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Impact of *Curcuma mangga* Val. Rhizome Essential Oil to p53, Bcl-2, H-Ras and Caspase-9 expression of Myeloma Cell Line

Endang Astuti[1], Retno Sunarminingsih[2], Umar Anggara Jenie[2], Sofia Mubarika[3] and Sismindari[2]

[1]Doctor Candidate, Biotechnology Study Program, Universitas Gadjah Mada, Yogyakarta
[2]Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta
[3]Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta

Abstract

Cancer is a disease, a public health problem, which is found in the world as well as in Indonesia. In general, some of cancer theraphies are ineffective, characterized by the resistance performance of cancer cell line, the exposed normal cell and by the side effects. Nowadays, studies to find the specific and safely anti-cancer drugs were increased by the time. Several studies revealed that *Curcuma mangga* Val. Rhizome contains some secondary metabolites, essential or non-essential oil, which has cytotoxic activities to the cancer cells. Based on these anti-cancer potentials, this study has several aims to recognize anti-cancer selectivity and molecular mechanism by inducting apoptosis and inhibiting myeloma cell proliferation. To *C. mangga* Val. essential oil, immunocyto chemical test was performed to determine the expression of p53, caspase-9, Bcl-2, H-Ras protein while TUNEL test was performed to determine the number of apoptosis cells.

The results of this study shown that anti-cancer molecular mechanism of *C. mangga* Val. essential oil to myeloma cell line was performed by increasing apoptosis; by increasing the expression of pro-apoptosis p53, caspase-9 protein and reducing protein which is increasing proliferation Bcl-2 and H-Ras.

Keywords: *C. mangga* Val., essential oil, myeloma cell line, apoptosis

Introduction

Several studies of *C. mangga* Val. revealed that *C. mangga* Val. contains a lot of non essential oil as well as the essential oil secondary metabolites with cytotoxic to cancer cell activities (Verlianara, 2004; Rumiyati *et al*., 2007). Based on the anticancer potent of *C. mangga* Val. essential oil secondary metabolites, a study to recognize the anticancer molecular action mechanism, by inducing apoptosis and inhibiting proliferation, is necessary to performed.

Cancer disease suffered since there is no equilibrium of cell addition rate (proliferation) and cell loss rate (especially through apoptosis). The changes occur since there is genetical changes especially on the growth gene i.e. oncogene (positive regulator) and oncosupressor gene (negative regulator). The changes of proliferation and apoptosis pattern could be also detected by observing the protein expression (Meiyanto, 1999). Multiple myeloma (MM) is a malignant B cell with an accumulation plasma cell characteristic in spinal column, bone lesion and immuno-deficiention (Hallek *et al*, 1998).

B-cell lymphoma-2 (Bcl-2) protein group contains of pro-apoptosis and anti-apoptosis proteins. Anti-apoptosis containing Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, Bcl-2A1 and Bcl-B cause pro-apoptosis BH3-domain protein (Bim or tBid) inactive (Huang, 2000; Solari *et al*., 2002; Fesik, 2005; Call *et al*., 2008; Kang and Reynold,

***Corresponding author:**
Endang Astuti
Chemistry Department, Faculty of Mathematic and Natural Sciences, Universitas Gadjah Mada, Yogyakarta, Ph. (0274) 545188, email: endangastuti@ugm.ac.id

2009; Wong, 2011l; Apraiz *et al*., 2011). Small number of p53 protein is expressed at normal condition (Wang *et al*., 2003; Lane and Levine, 2010). Concentration of p53 protein as well as the activity were controlled by the cell pressure, i.e. DNA breakage. Ras oncogene contains of H-Ras, K-Ras and N-Ras coding protein with GTPase activity. Ras has a role in cell growth and controling proliferation as well as cell apoptosis processes (Neri *et al*., 1989). Caspase-9 is an iniciator caspase, and apoptosom such as a complex containing Apaf-1, cytochrom c and cofactor dATP/ATP is required to activate it. Cytochrom c induces dATP binds to Apaf-1 (apoptotic protease activating factor 1) (Jiang and Wang, 2000). Cytochrom c has an important role in apoptosis, since the cytochrom c and dATP induce a Apaf-1/caspase-9 complex formation, in addition activating caspase efector i.e. caspase-3 and caspase-7. CARD (N-terminal caspase-recruitment domain) of Apaf-1 in the apoptosom interacts to procaspase-9 and makes zymogene procaspase-9 activated. Oncoprotein Bcl-2 will inhibit apoptosis and avoid the cytochrom c extrication from mitochondria (Tait and Green, 2010).

To study the anticancer molecular mechanism of *C. mangga* Val. essential oil to myeloma cell, the immunocytochemical test to p53, Caspase-9 protein expression and apoptosis tests to recognize the impact to apoptosis, as well as to Bcl-2 and H-Rasprotein for proliferation.

Material and Methods

Cancer cell lines: $NaHCO_3$ (Merck), trypsin (Gibco), washing solution 99% RPMI 1640 (Roswell Park Memorial Institute 1640)(Gibco), 1% penstrep (Penicillinand Streptomycin)(Gibco), culture media containing 89% RPMI, 1% penstrepand 10% FBS, nitrocellulose filter paper 0.22 μm (Whatman), MTT solution i.e. MTT (3-(4,5-dimethyltiazole-2-il)-2,5-dipheniltetrazolium bromide (Sigma) was diluted in PBS (phosphate buffer saline) at concentration of 5 mg/ml, formazan solution 10% SDS (sodium dodesylsulfate)in 0.01 N HCl, yellow tip, blue tip (Boeco, Brand), DMSO (dimethyl sulphoxide) (Merck). Apoptosis tests using TUNEL method (terminal deoxynucleotidyl transferase dUTP nick-end labeling): fixation reagent: 4% paraformaldehide (PFA) in PBS, PBS, ethanol, preparate glass containing APES (aminopropyl-triethoxysilane), TUNEL reagent i.e. TUNEL label mix (fluorescein-dUTPand –dNTP) and TdT (terminal deoxynucleotidyl transferase), anti fluorescein TUNEL POD (Roche Diagnostics Indianapolis), Cardassian DAB Chromogene Substrate (Sigma), Hematoxylin (Dako), Xilena (Sigma). Immunocytochemical tests: monoclonal antibody p53, Bcl-2, Caspase-9 and H-Ras (Lab Vission), micro platee24 wells (Iwaki), ethanol (Merck), hydrogen peroxide (Merck), phosphate buffersaline (PBS) (Novo Castra), background erasers (protein blocker) (Trekkie Universal Link), TrekAvidin-HRP (label), Cardassian DAB Chromogen Substrate (Sigma), hematoxylin (Dako), cylene (Sigma), absolute alcohol (95%), aquadest, micro slide poly-L-Lysine (Muto Glass), deck glasses.

Rhizome and branches of *C. mangga* Val. 9 month oldsamples were separated, rinsed and thinly sliced to enlarge the contact of surface area and water. In order to facilitate the water vapour to cell wall and evaporate essential oils, the samples were air dried for 3 days. Steam distillation was then performed. For isolation method, water-steam distillation for 6 hours were used.

Apoptosis test of *C. mangga* Val. essential oil samples were performed by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) method (Negoescu *et al*., 1996). Each of 24 wells was completed with glass slide (object glass) and added with 200 μL cancer cells with density of 20.10^4cells/well). In addition, plate was incubated at 37 °C and flowed by 5% CO_2 for 30 minutes to promote cell attachment on the object glasses. As much as 300 μL media were added to each well and incubated at 37

°C with 5% CO_2 for 24 h. The incubated plate was then added with sample at concentration of 500; 250; 125; 125; 62.5; 31.25 µg/mL and as a control cell, as much as 500 µL media were added to 1 well. Plate was then incubated at 37 °C with 5% CO_2 for 24 h. When incubation completed, supernatant of each well was removed. The object glasses were rinsed three times by immersing to chamber with 300 µL PBS, and followed with air dried. Cells on the object glass were fixed using 1mL PFA 4%, incubated for 20 minutes at room temperature, rinsed three times with 1 mL PBS for 2 minutes per each rinsing. In addition, the object glasses were rinsed with 1 mL ethanol 50% for 5 minutes, followed with 1 mL ethanol 70% for 5 minutes and 1 mL ethanol 95% for 5 minutes, and then air dried. The object glasses were placed on the 24 wells plate and stored in the freezer at -85 °C. In addition, the object glasses were rinsed with sterille H_2O, added with 20 µL Tunel label mix and TdT. The object glasses were placed on wet tissue as a base, closed with aluminum foil and incubated for 30 minutes at 37 °C. The object glasses were then rinsed with PBS two times, and deeply added with RNA solution, then incubated for 30 minutes at 37 °C. Another rinsing step with PBS was performed two times by adding 20 µL anti fluorescence (Tunel POD) with incubation for 30 minutes. Afterwards, the object glasses were rinsed with PBS three times, and then excess Cardassian DAB Chromogene Substrate was added to each cell and keep for 5 minutes. The object glasses were then rinsed with aquadest, the cell stained was performed with hemaxtocylin and keep for 30-60 seconds. The object glasses were then rinsed with tap water, 1%PBS solution and aquadest. The dried cells were then added with 95% ethanol. Xylene was added to each dried cell and covered with deck glass. The expression was then observed using light microscope. The apoptosis marked by dark-brown colorcell, while the un-apoptosis cell showed by blue color. Apoptosis cells percentage was then analyzed by calculating the number of apoptosis cells and the un-apoptosis cells. The calculation was performed by using microscope.

Immunocytochemical test was performed using p53, Bcl-2, Caspase-9 and H-Ras monoclonal antibody. Myeloma cells (density: 10^4/100 µL) were prepared in plate 96 wells, 100 µL media RPMI were added to one (1) well, another three wells added with the most sensitive of cancer cell extract/macerate/destilate with three (3) variation concentrations around of IC_{50} value. Afterwards, the plate was incubated at 37 °C with 5%CO_2 for 24 h. When incubation completed, media was discharged and the cells were removed by adding 100 µL trypsin, followed by re-suspension, then transfered to 1,5 mL micro tube, centrifuged at 1500 rpm for 5 min, and rinsed with 200 µL PBS two times. The supernant was discharged and the precipitate was then re-suspended with 30 µL RPMI media. First step of immunoctyochemical process in this study is myeloma cell fixation using 4%paraformaldehyde (PFA) for 20 min. Preparate was then incubated in hydrogene peroxide for 10-15 min. The cells were then rinsed with PBS two times, each for 3 min, added with monoclonal antibody (primary antibody) and incubated for 1 h at minimum, followed with cell rinsing by PBS four times. The cells were added with Biotinylated Goat Anti-Polyvalent (secondary antibody), incubated for 10 min at room temperature and rinsed with PBS four times. Afterwards, the cells were then added with Streptavidin Peroxidaseand also incubated for 10 min at room temperature, and rinsed with PBS four times. The cells were added with DAB (chromogene), incubated for 3-8 min, and rinsed with aquadest. The preparate was then embedded in hemaxtocylin for 3-4 min, rinsed with aquadest, and stick on the deck glass. Chromogene 3'-3' diaminobenzidine (DAB) was used as a horseradish peroxidaseenzyme substrate, the product visually can be seen as brown color (Graham, Jr. and Karnosky, 1966). Peroxidase and hydrogene peroxide (H_2O_2)

will oxidating DAB to brown unsoluble water polymer product (Dannenberg *et al*., 1994). Gene expressed cells showed a dark brown color, while the non-ones were blue. The results of immunocytochemical tests were presented as percentage, as a cells number of positive expressed gene to 100 cells calculation (Burry, 2010).

Results and Discussion

Results of this study are presented on Table 1 and 2, while the apoptosis myeloma cancer cell percentages by the addition of rhizome-branches mixture *C. mangga* Val. essential oil at various concentration are presented on Table 3.

In majority, MM patient (74-100%) and MM cancer cell have excess Bcl-2 expression. The high concentration of Bcl-2 protein causes MM cell be non-apoptosis and resistant to several medicines (Hallek *et al*., 1998). However, the results on Table 2 and Figure 1 showed that only 5.79%of myeloma cancer cells without *C. mangga* Val. essential oil (control) expressing Bcl-2.

This distinction is possible since the cell culture has grown repeatedly, then epigenetic changes may occurs. The epigenetic changes is a spontaneous changes caused by sensitive cell culture to enviromental changes (Rubin, 1993). The addition of *C. mangga* Val. essential oil at various concentrations as well as with various parts will only slighlty increase the Bcl-2 expression, therefore it is expected to increase the apoptosis. The low results of Bcl-2 expression by the addition of *C. mangga* Val. essential oil is possible caused by the BH3 protein activation which is inhibiting Bcl-2 protein anti-apoptosis or activating pro-apoptosis protein such as Baxor Bak.

The results of p53 protein expression on myeloma cells are presented on Table 1,2 and Figure 2. The results showed that without any addition of *C. mangga* Val. essential oil, p53 protein was only expresseed 10.17%. The low of p53 expression indicates *p53* gene mutation. This results is correspond to Ozaki and Nakagawara (2011) studies, which is found more than 50% cancer patient with p53 gene mutation and malfunction. Mutation on *p53* gene is usually occurs at final stadium (final level) with 20-40% prevalence which will inhibit apoptosis and cause MM cell differentiation (Hallek *et al*., 1998).

Myeloma cell by the addition 250 µg/mL of *C. mangga* Val. branch essential oil could increase the p53 protein expression to 64.79%. By the increasing of p53 protein, it

Table 1. Protein expression (%) of Bcl-2, p53, and Caspase-9 in myeloma cell by the addition of rhizome, branches, and rhizome-branches mixture essential oils of *C. mangga* Val.

Essential oil	Protein expression (%)					
	Bcl-2		p53		Caspase-9	
	+	-	+	-	+	-
Control	5.79	94.21	10.17	89.83	3.55	96.45
Rhizome	3.11	96.89	36.51	63.49	67.62	32.76
Branches	14.83	85.16	93.86	6.14	61.04	38.96
Mixture	26.32	73.68	70.08	29.91	33.77	66.23

Note; Sign (+) is positive expression cell (brown color) dan (-) is negative expression cell (blue color)

Table 2. Protein expression (%) of Bcl-2, p53, Caspase-9 and H-Ras in myeloma cell by the addition of rhizome-branches mixture essential oils of *C. mangga* Val. at various concentration

Essential oil concentration (µg/mL)	Protein expression (%)							
	Bcl-2		p53		Caspase-9		H-Ras	
	+	-	+	-	+	-	+	-
Control	5.79	94.21	10.17	89.83	3.55	96.45	5.50	94.50
250	6.03	93.97	64.79	35.21	100	0	4.52	95.48
125	3.21	96.79	6.77	93.23	26.58	73.42	8.67	91.33
62.5	22.26	77.74	3.83	96.17	8.68	91.32	28.53	71.47

Note; Sign (+) is positive expression cell (brown color)dan (-) is negative expression cell (blue color)

Table 3. Myeloma apoptosis percentage by the addition of rhizome-branches mixture essential oils of *C. mangga* Val. at various concentrations

Concentration essential oils of *C. mangga* Val. (µg/mL)	Apoptosis (%)	
	+	–
Control	0.08	99.12
500	1.53	98.47
250	16.39	83.61
125	40.38	59.62
62.5	0.07	99.93
31.25	4.01	95.99

Note; Sign (+) is positive expression cell (brown color) dan (-) is negative expression cell (blue color)

is expected to have p53 with a function as a normal cell as well as apoptosis increased.

In apoptosis, the p53 has a function to control Bcl-2 protein group activities, direct and indirectly. The Bcl-2 protein group controlling apoptosis by influencing mitochondria permeability and inhibiting cytochrome c extrication. The increasing of p53 expression is suppresing Bcl-2 as well as Bcl-xL expression and activity. Therefore the pro-apoptosis protein i.e. Bad, Bid, Baxand Bid are epressed in cytosol, and then transferred to mitochondria and promote cytochrome c extrication. Furthermore, p53 protein inducting Bax, Puma and Noxa which are directly inhibit Bcl-2 (Hemann and Lowe, 2006), therefore the increasing of p53 expression by the addition of *C. mangga* Val. essential oil in this study was not followed by the increasing of Bcl-2 expression. The increasing of p53 expression myeloma cell by the addition of *C. mangga* Val. essential oil will activate Bax protein and inhibit Bcl-2 expression.

Ras oncogene mutation of multiple myeloma (MM) patient is performed in *N-Ras* gene (32-54,5%) and*K-Ras* (27-54,5%), however there is no *H-Ras* mutation (Neri *et al*., 1989; Liu *et al*., 1996; Bezieau *et al*., 2001). Different result was mentioned by Crowder *et al*. (2003), there are mutation in nucleotide GTP binding on *H-Ras* gene MM

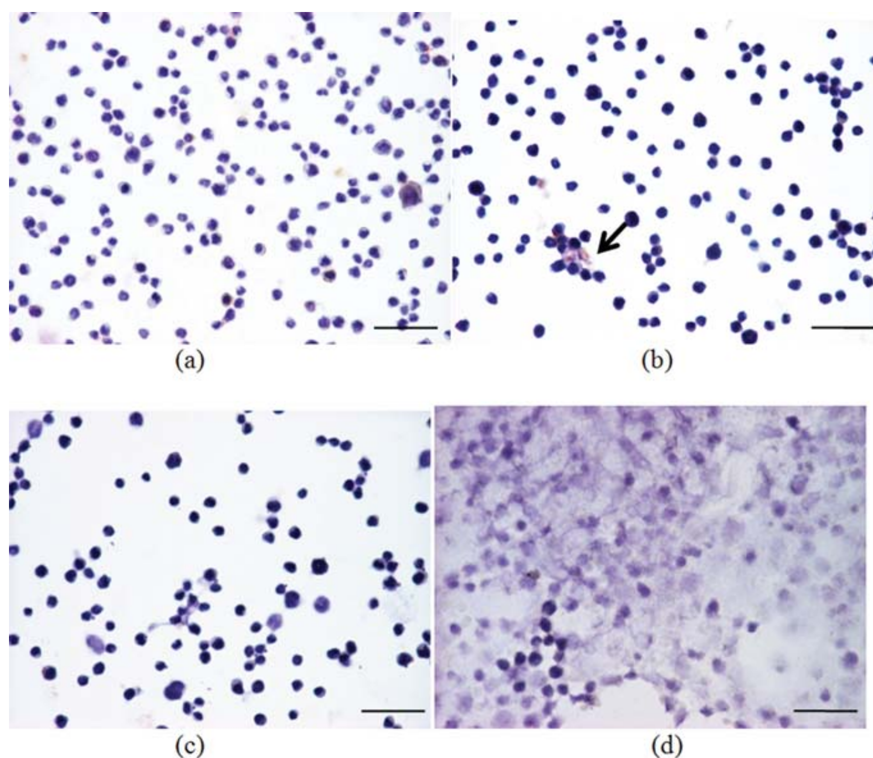

Figure 1. Protein expression of Bcl-2 myeloma cell by adding the rhizome-branches mixture essential oils ofC. *mangga* Val., (a) control, (b) 62.5 µg/mL, (c) 125 µg/mL and (d) 250 µg/mL (200x magnification). Sign (→) is positive expression cell (brown color) in cytoplasm and scale (—) is equal with 50 µm.

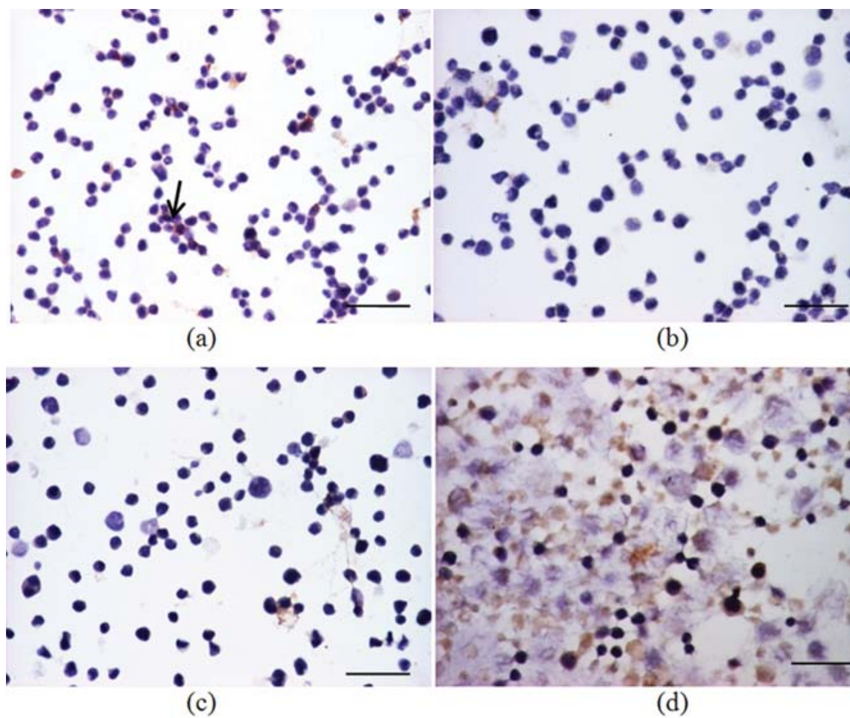

Figure 2. Expression of p53 protein myeloma cell by the addition of rhizome-branches mixture essential oil, (a) control, (b) 62.5 µg/mL, (c) 125 µg/mL and (d) 250 µg/mL. Magnification of 200x. Sign (→) is positive expression cell (brown color) in nucleous and cytoplasm and scale (—) is equal with 50 µm.

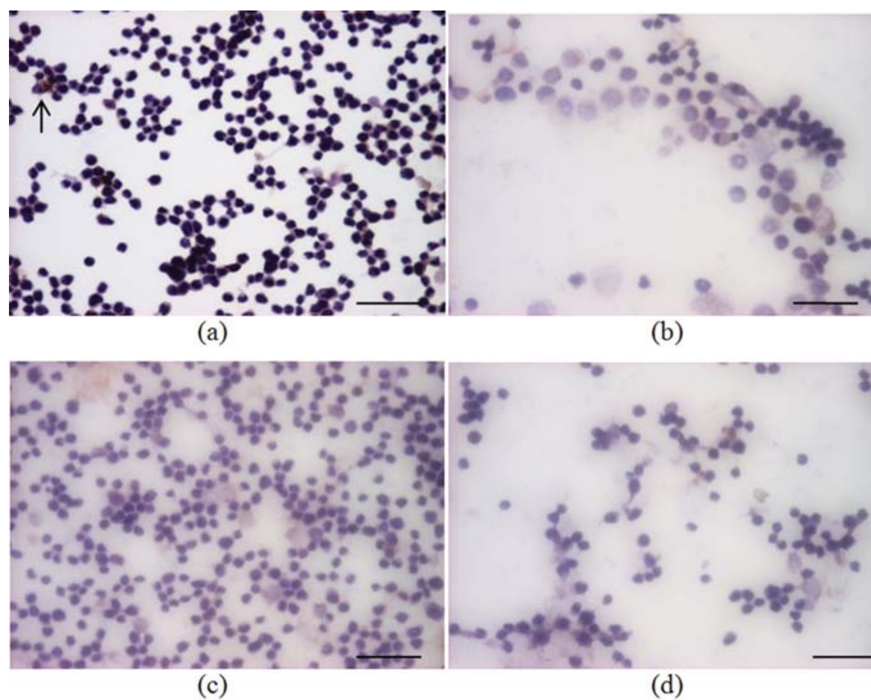

Figure 3. Expression of H-Ras protein myeloma cell by adding rhizome-branches mixture essential oil, (a) control, (b) 62.5 µg/mL, (c) 125 µg/mL and (d) 250 µg/mL. Magnification of 200x. Sign (→) is positive expression cell (brown color) on the cell membrane and scale (—) is equal with 50 µm.

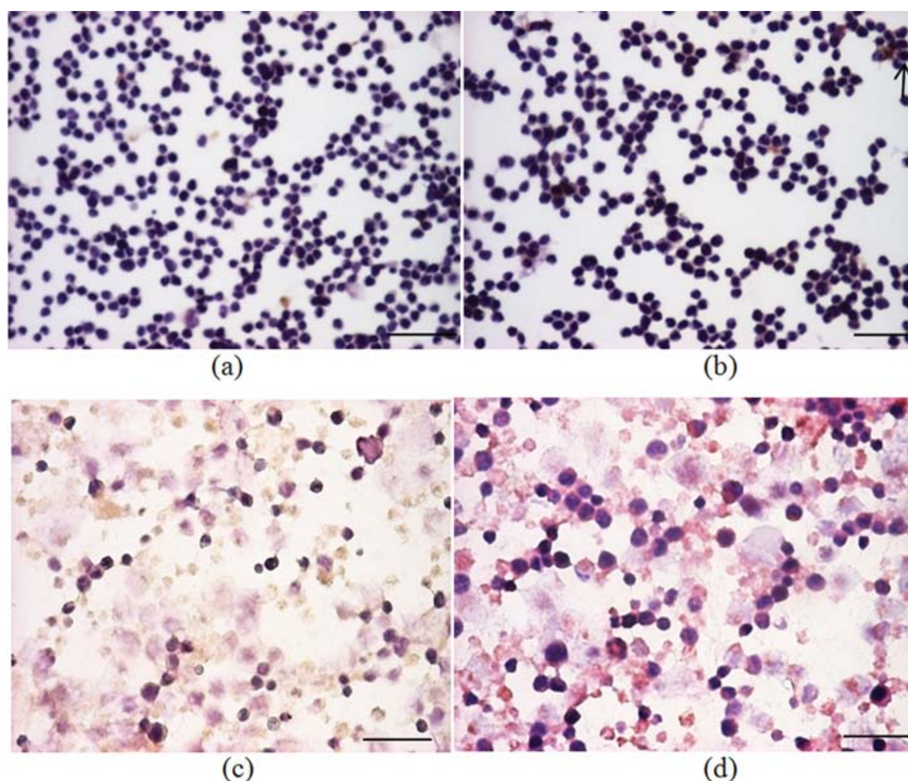

Figure 4. Expression of caspase 9 protein myeloma cell by adding rhizome-branches mixture essential oil, (a) control, (b) 62.5 µg/mL, (c) 125 µg/mL and (d) 250 µg/mL. Magnification of 200x. Sign (→) is the positive expression cell (brown color) on cytoplasm and scale (—) is equal with 50 µm.

cell. Rasis anti-apoptosis and pro-apoptosis through different ways. Rasis pro-apoptosis protein will activate Raf, MEK, ERK, RSK which inhibiting anti-apoptosis Bad, Bcl-2 and Bcl-xL protein activities. Contrary, as an anti-apoptosis protein, Ras inducting PI3-K and AKT which will inhibit caspase-9 and Bad activity (Cox and Der, 2003). H-Ras oncogene has a function as pro-apoptosis protein. Activity of H-Ras pro-activity colon cancer cell HT29 increases with the addition of deaceytlation hyston (HDACi) inhibition. HDACiassists caspase-3, -7 and -8 activations (Rajasekharan and Raman, 2013).

The study results (Table 1, 2 and Figure 3) shown that there are no mutation on *H-Ras* gene myeloma cell since the H-Ras expression on the cell without any addition of *C. mangga* Val. essential oil was only 5.5% and the addition of *C. mangga* Val. essential oil reach of 250 µg/mL concentration was not increase H-Ras significantly. Based on these results, the role of H-Rasas anti-apoptosis was low while it was high as pro-apoptosis protein on myeloma cell, without and with *C. mangga* Val. essential oil addition.

Immunocytochemical test results of caspase-9 antibody are presented on Table 1, 2 and Figure 4. The results of this study shown that the *C. mangga* Val. essential oil has a possibility to increase of caspase-9 expression in range of 3.55% without any treatment to 100% or all of the cell express caspase-9 by the addition of 250 µg/mL *C. mangga* Val. essential oil. The high expression is also caused by the low of Bcl-2 expression (6.03% of 250 µg/mL essential oil), therefore Bcl-2 blocks the cytochrom c extrication from mitochondria.

Two cell death types are apoptosis and necrosis. Apoptosis or cell death program is a program used to control the cell number and quality. The results of this study (Table

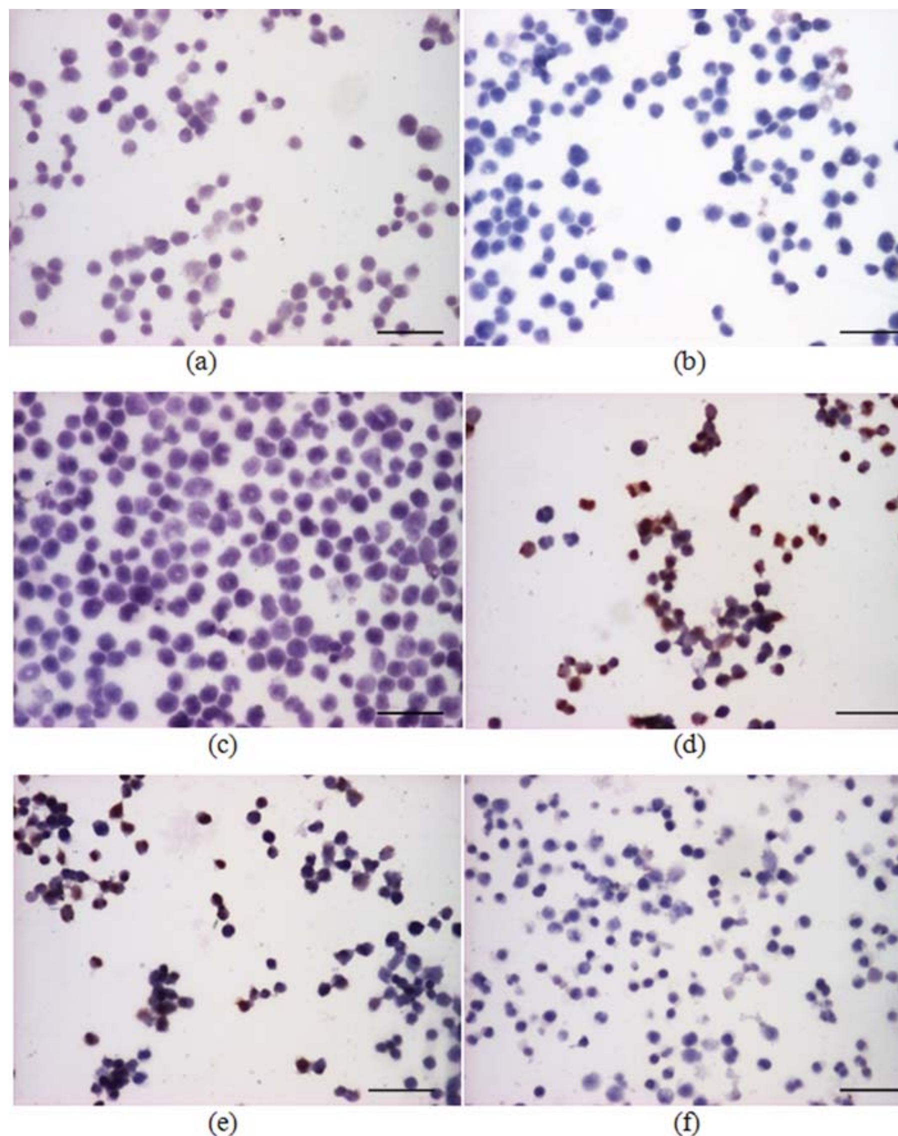

Figure 5. The apoptosis myeloma cell by the addition of rhizome-branches mixture essential oil, (a) control, (b) 31.25 µg/mL (c) 62.5 µg/mL, (d) 125 µg/mL, (e) 250 µg/mL and (f) 500 µg/mL. Magnification of 200x. Sign (→) is the positive expression cell (brown color) on cytoplasm and scale (—) is equal with 50 µm.

3 and Figure 5) shown that no apoptosis on the myeloma cell without any addition of *C. mangga* Val. essential oil.

The addition of 125 µg/mL *C. mangga* Val. essential oil causes the increasing of death cell 40.38%, while no apoptosis induce (0.07%) at concentration of 62.5 µg/mL. The addition of 62.5 µg/mL essential oil was not induce pro-apoptosis p53 protein expression by resulting result only 3.5% and for caspase-9 was 8.13%. Beside of that, the anti apoptosis Bcl-2 protein expression was high enough of 22.26% and H-Ras was 26.58%. The main apoptosis ways are extrinsic and intrinsic ways (mitochondria way). Characteristic of intrinsic way is the mitochondria malfunction through cytochrom c extrication, caspase-9 activation, followed by caspase-3 and 7. In addition, by the cell surface activation through death receptor which is activating caspase-8 or caspase 10, followed by the increasing of caspase-3 is a characteristic of extrinsic way.

Conclutions

The *C. mangga* Val. essential oil increased the protein expression of pro-apoptosis p53, caspase-9 and reduced the protein expression Bcl-2 and H-Ras which stimulate proliferation of myeloma cell line. The increasing of myeloma cell line apoptosis was observed by the addition of *C. mangga* Val. essential oil through mitochondria ways (intrinsic).

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