

Elevation of p53 level in human lung cancer cell line NCI-H23 apoptosis treated by *Morinda citrifolia* extract

(Peningkatan aras p53 dalam apoptosis NCI-H23 titisan sel kanser paru-paru manusia yang diberi ekstrak *Morinda citrifolia*)

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Key words: *Morinda citrifolia*, lung cancer, apoptosis, p53

Abstract

Effective p53 is a suppressor protein that acts as a key of transcription factor to multi cellular interaction. Its function is to balance between the cell growth and dead cells in the living system and plays an important role in deletion of cell growing and apoptosis induction. Studies have shown that p53 is activated when the signals are received by the cells due to stimulation from the inner or outer stressors. The accumulation of p53 resulted in either cell cycle arrest or apoptosis induction to the cells. An involvement of p53 in apoptosis has been reported in cells death treated by anticancer drug. Result showed that *M. citrifolia* extract inhibited and induced apoptosis in human lung adenocarcinoma. In mechanism study, apoptosis of NCI-H23 treated with *M. citrifolia* extract increased the expression of p53 level in the cells. Elevation of p53 induces apoptosis through either extrinsic or intrinsic pathway by depolarisation and increase sensitivity cells towards induction of apoptosis. The findings suggest that apoptotic event induced by *M. citrifolia* was through modulation of elevated p53 in the NCI-H23 cells and thus, promoting activation of down stream cascades involved in apoptosis mechanism.

Introduction

The World Health Organization (WHO) has estimated that approximately 80% of the world's population depends on traditional medicines for meeting their primary health care needs. Over the last decade, a growing number of people have become interested in the medicinal uses of *Morinda citrifolia*. Its therapeutic effects including anticancer activity had been reported in a few studies (Hirazumi and Furusawa 1999).

Modern scientific researches revealed the potential of *M. citrifolia* in fighting cancer directly or indirectly after taking

M. citrifolia supplement. Study performed at the University of Hawaii showed that the fruit juice contains a polysaccharide-rich substance called noni-ppt with antitumour activity. The administration of noni-ppt significantly enhances the survival duration of mice lung tumour (Hirazumi et al. 1996). This suggested the possibility of the fruit juice might react to suppress tumour growth through activation of the host immune system.

Other strategy used to study possible anticancer activity was by evaluating its ability to inhibit directly the cancer

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cell growth. The induction of apoptosis is known to be an efficient strategy for developing new anticancer drugs therapy. A number of cancer agents such as taxol (Parekh and Simpkins 1997), curcumin, flavanoids (Kawaii et al. 1999), vinblastine and vincristine (Jordan and Wilson 2004) reported inhibit proliferation of cancer cells by induced cell apoptosis.

Many studies have shown that 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). The antiproliferative properties of *M. citrifolia* extract in human cancer cells have been demonstrated to inhibit proliferation of human lung adenocarcinoma NCI-H23 cells (Kharis and Azimahtol 2003, 2004).

Genetic alterations resulting loss of apoptosis or disturbance of apoptosis-signalling pathways are likely to be critical components of carcinogenesis (Schulte-Hermann et al. 1994). Therefore, the induction of apoptosis is known to be an efficient strategy for cancer therapy. One of the main regulators of cell growth and death is the tumour suppressor protein p53. Normal cells express very low levels of p53 protein, but various cellular stress stimuli lead to a rapid increase of the p53 protein level which then accounts either for growth arrest or programmed cell death (Sionov and Haupt 1999). These results prompted

the authors to further evaluate the anticancer activity in vitro and clarify the mechanism of its antiproliferative activity. This study demonstrated the apoptosis mechanism induced by *M. citrifolia* was modulated through the elevation of p53 level in the cells.

Materials and methods

Preparation of *Morinda citrifolia* extract

The mature fruit of *M. citrifolia* was obtained from MARDI station, Kundang, Selangor. The fruit was washed and rinsed thoroughly before slicing followed by drying in the oven at 60 °C for 3 days. The dried slices were then ground to powder and stored at -20 °C. The extract was prepared by soxhlet extraction method with petroleum ether for 5–8 h. The remaining solid waste powder of *M. citrifolia* was re-extracted with methanol for another 6–8 h. After that, the methanol extract was partitioned with chloroform in separation funnel as described in Kharis and Azimahtol (2003). The fraction obtained was concentrated in a rotary evaporator and placed in fume hood to remove remaining solvent residue in the extract (Figure 1). The dried extract was then stored at -20 °C.

Cell culture

Human lung adenocarcinoma cell NCI-H23 was purchased from American Type Culture Collection (ATCC) and grown as

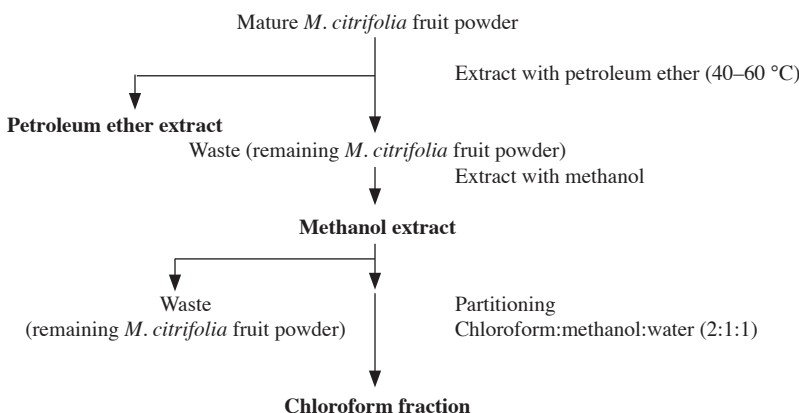


Figure 1. Extraction and partition scheme flow chart of *Morinda citrifolia*

monolayer in RPMI-1640 nutrient medium supplemented with 10% FBS and antibiotics. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C. The medium was changed for every 2 days intervals.

Antiproliferative assay

Cell viability for use in antiproliferative assay was measured employing 0.05% methylene blue according to Lin and Hwang (1991). The percentage of the viable cells after treatment was plotted against different extract concentrations. The EC₅₀ value of the extract was determined from the graph at 50% cell kill. Only the cell with 80% or higher viability would be used in the experiment.

Hoechst 33258 staining

The Hoechst 33258 staining assay was used according to Hishikawa et al. (1999). The lung adenocarcinoma (NCI-H23) cells grew to confluent on poly-L-lysine slide in the six wells plate. The confluent cells were treated with *M. citrifolia* extract at EC₅₀ value of concentration and incubated for 24 h. DMSO (1%) was used as negative control. After incubation, cell was fixed with 4% of cold p-formaldehyde. The cell was then rinsed twice with phosphate buffer saline. A few drops of 0.2% Triton-X was added and incubated for 10 min at 4 °C. Once again, the cell was rinsed with phosphate buffer saline twice followed by staining with 10 µM of Hoechst 33258 for one hour. The stained cell was then rinsed with distilled water prior to microscopic observation under 480 nm.

Treatment of *Morinda citrifolia* extract and protein isolation

NCI-H23 cells at a density of 2 x 10⁵ were grown monolayer in a 175 cm² culture flask until confluent. The confluent cells were then exposed to *M. citrifolia* extract for different time periods of 24, 48 and 72 h at EC₅₀ value concentration as reported earlier (Kharis and Azimahtol 2003). The treated

cells were harvested using cell scrapper and centrifuged at 4,000 g for 5 min. To prepare proteins for immunoblotting, untreated or treated cells with *M. citrifolia* extract were lysed in protein extraction buffer (0.1 M 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-thawing the cells pellet in liquid nitrogen and followed by sonication for 10 min. The cell was then centrifuged at 14,000 g for 10 min to isolate the protein for western blot. The protein concentration was determined according to Bradford (1976).

Western blotting analysis

Equal amounts of sample lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 28 mA for 90 min and electrophoretically transferred onto Polyvinylidene Difluoride (PVDF) membrane (Amersham Pharmacia Biotech), power supplied with 200 mA and 100 V for 3 h at 4 °C. Efficiency of transfer and equal loading of proteins were confirmed by staining membranes with Coomassie blue. The transferred membrane was blocked with 5% non-fat dry milk in phosphate buffer solution, PBS (pH 7.5, 0.1% Tween-20) overnight. After extensive washing with PBS, the blot was used for probing.

The expression of p53 protein was done by probing the transferred membrane with specific primary monoclonal mouse antibodies IgG (antihuman p53) for 2 h and mild agitated (50 rpm). After washing with PBS several times, the membrane was re-incubated with secondary antibody IgG conjugated anti-mouse HRP (Horseradish Peroxidase) at 1:20,000 dilutions for 2 h at room temperature. Signals of proteins were detected using 'Chemiluminescence Western Lightning Chemiluminescence Reagent Plus' (Perkin Elmer) and exposed to x-ray film using Kodax casset for 10 min in the dark room (Towbin et al. 1979).

Results and discussion

Results from the study found that *M. citrifolia* extract has the potential to inhibit the growth of human lung adenocarcinoma NCI-H23. The cell viability of NCI-H23 decreased significantly after treatment with *M. citrifolia* extract. The EC₅₀ value of human lung adenocarcinoma NCI-H23 after treatment with *M. citrifolia* extract for 36 h was 29 µg/ml. Complete loss of cells could be observed with higher dose or longer exposure of *M. citrifolia* extract. Damnachanthal, a known anticancer substance isolated from *M. citrifolia* root that was used as a control (positive) in the experiment, showed stronger inhibition to NCI-H23. To confirm that the inhibition effect of *M. citrifolia* was due to the induction of cell apoptosis, DNA fragmentation, the hallmark of cell apoptosis, was analysed in NCI-H23 cells after *M. citrifolia* treatment.

The cell death induction exhibited fluorescence signals in the cells nucleus as determined with the Hoechst 33258 staining assay. Morphological changes compatible with apoptosis (chromatin condensation and DNA fragmentation) were observed in the cells treated with *M. citrifolia* extract. Nuclear chromatin began to condense and could be seen as fluorescence signal after 24 h (Plate 1). The chromatin condensation observed in the cells is the important characteristic feature during early apoptosis, where the endonucleases process become active and will cleave DNA into fragments with a well-defined length of about 180–200 base pairs (Wyllie et al. 1980).

The cells treated with *M. citrifolia* extract increased the membrane permeability to Hoechst 33258 compared to non-treated cells (DMSO). The negative control treated with 0.1% DMSO did not react with Hoechst 33258 and the whole cells were dyed green without fluorescence (Plate 2). This indicated that no apoptosis occurred in the negative control cells as reported by Kim et al. (1999). Usually Hoechst 33258 staining is effectively used to determine

apoptosis at early stage. Therefore the results have proven the apoptosis induction in NCI-H23 by *M. citrifolia* extract.

Several evidences demonstrated the direct DNA damage by external factors that caused stresses which could lead in p53 protein accumulation and activation. To explore the possible role of p53, the expression of p53 protein was examined by Western blot analysis. In the study, confluent cells were used and treated with *M. citrifolia* extract. By trypan blue method, the cells viability used in the experiment were 90% viable. SDS-PAGE gel electrophoresis of

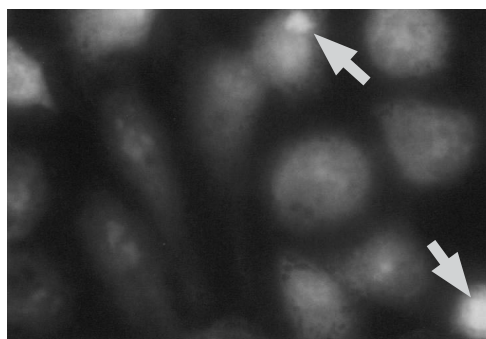


Plate 1. NCI-H23 cells treated with *Morinda citrifolia* extract at concentration EC₅₀ (29 µg/ml) demonstrate margination of chromatin condensation (shown by arrows) in cell nucleus after staining with Hoechst 33258. The cells viewed under Zeiss Axioskope fluorescence microscope using the 520 ± 20 nm filter under X1000 magnification

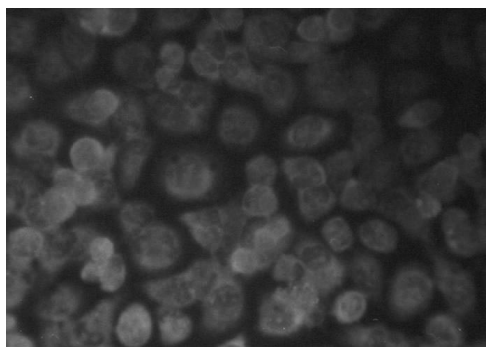
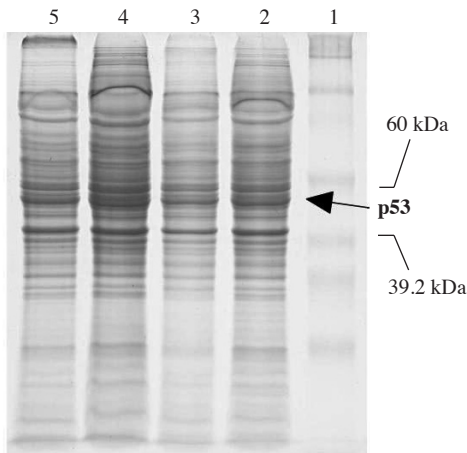


Plate 2. The negative control (0.1% DMSO) of NCI-H23 cells after staining with Hoechst 33258. The cells viewed under Zeiss Axioskope fluorescence microscope using the 520 ± 20 nm filter under X400 magnification

crude protein extract showed separation of p53 protein between 39.2–60 kDa using a mix protein marker BlueRanger (m.w 215kDa–18.3kD) (Plate 3).

The NCI-H23 cells treated with the *M. citrifolia* extract showed increment in expression level of p53 protein during apoptosis. The p53 levels were measured by quantitative Western blot analysis after normalizing with β -actin content. Monoclonal mouse antibody IgG (anti-p53)



Lane 1: Protein mix BlueRanger marker
 Lane 2: Negative control (0 h)
 Lane 3: *M. citrifolia* extract (24 h)
 Lane 4: *M. citrifolia* extract (48 h)
 Line 5: *M. citrifolia* extract (72 h)

Plate 3. SDS-PAGE electrophoresed of NCI-H23 cells crude protein extracts stained with Coomassie Blue. The p53 protein bands size separated between 39.2–60 kDa bands marker

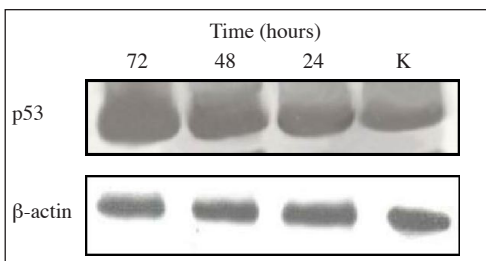


Plate 4. Treatment of *Morinda citrifolia* elevated the expression of p53 in a time dependent manners in NCI-H23 cells as compared to control. The blots were re-probed with anti- β -actin antibody to confirm equal protein loading

was used and detected by probing with secondary antibody IgG conjugated with HRP (Horseradish Peroxidase).

Upon 24 h of treatment with *M. citrifolia* extract at EC_{50} concentration of 29 μ g/ml, the results showed an increment of p53 in apoptosis cells compared to control at 0 h. After 48 h, the expression level of p53 was markedly increased. Further treatment with *M. citrifolia* extract at 72 h observed little increment in expression of p53 level as compared to former hours (Plate 4). Densitometric analysis of the p53 bands found the densitometric relative intensity values of p53 band concentration were 1.19 ± 0.082 (24 h), 1.90 ± 0.05 (48 h) and 2.05 ± 0.064 (72 h) compared to control (0 h) (Figure 2).

Tumour suppressor gene of p53 plays a central role in the cellular response to genotoxic stress. Activated wild-type p53 may lead to the onset of apoptosis or alternatively turn on the DNA repair machinery leading to the completion of the cell cycle. It is believed that induction of p53-dependent cell growth arrest permit DNA to repair. However, when damage is irreparable and the DNA repair machinery fails, the multi-step apoptotic process is induced. Therefore, p53 is essential for the maintenance of the normal genome integrity and stability.

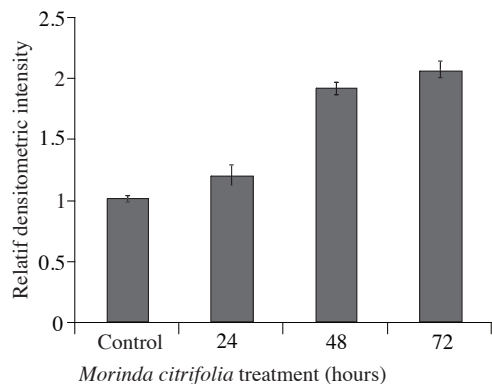


Figure 2. Relatif densitometric intensity value of p53 protein in NCI-H23 treated with chloroform fraction. Values are shown as the means of three separate experiments; $p < 0.05$

In normal unstressed cells, p53 protein exists at a low level as monomer or homodimer, most likely in the cytoplasm. The p53 plays a role in mediating the response to DNA damage, by orchestrating both G1 growth arrest and induction of apoptosis. Upon certain stresses, the level of p53 protein in the cell increases rapidly and its transcription factor activities are increased. Activation of p53 protein can be transcriptional, translational, post-translational, and subcellular distributional (White 1996).

Detailed studies on p53-network have already revealed hundreds of genes and proteins in fulfilment of p53 function. The p53 stimulates a wide network of signals that act through two major apoptotic pathways. It can induce apoptosis either by the sequence-specific transactivation (SST) function or by SST-independent pathways (Sionov and Haupt 1998; Fridman and Lowe 2003; Sax and El-Deiry 2003).

Proteins which are directly transcriptional activated by p53 and promote apoptosis are: Bax, Puma, Noxa, Bid and Apaf-1. All these proteins are involved in the intrinsic apoptotic pathway. The extrinsic apoptotic pathway is also regulated by p53, for example Fas/CD95, DR5 and Fas ligand (Fridman and Lowe 2003). The extrinsic, death receptor pathway triggers the activation of a caspase cascade, while the intrinsic, mitochondrial pathway shifts the balance in the Bcl-2 family towards the pro-apoptotic members, promoting the formation of the apoptosome, and consequently caspase-mediated apoptosis (Haupt et al. 2003). The impact of these two apoptotic pathways may be enhanced when they converge through Bid, which is a p53 target (Sax et al. 2002).

Furthermore, p53 can induce apoptosis independently of its transcription activity, for example by repressing the transcription factor E2F-1 (Sionov and Haupt 1999; La Thangue 2003) or by direct interaction with apoptosis inducing proteins such as the helicases XPB and XPD (Wang et al. 1996).

These pathways, however, are crucial for the apoptotic effects of *M. citrifolia* extract to be elucidated. Therefore, further experiments are required to validate the role of p53 towards targeted genes on the *M. citrifolia* extract-induced apoptosis in human lung NCI-H23 cell lines. Based on this study, it is reasonable to postulate that the increased expression of p53 by *M. citrifolia* extract may lead to increase in the activities related to apoptosis induction or to cause cell cycle arrest. It is suggested that the elevation of functional p53 is an important indication in determining its apoptosis pathway.

Conclusion

Treatment of the *Morinda citrifolia* extract found to inhibit proliferation of human lung adenocarcinoma NCI-H23 cells growth. Further analysis as determined with Hoechst 33258 staining assay demonstrated that the mode of cell death was through cell apoptosis. It was found that the mechanism in which the susceptibility of NCI-H23 to apoptosis involved the activation of p53. The p53 level in the cells increased dramatically with incubation time, thus promoted targeted genes to mediate apoptosis in human lung adenocarcinoma NCI-H23 cell lines.

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Abstrak

p53 merupakan protein perencat yang efektif bertindak sebagai elemen utama kepada faktor transkripsi pelbagai tindak balas selular. Ia bertindak mengimbangi pertumbuhan sel dengan kematian sel dalam sistem hidupan dan memainkan peranan penting dalam perencatan tumbesaran kanser dan induksi apoptosis. Kajian menunjukkan p53 diaktifkan apabila isyarat diterima akibat rangsangan daripada tegasan dalaman atau luaran. Ini menyebabkan p53 menahan pertumbuhan atau mengarahkan apoptosis sel. Penglibatan p53 dalam mekanisme apoptosis banyak dilaporkan sebagai punca kematian sel yang diberi perlakuan dengan dadah antikanser. Ekstrak *M. citrifolia* didapati merencat dan mengaruh apoptosis dalam sel adenokarsinoma paru-paru manusia. Kajian mekanisme apoptosis sel NCI-H23 yang diberi perlakuan ekstrak *M. citrifolia* mengesahkan perlakuan ekstrak *M. citrifolia* meningkatkan pengekspresan protein p53 di dalam sel. Peningkatan p53 dalam sel boleh mengaruh proses apoptosis sel sama ada melalui tapak jalan ekstrinsik atau intrinsik melalui proses nyahpolarisasi dan meningkatkan kepekaan sel terhadap pengaruh apoptosis. Penemuan ini mencadangkan aktiviti apoptosis oleh ekstrak *M. citrifolia* melalui modulasi aras p53 yang meningkat di dalam sel NCI-H23 dan seterusnya mengaktifkan kaskad-kaskad yang terlibat dalam mekanisme apoptosis.