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BCL-2 was Downregulated in G2/M-Arrest Breast Cancer Cells MCF-7-Treated with Nordamnacanthal

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Abstract:
Nordamnacanthal, an anthraquinone extracted from the root of *Morinda elliptica* has cytotoxic properties towards various cancer cell lines and antitumor-promoting activities. This study was conducted to determine the effects of nordamnacanthal on the cell cycle, and the expression of Bcl-2 and Bax in breast cancer (MCF-7) and acute t-lymphoblastic leukemia (MOLT-4) cells at 50% of the total cell population underwent apoptosis. Nordamnacanthal caused 50% of MCF-7 and MOLT-4 cells underwent apoptosis at 15 µg/ml and 70 µg/ml, respectively, as analyzed by using a fluorescence microscope following staining with the acridine orange (AO) and propidium iodide (PI). The apoptotic cells exhibited nuclear fragmentation, chromatin condensation in the nucleus and membrane blebbing. Cell cycle analysis by flow cytometry indicated that nordamnacanthal arrested MCF-7 cells at the G2/M phase. For MOLT-4, no cell cycle arrest was observed. Bcl-2 and Bax were downregulated in nordamnacanthal-treated MCF-7 cells. On the other hand, expression of the proteins in MOLT-4 was not significantly different ($p>0.05$) from the control. In conclusion, treatment of MCF-7 cells with nordamnacanthal at the concentration that caused 50% of the total cell population underwent apoptosis, induced the G2/M arrest with downregulation of Bcl-2.
Keywords: *Nordamnacanthal; apoptosis; cell cycle arrest; Bcl-2 family proteins*

1. Introduction:

Morinda elliptica is a small plant from the Rubiaceae family also known as "mengkudu kecil", is a native plant from Asia and Polynesia. This plant has been used in traditional folk medicine for treatment of cholera, diarrhea, piles, headache and to increase appetite (Ali *et al*., 2000). Roots of the plant are usually rich in anthraquinones (AQs), which most often occur as glycones and glycosides.

Nordamnacanthal or 2-formyl-1,3-dihydroxyanthraquinone ($C_{15}H_8O_5$) is one of the 11 anthraquinones which has been extracted from *Morinda elliptica* (Nor Hadiani *et al*., 1997) (Figure 1).

Some studies have indicated that nordamnacanthal has a number of biological properties, including antioxidant activities and antitumor effects on human B-lymphoblastoid cell lines (Jasril *et al*., 2003). Nordamnacanthal has also been reported to exhibit antiviral, antimicrobial and cytotoxic properties (Ali *et al*., 2000). The mechanisms on how the bioactive agents of this anthraquinone exert their cytotoxicity are still unknown. It could be due to the prominent chemical feature of quinones with ability to undergo redox cycling to generate reactive oxygen species which eventually cause damage to the tumor cell (Kalyanaraman *et al*., 1991; Schreiber *et al*., 1987).

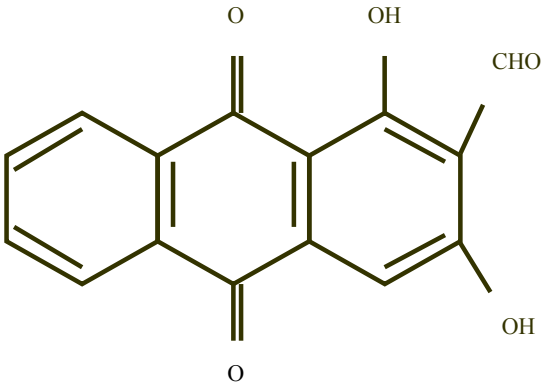


Figure 1: Chemical structure of nordamnacanthal (Rajendran *et al*., 2004)

Apoptosis or programmed cell death is genetically regulated as important to balance the proliferation and maintain the correct number of cells during development and pathological condition (Thompson 1995; Wyllie *et al*., 1980). This mode of cell death involves the removal of cells from tissues in a systematic and deliberate manner, without the inflammatory reaction (Kerr *et al*., 1972). There are two major pathways involve in apoptosis and have been

identified as extrinsic pathway (death receptor pathway) and intrinsic pathway (mitochondrial pathway) (Antonsson, 2004). Intrinsic pathway usually occurs when the cells are induced with anticancer drugs or cellular stresses such as hypoxia and induction of oncogenes by stimuli (DNA damages). This pathway involves mitochondria membrane to release cytochrome-c into cytoplasm, and binds to caspase adaptor Apaf-1 to trigger apoptotic cascade by activate the downstream caspases such as 3, 6 and 7 causing DNA fragmentation and cell death (Fernandez-Capetillo *et al*., 2002). Bcl-2 (B-cell leukemia/lymphoma 2) plays a central role controlling the intrinsic pathway. The family composed of suppressor of apoptosis such as *bcl-2, bcl-xl*, and *bcl-w*, and promoters of apoptosis such as *bax*, *bak*, *bok* and *bid* (Guo *et al*., 2001). In the apoptotic pathway, *bcl-2* will unstable the mitochondria barrier function that alter the permeability of the membrane to lead the release of cytochrome-c (Suzuki and Shiraki, 2001). Since apoptosis can be induced artificially by drugs, thus lead the development of cancer therapy that acts directly by switching on the cell death machinery in cancer cells. It is important to recognize the molecular mechanisms involved in apoptotic pathway, thus developing a drug targeting to inhibit or activate the anti-apoptotic and pro-apoptotic molecule, respectively.

In this study, we were comparing the effects of nordamnacanthal on the cell cycle, and the expression of Bcl-2 and Bax in MCF-7 and MOLT-4 cells when the population of apoptotic cells reached 50% of the total.

2. Materials and methods:

2.1. *Cell culture*

The human breast adenocarcinoma (MCF-7) and acute T-lymphoblastic leukaemia (MOLT-4) cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, USA. The cell lines were cultured in RPMI-1640 medium (PAA, Germany) containing 10% fetal bovine serum (PAA, Germany) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (PAA, Germany). The cells were incubated at 37 ^{o}C under 5% CO_2 in a humidified atmosphere. Cell number and viability were determined by staining the cells with trypan blue dye (Sigma, Germany) and counted using a hemocytometer viewed under a light microscope. The cells were subcultured when reach confluent at 99%.

2.2. *Compound*

Nordamnacanthal was kindly supplied by the Natural Product Laboratory, Institute of Bioscience, Universiti Putra Malaysia. The compound was dissolved in dimethyl sulphoxide (DMSO) to give the stock concentration of 10 mg/ml.

2.3. *Determination of the concentration of nordamnacanthal that caused 50% of the total cell population underwent apoptosis*

The cell density of 1 x 10^5 cells/ml was treated with various concentrations of nordamnacanthal until 50% of cell underwent apoptosis was achieved. Control which contains cell without compound was also included. Following the treatment, the cells were harvested by centrifugation (450 x g, 10 minutes), washed with phosphate buffered saline (PBS) twice before analysis. The cells were stained with 100 µg/ml of acridine orange (Sigma, USA) and 100 µg/ml of propidium iodide (Sigma, USA) at 1:1 mixture after treated with nordamnacanthal (Gorman *et al*., 1996). The suspension was placed onto a clean microscopic slide and viewed under a fluorescence microscope at 200 X magnification. Two hundred cells were counted in every sample. The number of viable, apoptotic and necrotic cells was counted and expressed as a proportion of the total cell number (%).

2.4. *Treatment with nordamnacanthal at the concentration that caused 50% of the cell population underwent apoptosis*

The MCF-7 and MOLT-4 cells were treated with 70 µg/ml and 15 µg/ml of nordamnacanthal, respectively, for 72 hours at 37 ^{o}C in 5% CO_2.

2.5. *Cell cycle analysis*

The cells were harvested by centrifugation at 644 x g for 10 minutes and washed twice with PBS. The fixation of the cells was done according to Klucar and al-Rubeai (1997) with some modifications. Briefly, the cells were fixed by 70% cold ethanol, and left at -20 ^{o}C for 2 hours. The cell pellet was then resuspended in PBS containing RNase A (1 mg/ml) on ice for 20 minutes in dark condition. Intracellular DNA was labeled with propidium iodide (PI) (1 mg/ml) and analyzed by flowcytometry using FACScan software (Cyan ADP, Dako, Denmark).

2.6. *Enzyme-linked Immunosorbent Assay for Quantitative Detection of Human Bcl-2*

The analysis was carried out using the Human Bcl-2 ELISA kit (Bender MedSystems, Austria). For the sample preparation, the cell was lysed with lysis buffer 1X. After 1 hour of incubation at room temperature, the sample was spun at 1000 x g for 15 minutes. The supernatant was taken as the lysate. Briefly, 80 µl sample diluent and 20 µl of sample was added to the wells coated with monoclonal antibody to human Bcl-2 after washed two times with the wash buffer. Next, 50 µl of Biotin-Conjugate was added to the wells. After 2 hours of incubation at room temperature on microplate shaker, 100 µl of Streptavidin-HRP was added. The sample was incubated again for 1 hour at room temperature on the microplate shaker. All the solution inside the wells was emptied, and washed 3X with the wash buffer. Immediately, 100 µl of TMB substrate solution was added. Finally, 100 µl of stop solution was added to each well to stop the enzymes reaction. The absorbance was read using an ELISA reader (Sunrise, Tecan) at 450 nm and 620 nm as a reference wavelength. The optical density was compared with the standard graph to determine the concentration of the protein.

2.7. *Human Bax Enzyme Immunometric Assay*

Bax protein concentration determination was carried out using the Human Bax Enzyme Immunometric Assay Kit (BD, USA). The lysate preparation was done according to the manual. Following centrifugation (16000 x g for 15 minutes), the cells were resuspended in modified cell lysis buffer 4 (0.5 µl/ml of Sigma Protease Inhibitor Cocktail and 1mM Phenylmethylsulfonyl fluoride (PMSF)). Next, 100 µl of the sample was added to the wells coated with monoclonal antibody to human Bax-α in triplicate. The plate was tapped gently to mix the contents. The sample was incubated at room temperature on a plate shaker for 1 hour. The wells were emptied and washed 5X with the wash buffer. After the final wash, the plate was tapped gently on a lint free paper towel to remove any remaining wash buffer. The sample was incubated again for 1 hour at room temperature on a plate shaker after added 100 µl of yellow Antibody (biotinylated monoclonal antibody to Bax-α) into each well. The wells were washed again for 5X with the wash buffer and emptied. Next, 100 µl blue Conjugate (streptavidin conjugated to horseradish peroxidase) was added to each well. The sample was left on a plate shaker for 30 minutes at room temperature. The wells were washed again as the previous step. A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide was added at 100 µl into each well. Finally, 100 µl of stop solution containing hydrochloric acid in water was added to stop the enzyme reaction. The optical density was read at 450nm and 570 nm as a reference wavelength by an ELISA reader

(Sunrise, Tecan). The optical density was compared with the standard graph to determine the concentration of the protein.

2.8. *Statistical analysis*

Student's *t*-test was performed to determine the significant difference between means of control and treated samples. A *p*-value of less than 0.05 ($p<0.05$) was considered as statistically significant.

3. Results:

3.1. *Morphological changes of MCF-7 and MOLT-4 treated with nordamnacanthal*

Morphological features of apoptosis were observed in MCF-7 and MOLT-4 cells treated with nordamnacanthal viewed under a fluorescence microscope following staining with AO/PI. They include condensation of nuclear chromatin and cytoplasm and membrane blebbing. Interestingly, some of the MCF-7-treated cells exhibited enlargement of cell volume and formation of multinucleated cells. The control cells appeared to be shiny, clear and healthy. There were also small numbers of necrotic cells with ruptured plasma membrane observed (Figure 2).

3.2. *Fluorescence analysis of mode of cell death*.

At the treatment of nordamnacanthal of 70 µg/ml and 15 µg/ml, 56% and 59% of MCF-7 and MOLT-4 cell population, respectively, underwent apoptosis. The percentages of necrotic cells were only 2% and 9%, for MCF-7 and MOLT-4 (Figure 3).

3.3. *Cell cycle analysis*

Figure 4 illustrates the changes in the cell cycle distribution of MCF-7 and MOLT-4 cells treated with nordamnacanthal at the concentration that caused 50% of the total cell population underwent apoptosis. The population of MCF-7 cells at the sub-G0 and G2/M phases increased significantly ($p<0.05$). Cell population at the G0/G1 and S decreased, but not significant as compared to the control ($p>0.05$). For MOLT-4 cells, there was a significant increased ($p<0.05$) at the apoptotic region (sub-G0). However, no cell cycle arrest was detected ($p>0.05$).

3.4. *Effect of nordamnacanthal on the level of expression of Bcl-2 and Bax in MCF-7 and MOLT-4*

Bcl-2 and Bax were downregulated in nordamnacanthal-treated MCF-7 cells. On the other hand, expression of the proteins in MOLT-4 was not significantly different ($p<0.05$) from the control (Figure 5 and Figure 6).

4.0 Discussion:

Natural product, a chemical compound produced by living organism, is the promising alternative strategies as a source of chemotherapeutic to kill the cancer cells (Newman *et al*., 2006). Examples of drugs having antitumor properties which derived from natural products are vincristine and vinblastine from *Catharanthus roseus* (Johnson *et al*., 1960). Both of these chemotherapeutic drugs have been implicated to be potent inducer of apoptosis in cancer cells by disrupting the microtubule (Forbes *et al*., 1992; Martin and Cotter, 1990). Apoptosis, not only vital for normal development of living things, but also has been claimed to be a very promising means for cancer therapy. Therefore, drugs that are inducing apoptosis are of preference over the ones causing necrosis due to the fact that the former will reduce side effects and inflammation by killing only the diseased affected cells without inflicting the surrounding healthy cells. In this study, nordamnacanthal, a natural product of *Morinda elliptica*, has been found to induce apoptosis in both the anchorage-dependent MCF-7 and the non anchorage-dependent MOLT-4 cells after viewing under a fluorescence microscope following staining with acridine orange and propidium iodide (AO/PI). Staining the cells with fluorescence dyes such as AO and PI is considered the correct method for evaluating the apoptotic cells with changed nuclear

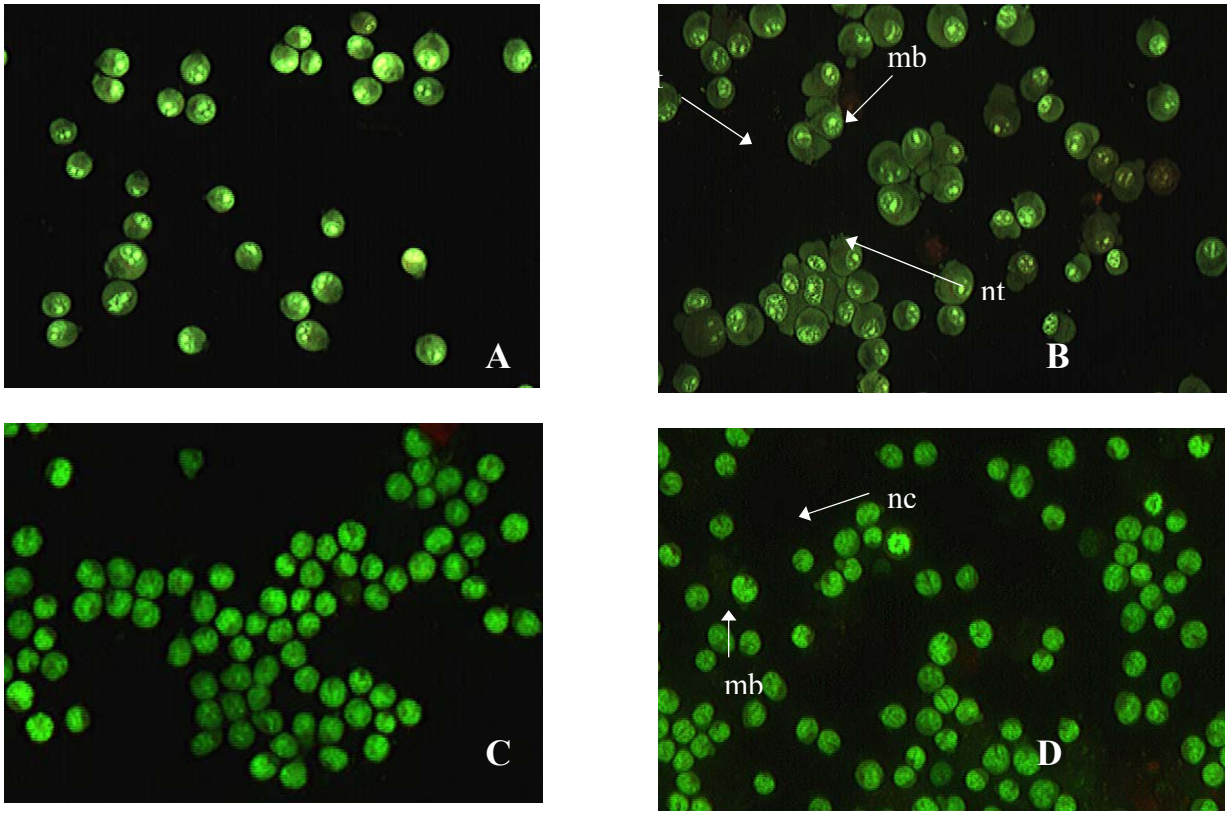
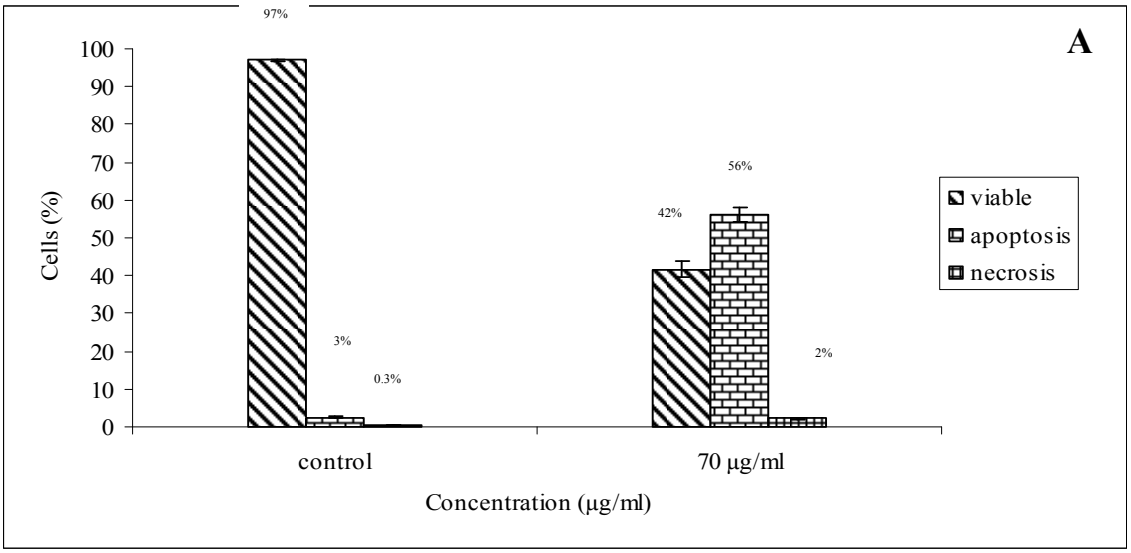


Figure 2: The morphology of (B) MCF-7 and (D) MOLT-4 cells treated with nordamnacanthal at the concentration that caused 50% of the cell population underwent apoptosis for 72 hours. Samples were stained with acridine orange and propidium iodide and observed under a fluorescence microscope. The cells showed apoptosis characteristics such as nuclear compaction (nc), chromatin condensation (cc), the membrane plasma blebbing (mb) and the mitotic cells (mt). Necrotic cells (nt) were observed (arrows). Controls were also included (A and C) (200 X magnification).

morphology (Savitskiy *et al*., 2003). AO/PI staining confers obvious characteristics such as nuclear chromatin condensation, nuclear fragmentation and finally leads to membrane blebbing. These characteristics allow differentiation of the subpopulation of apoptotic cells from viable and necrotic cells. AO is a cell-permeable DNA-binding dye was used in combination with plasma membrane-impermeable DNA-binding dye PI. AO and PI excite a green and red fluorescence, respectively, when they are intercalated into DNA. AO is taken up by both viable and nonviable cells, while PI is excluded by cells with intact membrane (viable and apoptotic). PI fluoresce red predominantly for necrotic cells (Ciapetti *et al*., 2002). The stained cells with AO/PI showed a combination of apoptotic cells with dense green areas in the nuclei, necrotic cells with red intact nucleus and viable cells with green intact nucleus. The 3 types of the cell were then easily identified, distinguished and counted to get the proportion of each in a given population.

Knowledge on the molecules involved in the programmed cell death is very important as to provide some insights on how they can be manipulated for a more selective and effective cancer cells killing activity. Nevertheless, it is very difficult and to our best knowledge there is yet a separation



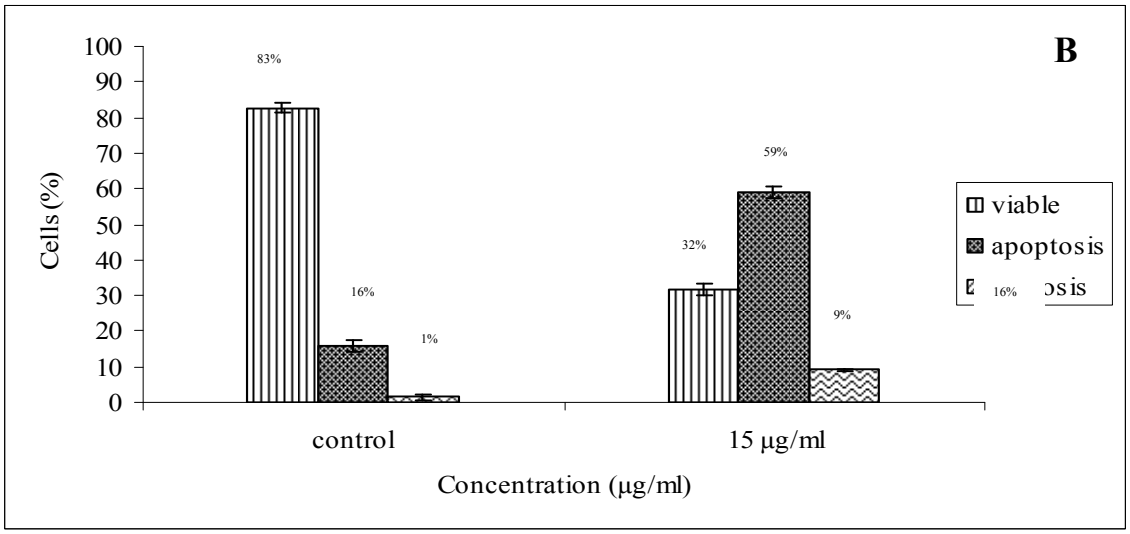


Figure 3: The percentage of viable, apoptotic and necrotic cells of (A) MCF-7 and (B) MOLT-4 treated with nordamnacanthal at the concentration that caused 50% of cells underwent apoptosis for 72 hours. Controls were also included. The counts were done under a fluorescence microscope following staining with acridine orange and propidium iodide. Each data is presented as mean ± SEM.

technique that can actually separate apoptotic cells from others. Due to the shortcoming, we decided to analyze the whole population of cells in a sample taking into account that approximately 50% of them underwent apoptosis. We chose 50% because it is an easy cut-off point to work with. Not only it is highly reproducible, but at that value (50%), the percentage of necrotic cells is very low (not significant), thus hindering the possibility of overshadowing the outcome. Higher concentration of nordamnacanthal was needed to cause approximately 50% of the MCF-7 cells (70 µg/ml) to undergo apoptosis compared to MOLT-4 (15 µg/ml). It indicates that MOLT-4 is more sensitive to the compound compared to MCF-7.

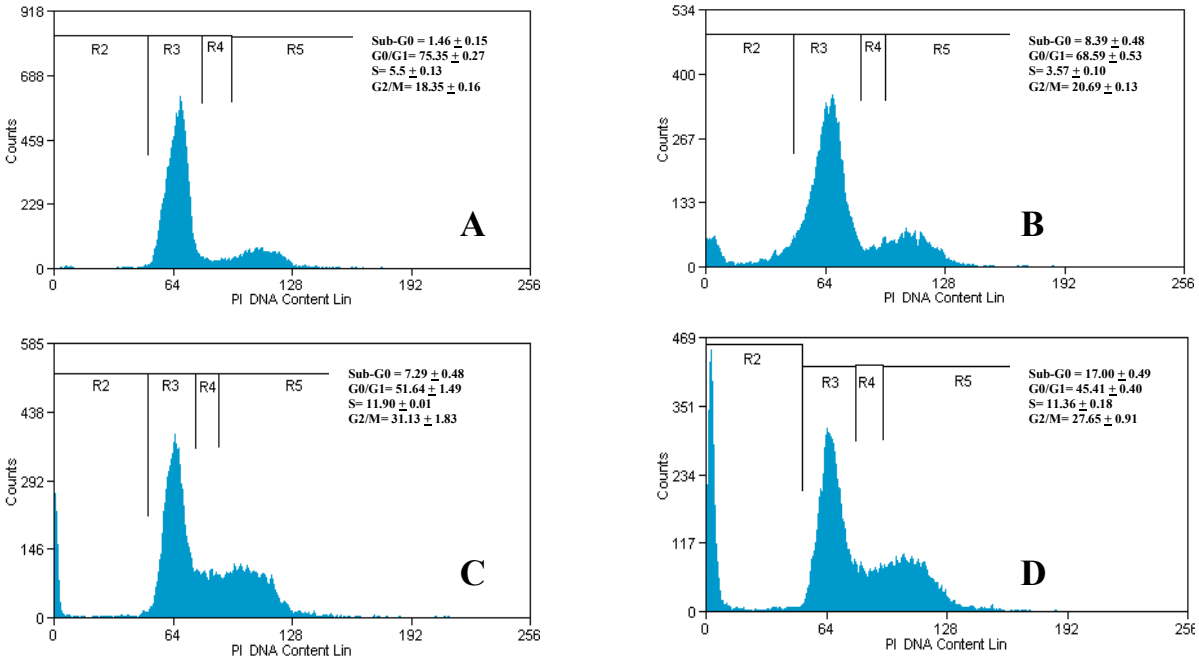


Figure 4: Cell cycle distribution from flowcytometric analysis of (B) MCF-7 and (D) MOLT-4 cells treated with nordamnacanthal at the concentration that caused 50% of the cell population underwent apoptosis for 72 hours. Controls were also included (A and C). (R2= sub-G0; R3= G0/G; R4= S; R5= G2/M).

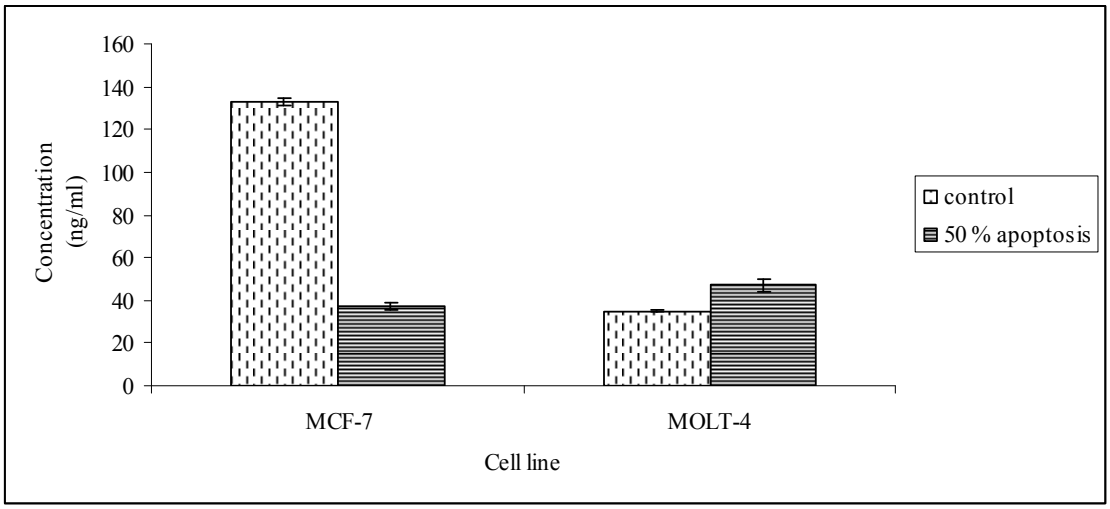


Figure 5: The concentration of Bcl-2 of MCF-7 and MOLT-4 treated with nordamnacanthal at the concentration that caused 50% of cell population underwent apoptosis cells underwent apoptosis for 72 hours. Controls were also included. Each data is presented as mean ± SEM.

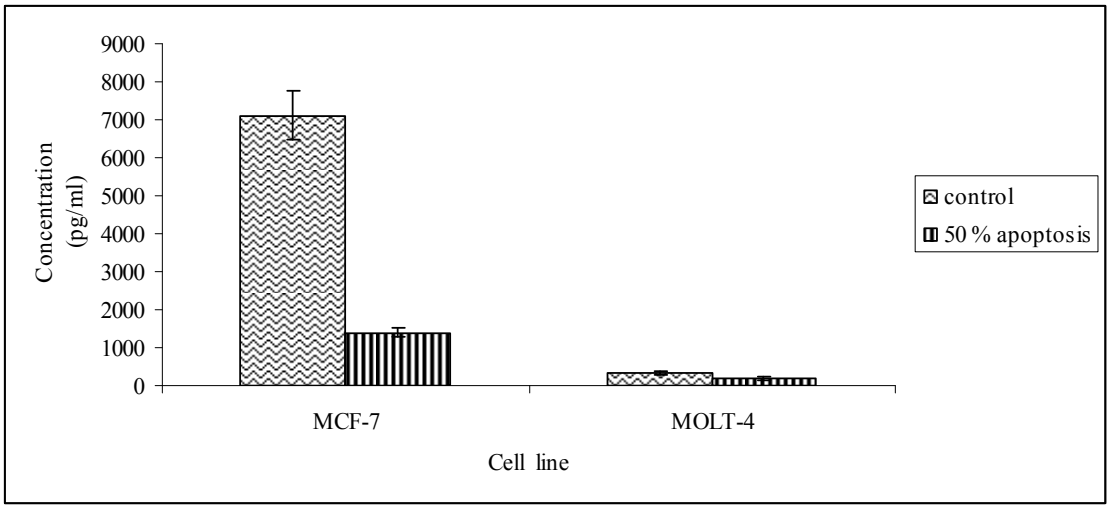


Figure 6: The concentration of Bax of MCF-7 and MOLT-4 treated with nordamnacanthal at the concentration that caused 50% of the cell population underwent apoptosis for 72 hours. Controls were also included. Each data is presented as mean ± SEM.

It is believed due to many factors in the microenvironment of solid tumor that are responsible for non-uniform and insufficient levels of anticancer agents being delivered. The abnormalities in the extracellular matrix (ECM) in tumors, for instance, lead to the deficiencies in interstitial transport (Wang and Yuan, 2006), which ultimately affect the bioavailability and efficacy of chemotherapeutic agents (Galmarini and Galmarini, 2003). The ECM is made up of a matrix of proteoglycans, collagens, and additional molecules, which are produced and assembled by stromal and tumor cells (Mow *et al*., 1984). In tumor, greater collagen content in the ECM required higher infusion pressure to initiate flow in the tumor interstitium (McGuire *et al*., 2006), thus obstructs the transport of anti-cancer agent (Wang and Yuan, 2006).

The incidence of apoptosis was further confirmed using flow cytometric analysis. Sub-G0 peak was detected in both cell lines after treatment with nordamnacanthal. The visibility of "sub-G0" peak (hypodiploid DNA peak) by flow cytometry with other supporting information can be taken as definite evidence for the apoptotic cell population (Ormerod *et al*., 1992). Besides inducing apoptosis, the compound was also found to arrest the MCF-7 cells at the G2/M phase. G2/M blockage stops the cells in that phase from dividing and bring about an increase in the DNA hypodiploid detected by the existence of sub-G0. It should be mentioned that some of the MCF-7 cells treated with nordamnacanthal were bigger in size compared to the others (apoptotic, necrotic and viable cells), which indicates a phenomenon called as mitotic arrest (Figure 2) (Ming-Jie *et al*., 2004). Besides apoptosis, cells may undergo mitotic catastrophe due to mitotic cell death, which is associated with the abnormal segregation of chromosomes and aberrant cytokinesis, resulting in cells of abnormal size and DNA content (Hendry and West, 1997). Mitotic cell death is defined as loss of reproduce integrity after inappropriate entry into mitosis and frequently characterized by the emergence of cells containing multinuclear fragments (micronuclei) (Muller *et al*., 1996). Nevertheless, the mechanisms on how cytotoxic stress causes MCF-7 cells to

undergo apoptosis at the G2/M checkpoint are still unclear. A study done by Hengartner (2000) drives a hypothesis that nordamnacanthal may be able to interact with the major component of microtubule assembly, suggesting that disruption of the microtubule network initiates events which lead to apoptosis (Martin and Cotter, 1990). Another speculation is that the compound induces inappropriate alteration in the expression or/and activation of Cdks and regulators, leading to blockage of cell cycle progression and induction of apoptosis.

Bcl-2 and Bax were downregulated in nordamnacanthal-treated MCF-7 cells (Figure 5 and Figure 6). Down-regulation of Bcl-2 is closely related to the occurrence of apoptosis in cells (Che-Jen *et al*., 2008). Bcl-2 is predicted to play a role and involved in the G2/M arrest (Figure 5) as it has been demonstrated to interact with a variety of proteins involved in regulating the G2/M transition (Dong-Oh *et al*., 2008). Apoptosis can be controlled by the degradation rate of proapoptotic proteins such as Bax (Li and Dou, 2000). The exact cause on downregulation of Bax in MCF-7-treated cells is still unclear. However, a recent study has shown that degradation or expression rate of Bax, at above or below some threshold value, respectively, is related to cell survival, where by, at a critical point that the amount of Bax might be associated with the onset of cancer in living cells (Bagci *et al*., 2006). On the other hand, the expression of Bcl-2 and Bax in the MOLT-4 cells treated with nordamnacanthal was not different from the control. It is suggested that other Bcl-2 family proteins are involved. Bcl-2 is the founding member of a family of proteins that regulates cell death. It includes the anti-apoptotic members (Bcl-2, Bcl-xL and Bcl-W) and the pro-apoptotic members (Bax, Bad, Bak and Bik) (Cory and Adams, 2002).

In short, there are differences in the regulation and involvement of certain molecules in the solid tumor (anchorage-dependent cells, MCF-7) and non-solid tumor (non anchorage-dependent cells, MOLT-4) in apoptosis induced by nordamnacanthal. It is obvious that the compound caused G2/M arrest and downregulation of Bcl-2 and Bax only in MCF-7 cells, but not in MOLT-4. The differences are important as to give some clues for the manipulation and design of a new drug (nordamnacanthal, in this case) with enhanced effectiveness in killing the cancer cells.

5.0 Acknowledgment:

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