Chong et al. BMC Complementary and Alternative Medicine 2012, 12:134
http://www.biomedcentral.com/1472-6882/12/134

BMC Complementary & Alternative Medicine

RESEARCH ARTICLE Open Access

In vitro evaluation of *Pandanus amaryllifolius* ethanol extract for induction of cell death on non-hormone dependent human breast adenocarcinoma MDA-MB-231 cell via apoptosis

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Abstract

Background: Our previous study had shown that *P. amaryllifolius* was able to selectively inhibit cell proliferation of hormone independent breast cancer cell line MDA-MB-231. To understand the mode of killing and mechanism of action for *P. amaryllifolius*, the ethanol extract was evaluated for the interruption of cell cycle progression, PS externalization, DNA fragmentation and expression of anti/pro-apoptotic related protein.

Results: Cell cycle progression analysis, Annexin V and Tunel assays suggested that IC_{50} of *P. amaryllifolius* ethanol extract induced G0/G1 cell cycle arrest, PS externalization and DNA fragmentation. On the other hand, ELISA for cytochrome c, caspase-3/7, 8 and 9 indicated that apoptosis was contributed by mitochondrial cytochrome c release via induction of caspase 3/7, 9, and p53 was associated with the suppression of XIAP in *P. amaryllifolius* treated MDA-MB-231 cells.

Conclusion: Our findings suggest that *P. amaryllifolius* ethanol extract induced apoptosis on hormone independent breast cancer cell line MDA-MB-231.

Keywords: *Pandanus amaryllifolius*, MDA-MB-231, Apoptosis, Cytochrome c, Caspase

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Background

The rising prevalence of cancers and health care costs are among propelling factors to researchers and the public for the search of plant based systems which exert health benefits towards reduction of cancer risks and modification of tumor behavior. Plants have been the basis for nearly all medicinal therapy since ancient times until the development of synthetic drugs in the 19th century [1]. Ample archaeological evidence indicated that medicinal plants were regularly employed by people in ancient cultures for biomedically curative and psychotherapeutic purposes [2]. Plants have played important roles in cancer research, and indeed, most new applications of phytochemicals over the last half century have been for combating cancer [3]. Plant nutrients or phytochemicals have potential as chemopreventive agents, since most of these products are powerful antioxidants [4], others are phenolic in nature and the remainder includes reactive groups that confer cancer protective properties [5].

Few plant species that provide medicinal herbs have been scientifically evaluated for their possible medicinal application [6,7]. A systematic search for anticancer plants began in the middle of the 20th century through the applications of appropriate biological screening assays. The chemopreventive properties of plants extracts are often investigated via screening against a panel of human cancer cell lines [8]. *Pandanus amaryllifolius* or commonly known as “daun pandan” or “pandan wangi” is from the screw pine family. It is a common

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culinary plant in South-east Asia used as an additive to give a pleasant fresh hay like aroma [9]. Besides its aromatic value, *P. amaryllifolius* has a long history of ethnopharmacological uses in eleviating minor ailments such as fever, headache, sore throat and toothache [10,11]. Ethnomedical information reveals that the oil of *Pandanus* leaves is used extensively for the treatment of epilepsy and leprosy. This oil described as purgative, stimulant and brings antispasmodic relief for rheumatism conditions. In Taiwan, *P. amaryllifolius* is used by local communities for its diuretic and cardiotonic properties [10] and to cure thyroid problems and fever [11]. Previously, methanol extracts of *P. amaryllifolius* revealed strong inhibitory rate toward tumor promoter 12-*O*-hexadecanoylphorbol-13- acetate (HPA)-induced Epstein-Barr virus (EBV) activation in Raji cells [12]. Lately, *P. amaryllifolius* extract was found to display selective antiproliferative activity against non hormone dependent breast cancer cells [13]. Therefore, an investigation of the chemopreventive activities of *P. amaryllifolius* towards induction of apoptosis was carried out.

Methods

Preparation of the ethanol extract of *Pandanus amaryllifolius*

Leaves of *Pandanus amaryllifolius* were collected from Serdang, Selangor. The herbarium voucher specimen was identified by the morphology and fragrance of the leaves by Mr. Lim Chung Lu from the Forestry Division of the Forest Research institute of Malaysia (FRIM Kepong Selangor, Malaysia). The voucher number of *P. amaryllifolius* is ATCL 0011. The leaves of *P. amaryllifolius* were then thoroughly rinsed with tap and distilled water, and were air-dried at room temperature for 2 weeks. The plant samples were later homogenized and ground to a fine powder and soaked for 72 *hr* in absolute ethanol for exhaustive ethanol extraction. The extracts were collected by filtration through Whatmann paper No 1 and the residues were then re-soaked with a fresh portion of ethanol twice before being subjected to evaporation under reduced pressure in a rotary evaporator. The dried residues of the plant extracts were resuspended in DMSO (Sigma, USA) for use in biological assays.

Cell line and cell treatment

Non-hormone dependent breast adenocarcinoma MDA-MB-231 cell line (Cat. No. HTB-26) were obtained from the American Type Culture Collection (ATCC, USA) and cultured in RPMI 1640 culture medium (PAA, USA) supplemented with 10% fetal calf serum (PAA, USA), and 1% of penicillin streptomycin (PAA, USA) at 37°C, 5% CO_2. Adherent cells at 80% confluency were harvested using Accutase (PAA, USA) for analysis.

MDA-MB-231 cells were grown to the exponential phase and treated with *P. amaryllifolius* ethanol extract at a concentration that inhibited 50% of cell growth in MTT assay (IC_{50} = 90 μg/mL) [13] at different time points as stated in the following assays. Then, 1 million of untreated control and *P. amaryllifolius* ethanol extract treated MDA-MB-231 cells were harvested by Accutase (PAA, USA), washed with cold phosphate buffer saline (PBS) and subjected to the following test.

Flow cytometry cell cycle progression quantification

After 24, 48 or 72 *hr* of incubation, the pelleted untreated and *P. amaryllifolius* ethanol extract treated MDA-MB-231 cells were fixed in 80% ethanol at −20°C for overnight. After that, the samples were washed twice with 1 ml of PBS, resuspended in 100 μl of RNAse A (200 μg/ml) and incubated for 30 minutes. Then, 100 μl of propidium iodide (1 mg/ml) was added to the cells and incubated for another 30 minutes at room temperature. Flow cytometry was performed with a FACS Caliber (BD Biosciences, USA).

Flow cytometry Annexin V-FITC/ Propidium Iodide analysis

Both untreated and MDA-MB-231 cells treated with *P. amaryllifolius* extract for 48 *hr* at a concentration of 1×10^6 cells per ml were collected. The cell pellet was then resuspended in binding buffer and stained with 5 μl of both Annexin V FIT-C and propidium iodide using the BD Pharmingen Apoptosis Detection Kit I (BD Biosciences, USA) and incubated for 15 minutes. Flow cytometry was then performed with a FACS 440 (Becton-Dickinson, Mountain View, CA) using a 488 nm argon ion laser. The lower left quadrant indicated viable cell, the lower right early apoptosis and the upper right late apoptosis population.

Flow cytometry TUNEL DNA fragmentation analysis

DNA fragmentation of extract treated MDA-MB-231 cell was tested using TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) assay (BD Biosciences, USA). Briefly, after 48 and 72 *hr* of incubation, untreated and *P. amaryllifolius* ethanol extract treated MDA-MB-231 cells were fixed with 1% (w/v) paraformaldehyde in PBS for one hour followed by incubating with 70% (v/v) ice cold ethanol at −20°C for 30 mins. After that, samples were washed with ice cold PBS and added with DNA labeling reagent one hour before adding BrdU (Bromodeoxyuridine) antibody conjugated with FITC (BD Biosciences, USA). Finally, the samples were subjected to flow cytometry analysis using FACS Caliber (BD Biosciences, USA).

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ELISA cytochrome c release and activation of caspases 3/7, 8 and 9 detection

The release of cytochrome c to cytosol (Bender MedSystems, Austria) and activation of caspase 3/7, 8 and 9 (Promega, Madison, WI) were tested using Enzyme Link Immunosorbant Assay (ELISA). Release of cytochrome c was tested on the control and extract treated MDA-MB-231 cell after 12, 18 and 24 *hr* of incubation. On the other hand, activation of caspase 3/7 was tested at 24, 48 and 72 *hr* while caspase 8 and 9 at 3, 6, 9, 12, 15, 18, 24 and 36 *hr* incubation times. Briefly, both untreated and MDA-MB-231 cells treated with *P. amaryllifolius* ethanol extract at IC_{50} concentration at the concentration of 10^5 were lysed by addition of Triton-X 100 lysis buffer from each kit. The cytochrome c, caspases 3/7, 8 or 9 present in the samples were bound to antibodies adsorbed to the surface of the microwells. Fifty microliter biotin-conjugated antihuman cytochrome c antibody was added to all wells prior incubation for 2 *hr* at room temperature. Unbound biotin conjugated antihuman cytochrome c antibody was removed during the washing steps. Streptavidin-HRP was added to bind the biotin-conjugated anti-human cytochrome c, caspases 3/7, 8 or 9 antibodies and further incubated for one hour. Unbound Streptavidin- HRP was removed using wash buffer and 100μl substrate solution reactive with HRP was added to all wells. Coloured product were formed in proportion to the amount of human cytochrome c present in the cells. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. The relative concentrations of cytochrome c, caspases 8 and 9 were obtained by comparisons to their plotted respective standard curves while the activity of caspases 3/7 for each respective time point was expressed as the fold change obtained using the following formula:

$$\text{Fold change of caspase 3/7} = \times (\text{Absorbance of treatment} - \text{Absorbance of control})/\text{Absorbance of control}$$

which divided the absorbance of extract treated cell to absorbance of untreated control cell for each time point.

Flow cytometry apoptosis and cell cycle regulator protein quantification

The collected untreated control and extract treated cells after 24 and 48 hr of incubation were fixed and permeabilized using cytofix-cytoperm solution (Becton Dickinson, CA, USA) for flow cytometry intracellular protein analysis. After that, the cells were stained with either p53 (Santa Cruz, CA) or XIAP (Cat no ab26148, Abcam, UK) primary antibodies. Then, all the cells were washed and stained with 5 μg/10 μL of FITC goat anti-mouse Ig (BD Biosciences, USA) secondary antibody and analysed with FACS Caliber (BD Biosciences, USA). The results were expressed as population of cell (%) expressing p53 and XIAP for each time point respectively.

Statistical analysis

All experiments were assayed in triplicates (n = 3). Data were expressed as means ± S.D. All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 15. Treatment effects were determined using one-way ANOVA post hoc analysis. A value of $p < 0.05$ was considered significant unless indicated otherwise.

Results

Cell cycle progression, Annexin V/PI staining and DNA fragmentation of *P. amaryllifolius* ethanol extract treated MDA-MB-231 cells

Our previous study showed that *P. amaryllifolius* ethanol extract achieved 50% of inhibition against MDA-MB-231 at a concentration of 90 μg/mL [13]. This concentration was used in this study to confirm the mode of cell death and the related mechanism induced by this extract. Flow cytometry DNAse/PI staining was conducted to examine changes in cell cycle distributions following *P. amaryllifolius* ethanol extract exposure. After 24 hr, treatment of *P. amaryllifolius* ethanol extract resulted in an accumulation of 68% cells in G0/G1 phase (Figure 1a). This result suggested that the growth inhibitory effect of *P. amaryllifolius* ethanol extract at 24 hr was due to an arrest of DNA replication in the cell cycle. Thus, exposure of *P. amaryllifolius* ethanol extract demonstrated inhibitory effects in cell proliferation by arresting cells in G1/G0 at 24 hr.

MDA-MB-231 cells exposed to 48 hr incubation with PA extract resulted in detection of early apoptotic and secondary apoptotic populations in the Annexin V/PI study (Figure 1d). MDA-MB-231 cells exposed to 48 hr incubation with PA extract resulted in detection of 8% early apoptotic as well as 18% of secondary apoptotic populations respectively while control MDA-MB-231 cells only had <5% of total apoptotic cells (Figure 1d).

Also, treatment of *P. amaryllifolius* extract in cell cycle analysis, increased subG1 population in treated MDA-MB-231 cells indicative of apoptosis (Figure 1b, 1c). At 48 hr, 12% ($p < 0.05$) of the treated cells were present in subG1 peak compared with 3% of the cells in control medium. At 72 hr, 40% ($p < 0.05$) of treated MDA-MB-231 cells were detected as subG1 population. The accumulation of subG1 population was directly related to the decrease of cell populations in other phases of the cell cycle, indicating apoptosis.

Flow cytometry TUNEL assay was carried out to confirm induction of apoptosis by *P. amaryllifolius* ethanol extract in MDA-MB-231 cells. TUNEL-positive cells

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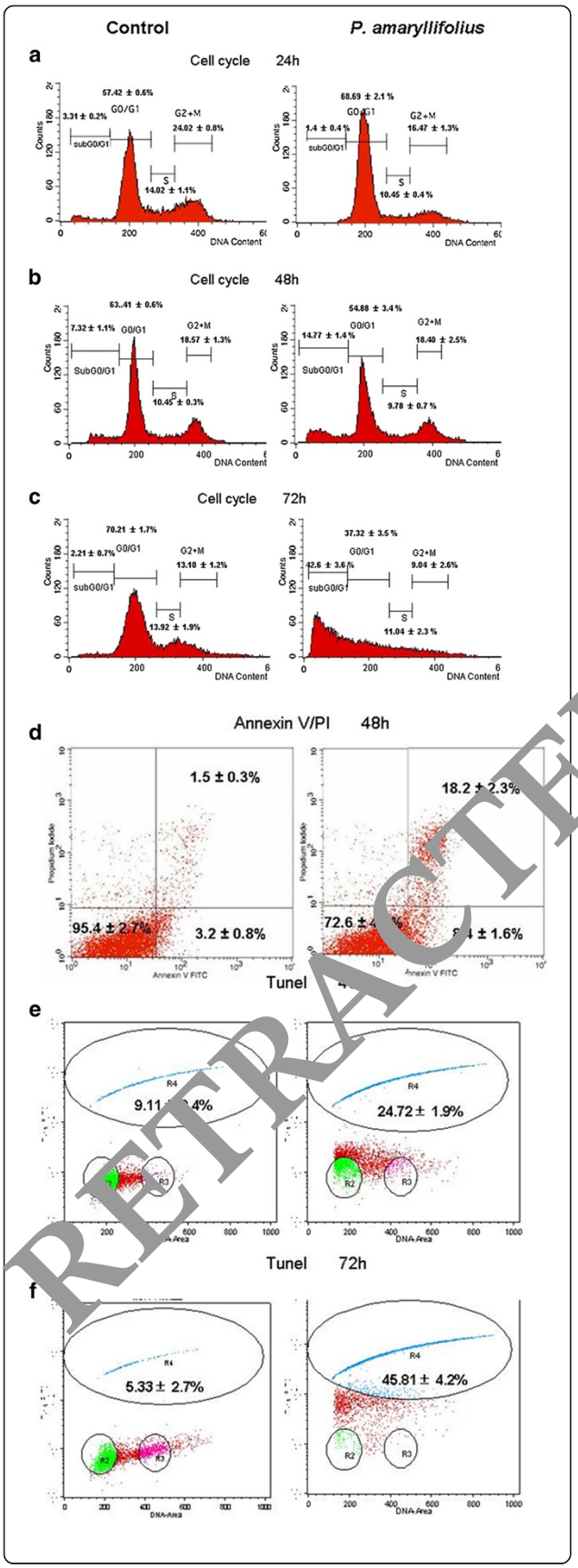


Figure 1 Effects of *P. amaryllifolius* extract on MDA-MB-231 cell DNA fragmentation. (**a**) *P. amaryllifolius* altered cell cycle distributions (%) but not sub-G0/G1 population in treated breast cancer MDA-MB-231 cells as compared to controls after 24 hr of treatment in cell cycle PI study. (**b**),(**c**) *P. amaryllifolius* induced subG0/G1 population of MDA-MB-231 cell after 48 and 72 hr treatment exposure detected by cell cycle PI staining assay. (**d**) *P. amaryllifolius* increased cell population of early and secondary apoptosis after 48 hr of treatment in AnnexinV/PI study (**e**), (**f**) *P. amaryllifolius* induced DNA fragmentation of MDA-MB-231 cell after 48 and 72 hr treatment exposure detected by TUNEL assay. Values are presented as means (n = 3) ± S.E. *significant ($p < 0.05$).

were apoptotic cells detected with DNA strand breaks which exposed free 3'-hydroxyl ends of double or single stranded DNA fragments labeled with a tracer dUTP. In TUNEL assay, after exposure of 48 hr to *P. amaryllifolius* ethanol extract, nearly 25% of treated MDA-MB-231 cells were detected as TUNEL positive (Figure 1e, 1f). This percentage was similar to the secondary apoptosis population in the Annexin V/PI study (Figure 1d) and the subG0/G1 population in the cell cycle study of MDA-MB-231 cells treated with extract for 48 hr. At 72 hr, nearly 45% ($p < 0.05$) of treated MDA-MB-231 cells were labeled as TUNEL positive cells when compared to ~5% in control cells.

Translocation of cytochrome c and activation of caspase 9 and effector caspase 3/7 in *P. amaryllifolius* ethanol extract treated MDA-MB-231 cells

Exposure of *P. amaryllifolius* ethanol extract resulted in detection of increased relative concentration of cytochrome c in the cytosol of MDA-MB-231 cells. The increase in cytochrome c level started after 12 hr exposure and the peak increase ($p < 0.05$) was observed at 36 hr after the exposure to *P. amaryllifolius* ethanol extract in MDA-MB-231 cells (Figure 2). The concentration of cytochrome c in control MDA-MB-231 cells remained rather constant throughout the study period.

Increased relative concentration of initiator caspase 8 and 9 was also observed following exposure to *P. amaryllifolius* ethanol extract of MDA-MB-231 cells. Although not statistically significant ($p > 0.05$), a steady increase in the level of both initiator caspases was observed after 6 hr exposure to *P. amaryllifolius* ethanol extract. A larger increase in the level of intrinsic pathway initiator caspase 9 was observed at 36 hr of exposure (Figure 2). Significant one fold increment ($p < 0.05$) in activity of effector caspase 3/7 was detected in MDA-MB-231 cells at 24 and at 48 hr, more than four fold increment of caspase 3/7 was noted when compared to control cells, following exposure to *P. amaryllifolius* ethanol extract (Figure 2).

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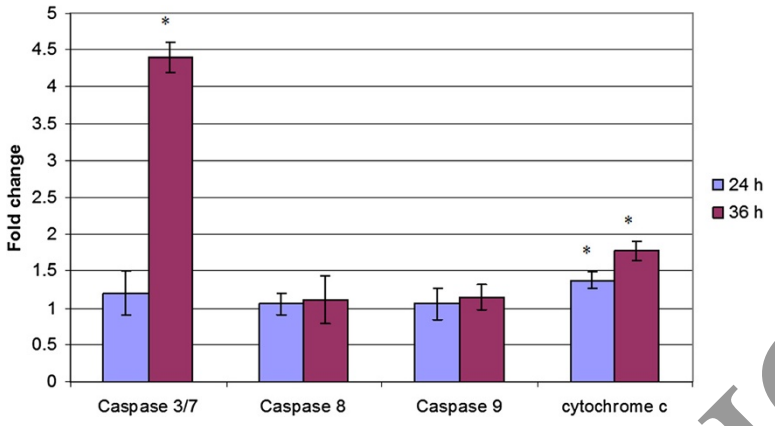


Figure 2 *Pandanus amaryllifolius* extract induced translocation of cytochrome c into the cytosol, activation of initiator caspase 8, 9 and effector caspase 3/7 (fold change) in treated MDA-MB-231 cells as compared to control cells. Values are presented as means (n = 3) ± S.E. *signified ($p < 0.05$).

Induction of apoptosis by *Pandanus amaryllifolius* in MDA-MB-231 cells involved tumour suppressor p53 protein and apoptosis inhibitor protein XIAP

A marked increase in tumour suppressor p53 protein level was also observed in *P. amaryllifolius* ethanol extract treated MDA-MB-231 cells. A significant ($p < 0.05$) increase of the population of cell expressing p53 was detected at 48 hr. Meanwhile, exposure to *P. amaryllifolius* ethanol extract resulted in down regulation of anti-apoptotic XIAP protein during 24 and 48 hr post treatment. A significant ($p < 0.05$) decrease in the level of XIAP was observed at these three incubation periods with the largest decrease of four fold noted during 24 *hr* (Figure 3).

Discussion

A previous study had reported that ethanolic extract of *P. amaryllifolius* was able to selectively inhibit 50% of cell viability on estrogen independent MDA-MB-231 but not estrogen dependent MCF-7 breast cancer cell line [13]. This study was aimed to understand the regulation on cell cycle progression, mode of cell death and related mechanism of this cytotoxic effect.

Modulations of cell cycle events and apoptosis are amongst the modes of anti-proliferation strategies displayed by many natural products. Modulation of cell cycle by *P. amaryllifolius* extract was evidenced by the changes in cell cycle distributions and accumulation of G1 population in RNAse/PI staining assayed using flow cytometery. Individual cells in a population, can be assigned to a cell cycle position based on DNA content, as defined by fluorescence intensity. The proportions of cells in the various stages of the cell cycle could therefore be, identified through the measurement of relative DNA content [14]. Based on the flow cytometry analysis, there was a reduction of cells observed in the S, G2/M phase; and accumulation of MDA-MB-231 cells at G1 phase following exposure to *P. amaryllifolius* extracts at 24 hr (Figure 1a). Accumulation of cells at G1 phase could have contributed to G1 arrest. G1 checkpoints operate in the transition of G1 to S phase that restrain further cell-cycle progression if there is incomplete DNA replication process or sustained DNA damage. The arrest in G1 allows for repair of DNA damage, thereby avoiding the propagation of genetic lesions to progeny cells [15].

Unlike 24 hr, *P. amaryllifolius* treatment for 48 and 72 hr were found to induce apoptosis on MDA-MB-231 cells. Apoptosis or programmed cell death is a distinctive form of cell death exhibiting specific morphological and biochemical characteristics [16]. Biochemical changes occurring in mitochondria and cytoplasm during apoptosis were assayed to portray different apoptotic cell features. A classical feature of apoptosis, the degradation of DNA which signifies apoptotic events at the mitochondrial level, was also detected in breast cancer cells exposed to *P. amaryllifolius* extracts. The appearance of the hypodiploid sub-G0/G1 population (Figure 1c) is a specific marker of apoptosis as necrotic death did not induce sub-G1 peak in the DNA histogram [17]. On the other hand, since strands/nicks occurrence that can be detected by Tunel assay happens at a far higher rate in apoptosis than necrosis, while translocation of membranne phosphatidylserine is an early event of apoptosis, terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) and Annexin V/PI assay were used to support the apoptosis induction by *P. amaryllifolius* in MDA-MB-231 cells. Translocation of membrane phospholipid phosphatidylserine in Annexin V/PI study (Figure 1b) and double or single stranded DNA fragments (Figure 1c) were detected indicating the induction of apoptosis by *P. amaryllifolius* extracts in MDA-MB-231 cells [18,19].

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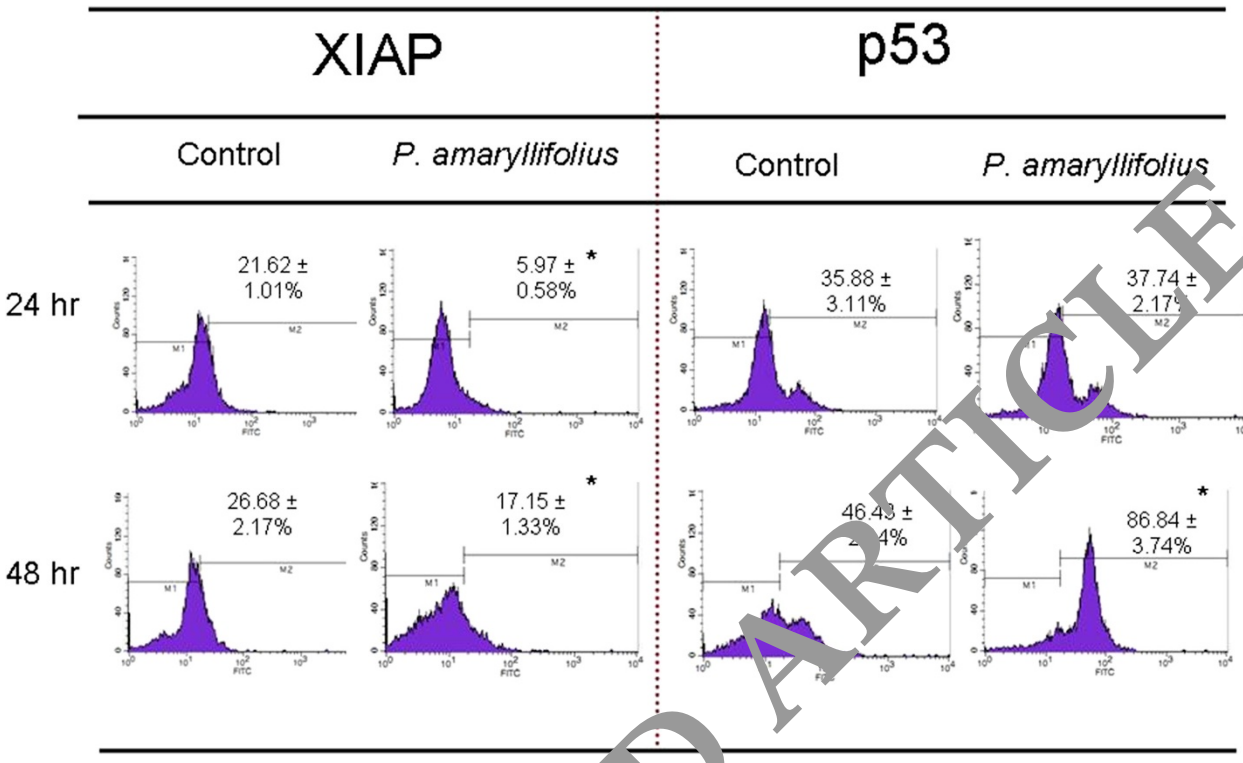


Figure 3 *P. amaryllifolius* extract induced the population of MDA-MB-231 cells that expressed tumour suppressor p53 protein while downregulated the population that expressed apoptosis inhibitor protein XIAP as compared to control cells. Values are presented as means (n = 3) ± S.E. *signified ($p < 0.05$).

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Further evidences in which *P. amaryllifolius* induced apoptosis in MDA-MB-231 cells were illustrated by the release of cellular apoptogenic factors into the cytoplasm. To determine whether cell death induced by *P. amaryllifolius* towards MDA-MB-231 cells involved intrinsic/mitochondrial activated apoptosis pathway, detection of cytochrome c was carried out. Mitochondrial activated apoptosis pathway is mediated by the mitochondrial release of cytochrome c. The onset of the mitochondrial activated apoptosis pathway is often by the occurrence of damaged DNA not sensed or repaired by checkpoint genes [20]. Additionally, caspases detection was performed to substantiate the execution of cell death. A rise in cytoplasm cytochrome c was detected alongside with increase in caspases 3/7 activity. Increased in the concentration of caspase 9, the caspase involved in the intrinsic apoptosis pathway, was also noted. The study hypotheses is that cytosolic cytochrome c induces the formation of the multisubunit apoptosome and may trigger the activation of procaspase 9. Activated caspase 9 is cleaved later to caspase 3, resulting in an amplifying proteolytic cascade. Active caspase 3 then mediates the apoptotic cascade [21]. Hence, cell death resulting from exposure of *P. amaryllifolius* towards MDA-MB-231 cells was found to involve the mitochondrial activated pathway.

It has been reported that the response of the mitochondrial activated apoptosis pathway may or may not be dependent on the presence of the nuclear transcription factor, p53 at 24 hr. However, Figure 3 also suggested that DNA damaging agents act to elevate p53 protein levels and/or p53-specific DNA binding activity in cells. The mechanism of p53 induction is however unclear. The increases in p53 protein levels caused by DNA-damaging agents could be triggered by a specific DNA lesion (or by several specific DNA lesions), by any general DNA helix distortion, or by some other signal transduction process independent of DNA template damage [22]. Induction of apoptosis by *P. amaryllifolius* in MDA-MB-231 cells was found to be transcription-dependent p53-mediated at 48 *hr*. Additionally, p53 was also reported to accumulate on the outer surface of the mitochondria which lead to alterations in inner mitochondrial transmembrane potential. Changes in the inner mitochondrial transmembrane potential facilitate cytochrome c release and procaspase 3 activations [23]. These observations indicated that p53 not only play a role in transcription but it itself may direct activation of the apoptosis machinery.

Repression of the inhibitor of apoptosis protein family (IAPs) is another emerging strategy against cancer.

Repression of IAPs is thought to lift the brakes and promotes cell death. Here, exposure to *P. amaryllifolius* had resulted in decreased level of one of the caspase inhibitor proteins, XIAP. XIAP was also shown to induce $NF_{\kappa}B$ activation, which contributes to pro-survival effect and inflammatory stimulation [24]. Thus, a decrease in XIAP level allows the activation of procaspase 9 and effector caspases and may have as well signaled the activation of mitogen-activated protein (MAP) Jun kinase 1 (JNK1) signal transduction pathway.

In our previous report, *P. amaryllifolius* was found to contain abundantly other bioactive phytochemicals and the phytosterol stigmasterols [25]. Several studies have indicated that certain types of phytosterols may possess anticancer activity. The cytotoxicity of stigmasterol against cancer cells evidenced towards breast cancer cells [26]. Cytotoxicity of other plant sterols was also observed towards colon cancer cells [27,28]. Stigmasterols or generally phytosterols were hypothesized to exert their anticancer properties through multiple pathways inclusive of modulations of signal transduction pathways and apoptosis. Phytosterols were found to inhibit tumor growth of non-hormone dependent breast cancer cells via the sphingomyelin pathway. Stigmasterol was reported to induce four to six fold increases in apoptotic death in MDA-MB-231 cells as evidenced by measuring the release of nucleosomes into the cytoplasm. The molecular targets in apoptosis induction by stigmasterols were found to involve down regulation of oncogenes myc and transcription factor p53. These bioactive compounds present in *P. amaryllifolius* extracts work synergistically in inhibiting proliferation of breast cancer cells. Stigmasterols as one component of *P. amaryllifolius* extracts may have contributed to the induction of the p53 mediated apoptosis pathways in MDA-MB-231 cells.

Conclusion

Ethanol extracts of *P. amaryllifolius* mediated mitochondrial activated apoptosis pathways in MDA-MB-231 cells. The molecular mechanisms in the induction of apoptosis by *P. amaryllifolius* and its bioactive components were found to involve upregulation of the tumour suppressor protein p53 and a reduction in the inhibitor of apoptosis protein XIAP. However, XIAP is not the only member of IAP family that involve in anti-apoptotic effect and the involvement of extrinsic apoptosis pathway to induce this mitochondrial activated apoptosis pathway is still unknown. Thus, further studies including isolation of individual stigmasterols and confirmation of both intrinsic and extrinsic apoptosis mediated by the isolated stigmasterols should be the direction to confirm the role of stigmasterols in the cytotoxicity effect of *P. amaryllifolius* ethanolic extract against MDA-MB-231 cell.

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Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HZC conceived the study, carried out the experimentation, acquisition and analysis of data and drafting of the manuscript. SKY assisted with the concept, analysis of data and drafting of the manuscript. AR provided funding. AR, AMA, NBA, FO and CLG-E conceived, designed and supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgements
The authors would like to thank University Putra Malaysia for providing research funding under Research University Funding (RUGS). The authors would like to thanks Prof S.G. Tan for proof-reading of this manuscript.

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Received: 6 April 2012 Accepted: 14 August 2012
Published: 22 August 2012

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doi:10.1186/1472-6882-12-134
Cite this article as: Chong *et al.*: [illegible]luation of *Pandanus amaryllifolius* ethanol extract for induction of cell death on non-hormone dependent human breast adenocarcinoma MDA-MB-231 cell via apoptosis. *BMC Complementary and Alternative Medicine* 2012 **12**:134.

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