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Morinda citrifolia extract inhibits proliferation of non-small human lung cancer cell line NCI-H23 via apoptosis by modulating the Bax:Bcl-2 ratio

(Ekstrak *Morinda citrifolia* merencat pembiakan sel besar kanser paru-paru manusia NCI-H23 melalui apoptosis dengan memodulasikan nisbah Bax:Bcl-2)

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Keywords: *Morinda citrifolia*, lung cancer, apoptosis, Bax, Bcl-2

Abstract

The involvement of Bcl-2 gene family in cells apoptosis mechanism is well described in many studies against cell death after treatment with anticancer drugs. Previously, the potential of *M. citrifolia* extract to inhibit proliferation and induce apoptosis in human lung adenocarcinoma cell growth was carried out. The mechanism involved in apoptosis of NCI-H23 cells after treated with *M. citrifolia* extract was studied. A search for the molecular mechanism revealed that *M. citrifolia* extract burden decrease the expression of the pro-apoptotic protein Bcl-2 but the level of Bax protein remain. These in consequence elevated the ratio of Bax:Bcl-2 in apoptotic cell. These findings indicated that the apoptotic events by *M. citrifolia* extract were associated with down-regulation of Bcl-2 protein expression in NCI-H23 cells.

Introduction

Over the last decade, there is a growing number of interest in the medicinal uses of *Morinda citrifolia*. The therapeutic effects of this plant extract including anticancer activity have been reported in some studies. Modern scientific researches revealed the potential of *M. citrifolia* in slowing down the progress of cancer activites in direct and indirect ways after taking the mentioned supplement. A study reported by Hirazumi et al. (1996) showed that the fruit juice contains a polysaccharide-rich substance called noni-ppt with antitumour activity. The administration of noni-ppt significantly enhanced the duration of survival of mice lung tumour through the activation of the host immune system.

Meanwhile, a number of anticancer agents such as taxol (Parekh and Simpkins 1997), curcumin, flavanoids (Kawaii et al. 1999), vinblastine and vincristine (Jordan and Wilson 2004) were reported to inhibit proliferation of cancer cells by inducing cell apoptosis. The induction of apoptosis is known to be an efficient strategy for developing new anticancer drugs therapy. Recently, extracts prepared from a variety of plants exhibit the ability to trigger apoptotic process (Bussing et al. 1999; Yoon et al. 1999; Seo et al. 2001). Previously, *M. citrifolia* extract showed potential antiproliferative activity by inducing apoptosis on human lung adenocarcinoma NCI-H23 cells (Kharis and Azimahtol 2003; 2004). These results prompted us to

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further evaluate the anticancer activity *in vitro* study and clarify the mechanism of its antiproliferative activity. One of the main regulatory steps of apoptotic cell death is controlled by the ratio of anti-apoptotic (Bcl-2, Bcl-XL, Bag-1, Bcl-W, Mcl-1 and A1) to pro-apoptotic (Bax, Bad, Bak and Bcl-Xs) members of the Bcl-2 family of proteins as a rheostat that determines the susceptibility to apoptosis (Gross et al. 1999; Haefen et al. 2002). In this study, the apoptosis mechanism induced by *M. citrifolia* was regulated through changes in the Bax:Bcl-2 ratio.

Materials and methods

Preparation of Morinda citrifolia extract

The fruits of *M. citrifolia*, used in the study were obtained locally from MARDI station, Kundang, Selangor. The fruits were washed and rinsed thoroughly, sliced and followed by drying in the oven at 50 °C. The dried slices were then ground into powder and stored at –20 ºC until further use.

Extract preparation was done by soxhlet extraction method with petroleum ether for 5–8 h (*Figure 1*). The remaining solid waste powder of *M. citrifolia* was re-extracted with methanol for another 6–8 h or until the solvent looked clear. Then, the methanol extract obtained was concentrated in a rotary evaporator and used to solvent partition with chloroform in a separation funnel at 1:1 ratio (Kharis and Azimahtol 2004). The chloroform fraction at the bottom layer was collected and evaporated to dryness in vacuum to remove the remaining solvent residue in the extract. The dried extract was stored at –20 ºC prior used.

Cell culture

Human lung adenocarcinoma cells (NCI-H23) were purchased from American Type Culture Collection (ATCC) and grown as monolayer in RPMI-1640 nutrient medium supplemented with 10% FBS and antibiotics. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 ºC. The medium was replaced every 2 days, in order to maintain cell viability.

Cell viability

Cell viability for use in antiproliferative assay was measured according to trypan blue staining method (Nurhanan 2001). The confluent cells were trypsinized (0.05% trypsin) and cells concentration was determined by counting the cells under microscope (100 x) using a hemacytometer. Only those with 80% viability or above are used in the experiment.

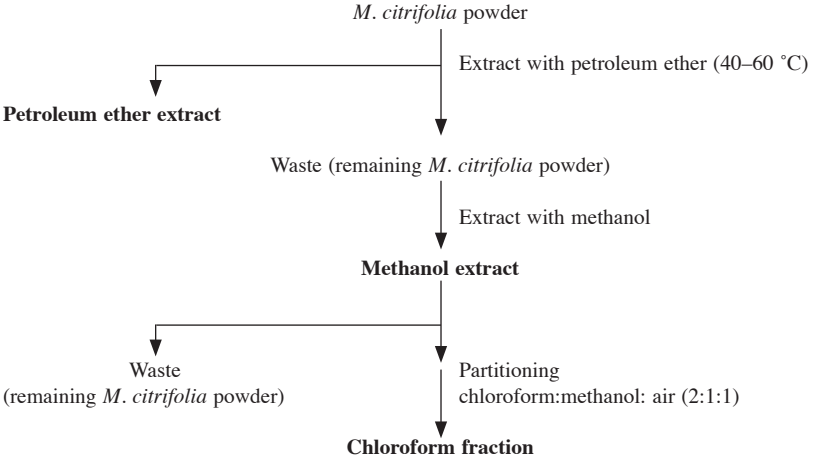


Figure 1. Flow chart of organic extraction and partition scheme of ***Morinda citrifolia***

Detection of apoptosis

Apoptosis cells were identified using DeadEnd™ Colorimetric TUNEL System kit from Promega based on Terminal deoxynucleotide Transferase-mediated dUTP-biotin Nick End-Labeling (TUNEL) assay method (Gavrieli et al. 1992). Cells were plated at a density of 2 x 10^5 on poly-L-lysine slide in the 6 wells plate until confluence. The confluent cells were then exposed to *M. citrifolia* extract at 100 µg/ml for 72 h (Kharis and Azimahtol 2003). The signals of DNA fragmentation were determined following the manufacturer's protocol and observed under fluorescence microscope.

Treatment of Morinda citrifolia extract and protein isolation

NCI-H23 cells at a density of 2 x 10^5 were grown monolayer in a 175 cm^2 culture flask until confluence. The confluent cells were then exposed to *M. citrifolia* extract at different duration i.e. 24, 48 and 72 h at 100 µg/ml (Kharis and Azimahtol 2003). The treated cells were then harvested and centrifuged at 4,000 g for 5 min. All samples were then lysed in protein extraction buffer (0.1M Tris-HCl, pH 7.2, 1% triton-X 100, 0.01% SDS, 100 µM phenylmethylsulfonylfluoride 10 µg/ml leupeptin, 10 µg/ml aprotinin) by freeze-thaw the cell pellets in liquid nitrogen followed by sonication for 10 min. The lysed cells were then centrifuged at 14,000 g for 10 min to isolate the protein for Western blot analysis. The protein concentration was determined using Bradford method (Bradford 1976).

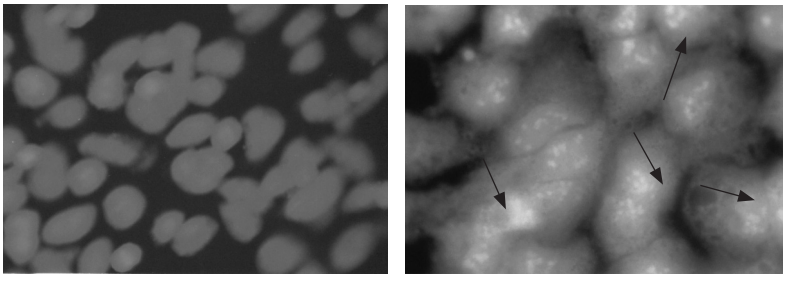
Western blot analysis

Equal amount of sample lysates (70 µg) were separated by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) at 28 mA for 90 min and electrophoretically transferred onto PVDF membrane (Amersham Pharmacia Biotech) for 3 h at 4 ºC with continuously power supplied with 200 mA and voltage 100 V. The membrane was blocked with 5% non-fat dry milk in PBS buffer pH 7.5, 0.1% tween-20 overnight. Blots were extensively washed with PBS-tween buffer and probed with specific primary monoclone mouse antibodies IgGs (anti-human Bax and Bcl-2) from Invitrogen at 1:1500 concentration for 2 h with mild agitatation (50 rpm). After washing with PBS-tween buffer several times, the membrane was re-incubated with secondary antibody IgG conjugated anti-mouse HRP (Horseradish Peroxidase) at 1:20000 for 2 h at room temperature. Signals of proteins were detected using 'Chemiluminescen Western Lightning™ Chemiluminescence Reagent *Plus*' (Perkin Elmer) and exposed to x-ray film using Kodax cassett for 10 min in the dark room.

Results and discussion

Genetic alternations in disturbance of apoptosis-signalling pathways are likely to be the critical components of carcinogenesis (Schulte-Hermann et al. 1994). Therefore, the induction of apoptosis is known to be one of the efficient strategies for cancer therapy. In addition, apoptosis is regulated and executed by different interplay of many genes responsive to different pathways such as Bcl-2 family related pathway. The Bcl-2 family members have recently been invoked to maintain cell viability by preventing the loss of mitochondrial membrane potential and play a critical role in mitochondrial cytochrome *c* release.

Therefore, a study was conducted to determine the effect of *M. citrifolia* extract on cell death induction. The molecular mechanism of Bcl-2 family genes expression on modulation of Bax:Bcl-2 protein ratio levels, which are involved as mediators of apoptosis, was examined. The expression of Bax and Bcl-2 level was investigated through Western blot analysis and normalized with β-actin. The confluent cells treated with *M. citrifolia* extract were 87% viable when analysed through cell viability assay using trypan blue method. When the cells were treated



Control (DMSO) *M. citrifolia* extract

Plate 1. Apoptosis induction in lung adenocarcinoma (NCI-H23) cells (shown by fluorescence signal) after treated with ***Morinda citrifolia*** *extract. The cells viewed under Zeiss Axioskope fluorescence microscope using 520 ± 20 nm filter*

with the *M. citrifolia* extract, the results showed apoptosis induction in lung adenocarcinoma NCI-H23 cells. The effect of *M. citrifolia* extract showed nuclear fragmentation and disintegration with intense fluorescence signal in cell nucleus of lung adenocarcinoma (NCI-H23) observed under fluorescence microscope.

The negative control treated with 0.1% DMSO did not react with propidium iodide and the whole cells were dyed red without fluorescence indicated that no apoptosis occurred in the negative control cells (*Plate 1*). The presence of DNA fragmentation in the cell treated with *M. citrifolia* extract was an important characteristic and a hallmark of apoptosis event (Wyllie et al. 1980). Therefore, the results proved that *M. citrifolia* extract induced apoptosis in lung adenocarcinoma (NCI-H23) cells.

In order to explore the potential signalling pathways in which the extract induced apoptosis, Western blot analysis was used to evaluate the expression of the genes for the transcription factor of Bcl-2 family of proteins. After 24 h treatment with *M. citrifolia* extract, the results showed a marked down-regulation of Bcl-2 protein in apoptosis cells compared to control (0 h) (*Figure 2*). At 48 h of *M. citrifolia* extract treatment, the expression of Bcl-2 protein level was much reduced and the level was very low compared to control. Similar trend was also observed at 72 h in which the Bcl-2 protein was almost not detected during

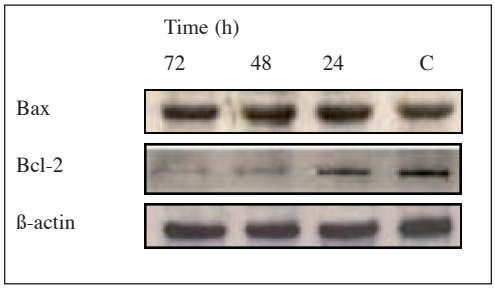


Figure 2. Effect of ***Morinda citrifolia*** *extract on the expression of Bax and Bcl-2 in NCI-H23 cells*

analysis. However, the expression level of Bax protein remained unchange during apoptosis.

Regardless of the incubation duration, there was no significant changes in the Bax expression after treated with *M. citrifolia* extract. Densitometry analysis showed no significant difference in Bax level during the incubation period after treatment with *M. citrifolia* extract (*Figure 3*). These results showed that the treatment did not affect the Bax protein in the cells.

The significant reduction of Bcl-2 protein expression level in apoptosis cells affect the normal distribution of Bax:Bcl-2 ratio. Low Bcl-2 expression increased cell sensitivity towards chemotherapeutic agent (Berchem et al. 1995). Therefore, these findings indicated that the increment of Bax:Bcl-2 ratio in *M. citrifolia* extract treated cells resulted in apoptosis induction in NCI-H23. The pro-apoptotic and anti-apoptotic members of the Bcl-2 family act as a rheostat in regulating programmed cell

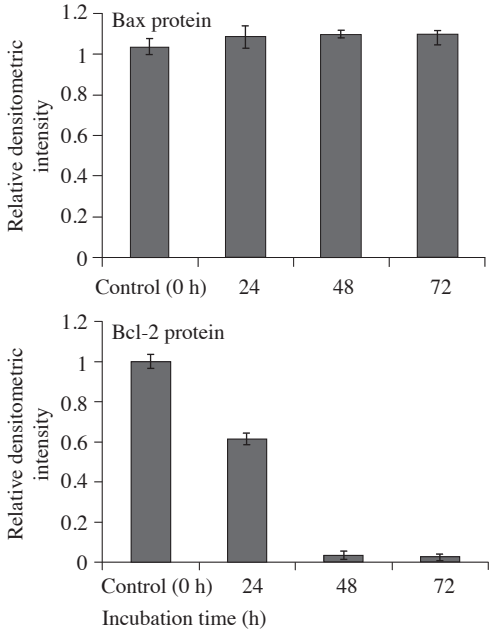


Figure 3. The relative densitometric intensity value of Bax and Bcl-2 protein in NCI-H23 treated with ***Morinda citrifolia*** *extract. Values are shown as means of three separate experiments; bars, SEM, p <0.05*

death and as a target of anticancer therapy (Baell and Huang 2002; Goodsell 2002). The ratio of death of antagonists such as Bcl-2 to agonists (such as Bax, Bcl-xs, Bad, Bid) determines whether a cell will respond to an apoptotic stimulus.

It has been reported that Bcl-2 protects against multiple signals that lead to cell death, indicating that Bcl-2 regulates a common cell death pathway and functions at a point where various signals converge (Vaux et al. 1988; Hockenbery et al. 1990; Nuriez et al. 1990). The balance of pro-apoptotic Bax and anti-apoptotic Bcl-2 protein expression regulates its own susceptibility to apoptosis (Korsmeyer et al. 1993). Down-regulation of the death suppressor Bcl-2 could repress tumour growth via promoting programmed cell death (Kluck et al. 1997; Zhivotovsky et al. 1998).

In another study, Huang et al. (2003) reported that the increasing Bax:Bcl-2 ratio induced apoptosis through down regulation of Bcl-2 proteins which are predominantly localized in the outer mitochondrial membrane. The Bcl-2 proteins mediate anti-apoptotic effect by stabilizing the mitochondrial membrane, inhibit permeability of transition pore ability and the release of cytochrome *c* (Egan et al. 1999). Inhibition of cytochrome *c* translocation from mitochodria to cytoplasm (Tsujimoto and Shimizu 2000) blocks the caspase activation step of the apoptotic process (Pan et al. 1998).

In contrast, Bax proteins predominantly localized in the cytosol, upon activation, translocated to the mitochondria, trigger the loss of mitochondria membrane potential, and mediate the release of cytochrome *c* to activate the caspase cascade involved in apoptosis (Priault et al. 1999). Thus, it can be concluded that the elevation ratio between the level of pro-apoptotic Bax protein and anti-apoptotic Bcl-2 modulate apoptosis induces by *M. citrifolia* extract in NCI-H23 cells.

Conclusion

The *M. citrifolia* extract tested on human lung adenocarcinoma (NCI-H23) cells showed growth inhibition through cell apoptosis. *In vitro* study revealed that the susceptibility of NCI-H23 cells to apoptosis involved down-regulation of Bcl-2 proteins. Inhibition of Bcl-2 proteins is linked to cytochrome *c* release which in turn activates the caspase cascade involved in apoptosis process.

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Abstrak

Penglibatan gen famili Bcl-2 dalam mekanisme apoptosis sel telah dijelaskan dalam pelbagai kajian berkaitan kematian sel yang diberi perlakuan dengan dadah antikanser. Sebelum ini, kajian mendapati ekstrak *Morinda citrifolia* berpotensi untuk merencat pembiakan dan mengaruh apoptosis terhadap sel adenokarsinoma paru-paru manusia. Mekanisme yang terlibat semasa apoptosis sel NCI-H23 setelah dirawat dengan ekstrak *M. citrifolia* telah dikaji. Hasil keputusan mendapati rawatan dengan ekstrak *M. citrifolia* merendahkan ekspresi protein pro-apoptotik Bcl-2 tetapi aras protein anti-apoptotik Bax tidak berubah. Peningkatan nisbah Bax:Bcl-2 mengaruh proses kematian sel melalui apoptosis. Penemuan ini menunjukkan tindak balas apoptotik oleh ekstrak *M. citrifolia* berkaitan dengan pengaturan menurun ekspresi protein Bcl-2 di dalam sel paru-paru manusia NCI-H23.

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