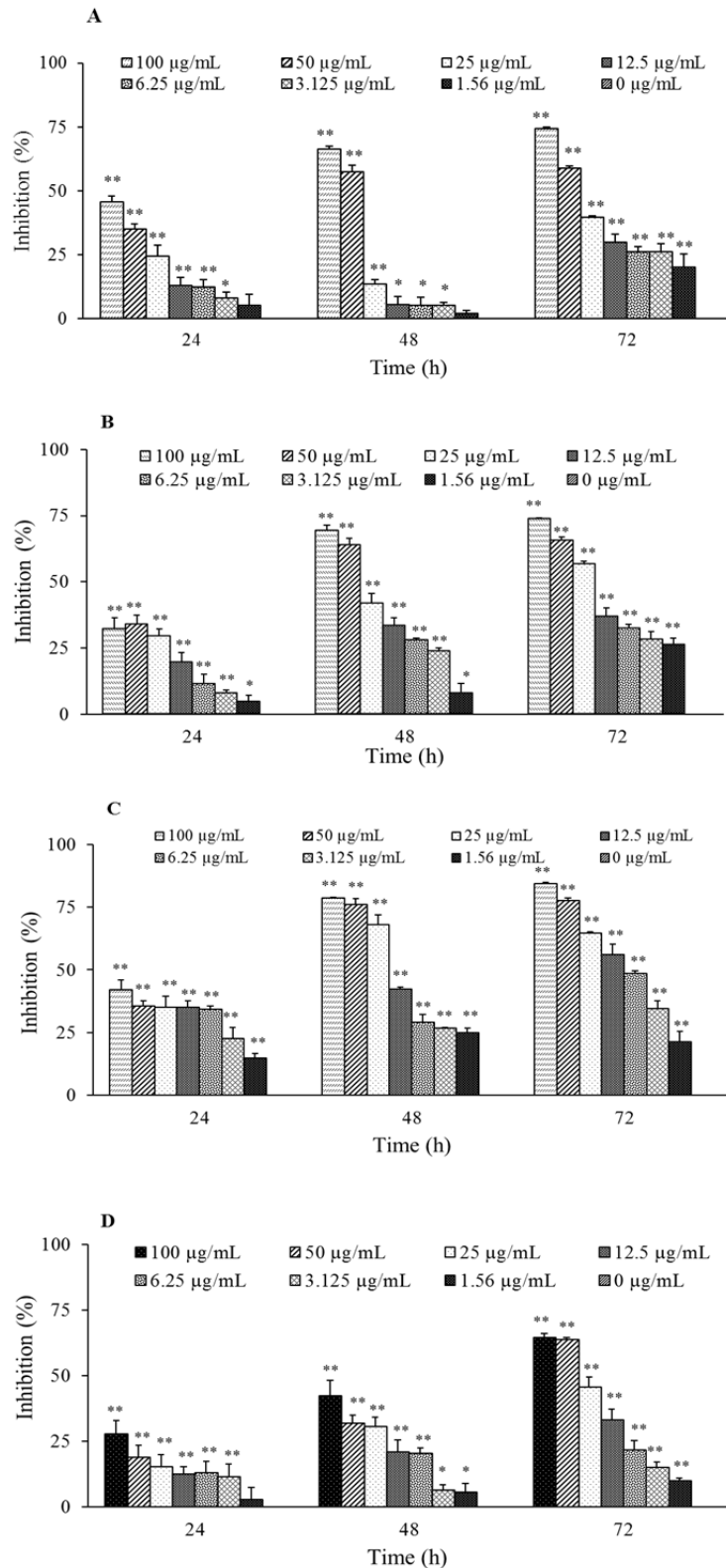
#### **Statistical analysis**

Cytotoxicity experiments performed in triplicates and results presented as percentage growth inhibition. IC<sub>50</sub> 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**

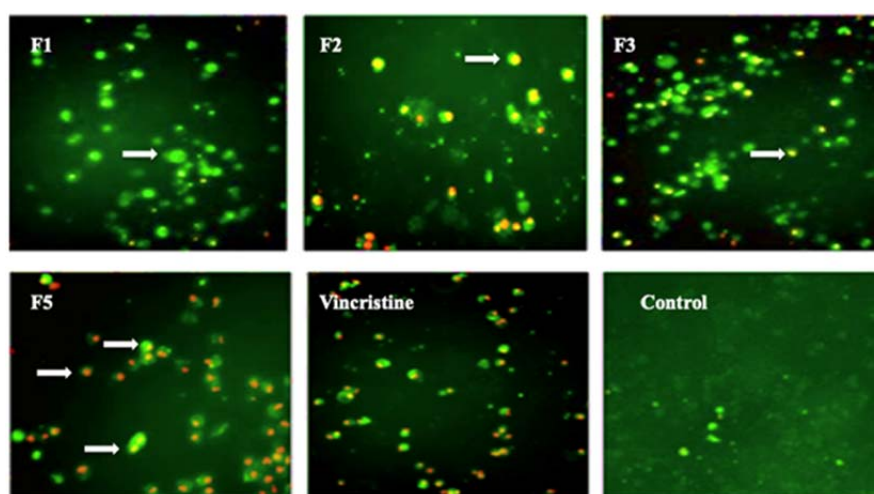
In this study, the cytotoxic effects of the crude methanol extract and fractions prepared from *V. rotundifolia* on human breast cancer cell line MCF-7 investigated by using the MTS assay. The crude methanol extract of *V. rotundifolia* exhibited cytotoxic activity against MCF-7 cell line in a concentration-dependent manner. In this study, IC<sub>50</sub> was determined. At 72 h incubation, the crude methanol extract was able to inhibit 50% of MCF-7 cells proliferation at the IC<sub>50</sub> value 63.09 µg/mL. On treatment with F1, the MCF-7 cell line showed an increase in cell death rate with an IC<sub>50</sub> value 29.51 µg/mL. Similarly, F2 exhibited significant activity with an IC<sub>50</sub> value 19.95 µg/mL. Interestingly, F3 exhibited considerable cytotoxicity in MCF-7 cell line with a lower IC<sub>50</sub> value of 6.30 µg/mL than F2 and F1 after 72 h treatment exposure. Furthermore, F5 exhibited the cytotoxic effect with an IC<sub>50</sub> value of 26.30 µg/mL comparable to F1, and F4 exhibited activity with IC<sub>50</sub> value 33.11 µg/mL. However, F6 and F7 exhibited an IC<sub>50</sub> 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.



**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, 12.50, 6.25, 3.125, 1.56, and 0 µg/mL after 24, 48, and 72 h incubation. Each value represented mean  $\pm$  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<sub>50</sub> values of *Vitex rotundifolia* methanol extract and fractions, n= 03.

| Samples     | IC <sub>50</sub> (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        | 26.30 ± 1.66 |
| 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<sub>50</sub> value of 0.625 µg/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<sub>50</sub> value < 30 µg/mL after 72 h) (18). Table 1 shows the IC<sub>50</sub> values of *V. rotundifolia* 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<sub>50</sub> (72 h) successfully induce the translocation of phosphatidylserine (annexin V<sup>+</sup>) 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<sup>+</sup>/PI<sup>+</sup>) 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<sup>-</sup> control) MCF-7 cells were not stained by both annexin V and PI (annexin V<sup>-</sup>/PI<sup>-</sup>), 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

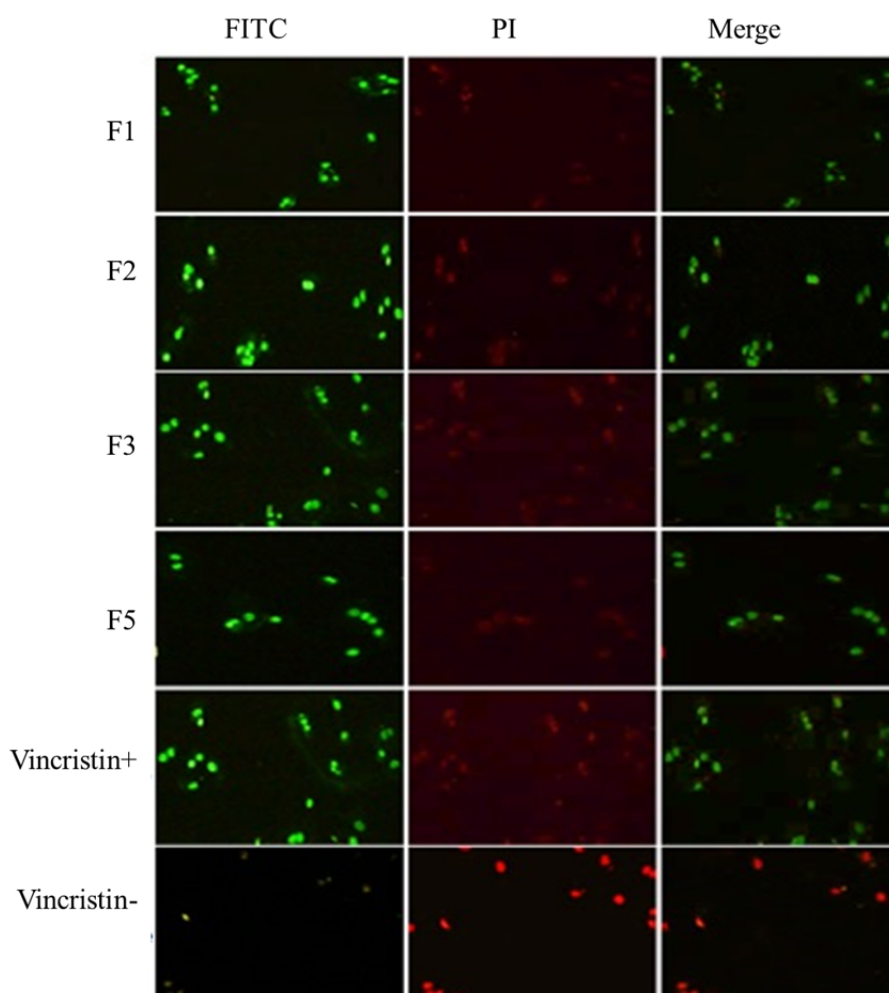
cytotoxicity effects on MCF-7 cell line via early apoptosis.

**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<sub>50</sub> (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.

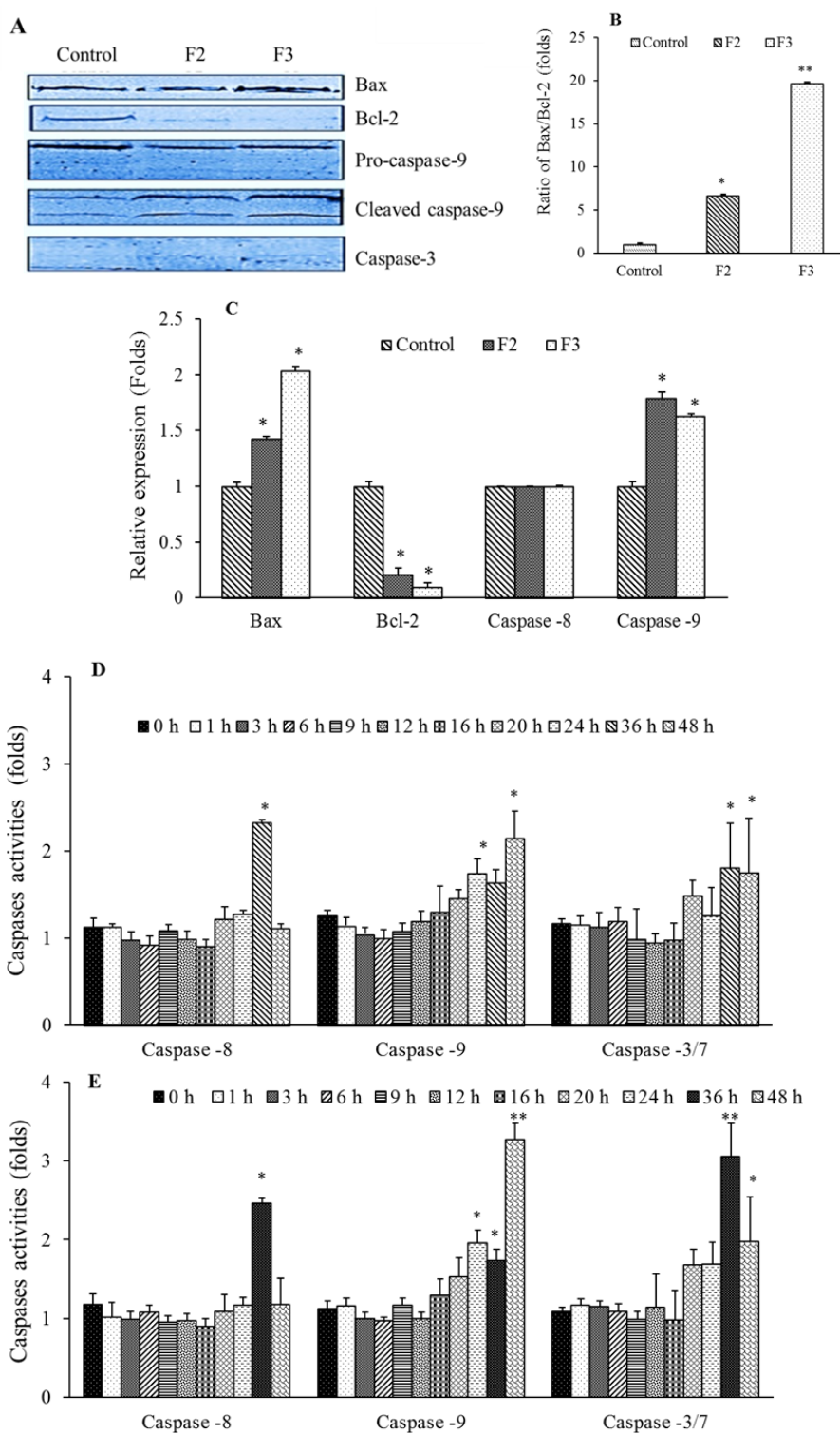
**Mechanism of apoptotic pathway activation in MCF-7 cell line**

To find out the potential mechanism of fractions induced apoptosis caspase Glo<sup>TM</sup> 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<sup>TM</sup> 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  $\pm$  SD of six replicates. \* And \*\* indicate significant differences ( $P < 0.05$  and  $P < 0.01$ , respectively) in comparison with negative control (0 h).

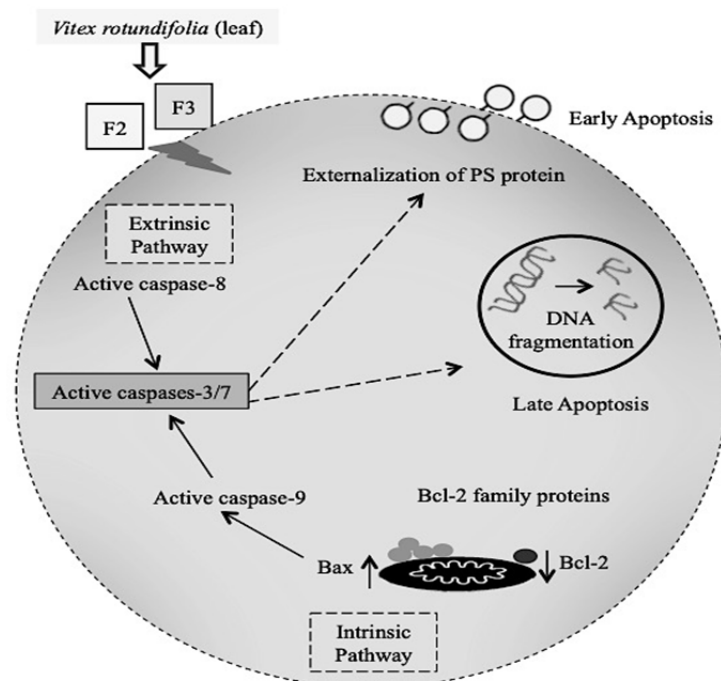


The findings obtained by western blot analysis demonstrate that fractions F2 and F3 induced the activation of Bax in MCF-7 cells as compared to control cells throughout 24 h. While the reduction in activation of Bcl-2 observed in MCF-7 cells treated with fractions F2 and F3 at 24 h. The ratio of Bax/Bcl-2 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.

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 ( $R_f$ ) value of the compounds fractions with the  $R_f$  value of standard compounds. The standard compounds S4, S1, S3, and S2 were separated as four bands at  $R_f$  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  $IC_{50} < 30 \mu\text{g/mL}$  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

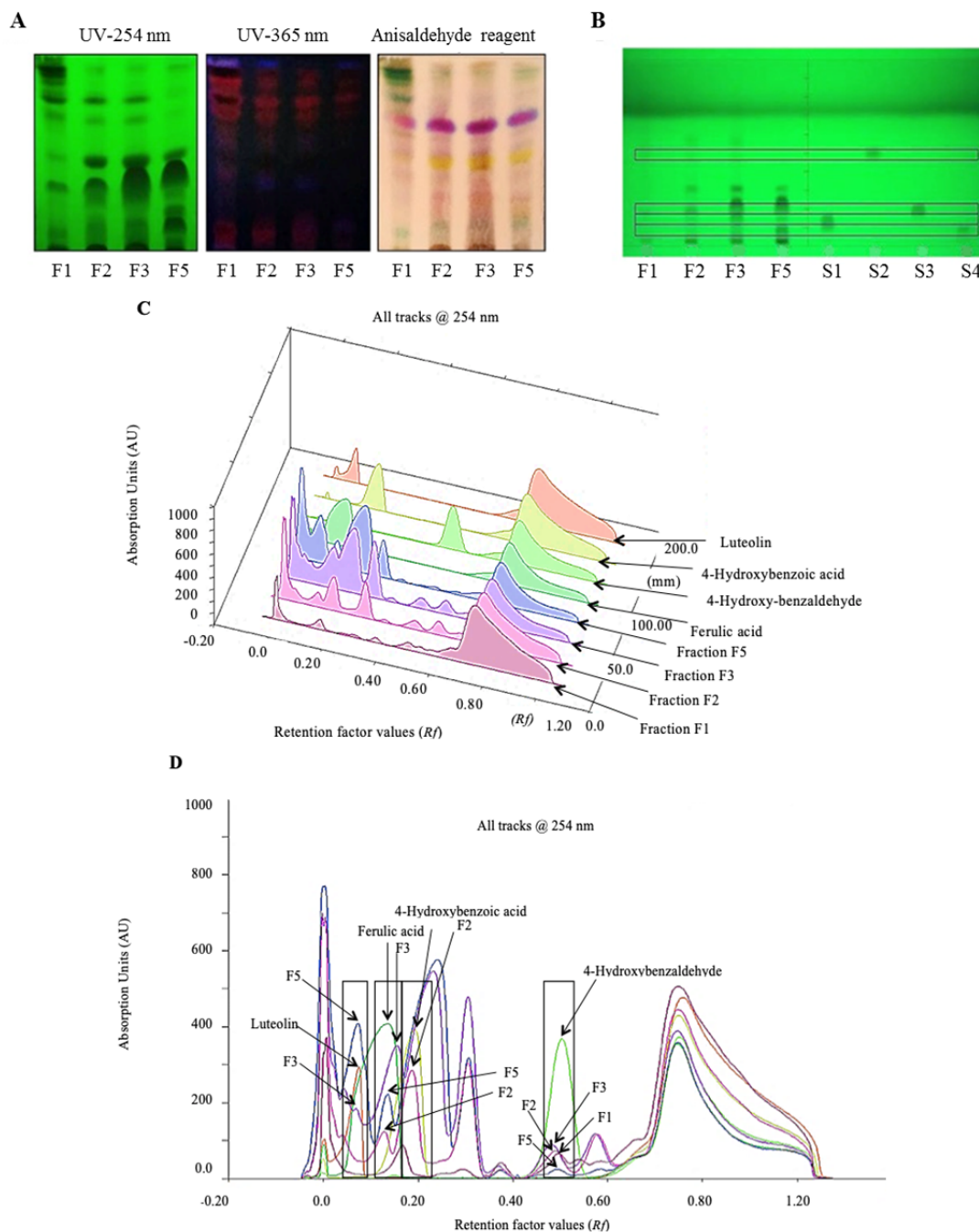
and late (DNA fragmentation) apoptosis in MCF-7 cell line at 36 h. Apoptosis is a fundamental regulatory mechanism of normal cells, any dysregulation in apoptosis could trigger the uncontrol multiplication of cells (21). The *V. rotundifolia* fruit extract suppress the cell viability and induce apoptotic 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 cells treated 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.

## ACKNOWLEDGMENTS

The authors wish to thank Universiti Malaysia Terengganu for the allocation provided to the Institute of Marine Biotechnology.

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