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The expression of p53 and hsp70 proteins after treatment with Annona muricata Linn leaf for activating apoptotic and lead to homeostasis program of Raji cells

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Technical Report

Abstract

Purpose: Organic extracts of plant Annonaceae enhances apoptosis in animal cells and get the drives to reach a new homeostasis. The incidence rate of nasopharyngeal cancer in Indonesia is quite high. Protein 53Kd (p53) play a role in apoptosis process, being heat shock protein 70 (hsp70) play a role in homeostasis. The aim of this research is to identify the apoptotic effects of Annona muricata Linn leaf toward Raji cells by observing the p53 and hsp70 expression. **Methods**: Apoptotic assay was performed in 24 wells micro-culture plate. Raji cells were prepared as 2×10^4 cells in 100 ml RPMI media per well. Roswell Park Memorial Institute (RPMI) medium was created and solvent was controlled with Dimethyl Sulfoxide (DMSO) solvent 0.25. Apoptotic test was performed by calculating trypan-blue-dye exclution. The cells were then grown in micro-culture plate with media plus extract non-lethal concentration of partition and fractionation of Annona muricata Linn leaf. The sampling was performed for 24 hours. The number of living cells was calculated in each of these well and incubation time were determined. Immunohistochemical staining was done to identify the expression of p53 and hsp70. **Results:** The results showed that Raji cells treated with partition of Annona muricata Linn leaf in ethyl acetate solvent 133.00 % resulted in higher apoptosis. Another results showed that Raji cells treated with fractionation Annona muricata Linn leaf in ethyl acetate solvent 103.20 % resulted in higher apoptosis. The expression of p53 after treatment with fractionation Annona muricata Linn leaf was higher than before while hsp70 expression after treatment with fractionation Annona muricata Linn leaf was lower. **Conclusion**: The conclusion is the higher the dose of Annona muricata Linn the higher the p53 expression thereby activates apoptosis process The higher dose of Anonna muricata Linn also leads to lower hsp70 expression indicating stable homeostasis of Raji cells.

Keywords: Annona Muricata Linn Leaf; p53; hsp70; Apoptosis; Homeostasis; Raji Cell

Introduction

Drug substance from herbs interacted with virus in the development of cancer do not so by interacting by the agent but rather through a direct effect on cell transformed apoptosis and proliferation. Although the interaction between virus and herbs medicines is plausible larger more biological details studies need conducted to validate this preliminary finding.[1] Annona muricata Linn has been shown to have strong anticancer effect on cancer cells of reporting via apoptosis. However the fundamental mechanism are still unclear.[2] So molecular targeting for the cancer therapy including inactivating virus gene product has been developing and on the way to clinical use.[3] Considering the high potential herbs in managing various disease, need to be researched the Annona muricata Linn, especially in kill nasopharyngeal cancer cells.

Nasopharyngeal carcinoma (NPC) is rare malignancy with an incidence under 1 per 100,000 person-year. The distribution

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of NPC suggest that both environmental and genetic traits factors contribute to its development.[4] This carcinoma has complex aetiology.[5] Nasopharyngeal carcinoma has remarkable epidemiological features including racial and familial aggregations.[6] Prevalence was nearly 60 % of malignant head and neck cancer. Cancer begin with a scene gene expression imbalance in apoptosis and cell proliferation, and DNA repair.[7, 8] Epstein Barr virus infection in early age combined with frequent exposure to carcinogenic co-factor is suggested to cause the NPC development. Higher LMP-1 EBV expression was related to more loco regional progressivity.[9] Given the high number of pain due to nasopharyngeal cancer we need to be controlled in order to keep the quality of human resources.

The role of Annona muricata Linn extract, expression of p53 and hsp70 proteins may be explained with path biological examination.[10, 11] Concept above may provide explanation on the decrease of NPC.

Aetiology of NPC is complex involving genetic susceptibility factor including EBV infection and exposure to chemical carcinogens. Development of the disease is started by a viral infection and continue to change in gene level then occurring change in epigenetic disrupt significantly.[12] Molecular description of NPC are the anti-apoptotic bcl2 is over-expressed and latent membrane protein-1 (Lmp-1) EBV is detected so the last a decline in the expression of p53 significantly.[13] Other studies demonstrate that p53 induce apoptosis is important in cell death after enjury.[14] Heat shock protein 70 also suspected of facilitating a groove apoptosis.[15] As known protein heat shock in a continuous manner and thorough expressed on majority of normal tissue and cancer.[16] An increase of expression hsp70 responsible on protein folding impact on function that lead and play a role in sick pathogenesis.[17, 18, 19] All, it is hope that groove scene carcinoma of the nasopharynx can be controlled using extract leaves Annona muricata Linn with an increase occurrence of an expression of p53 so that mediated apoptosis viable and the decline of expression hsp70 so that homeostasis cells can be achieved.

Methods and Materials

The first stage was to develop the Raji cells culture using Fresney method with many modifications.[20] Apoptotic assay was performed on 24well micro-culture plate. Raji cells were prepare as 2×10^4cells in 100ml Roswell Park Memorial Institute (RPMI) Media. This was followed by the creation of RPMI control and solvent plus Dimethyl Sulfoxide (DMSO) 0.25 %.

Subsequently a precipitate during 24hr in culture medium than the Raji cell were grown in microplate with media plus extract with a non lethal concentration of partition and fractionation *Annona muricata Linn* leaf. Of sampling was peformed for 24hrs. Apoptotic test was performed by calculating tryphan blue dye exclution. The number of living cells were calculate in each of these wells and incubation time were determined.[2, 21]

Immunohistochemical stainings was done with TSA-indirect method (Nen life science product, Renaissence) using monoclonal antibody against p53 (1:500) produce by Dako and hsp70 (1:500) was produced by Stressgen. Photo microscopic was collected using aX100 objective lens (Nikkon).[22]

Results

The first result showed (**Figure 1A and B** as well as **Table 1**) that Raji cell are treated with partition of *Annona muricata Linn* leaf in ethyl acetate the apoptotic rate was higher (2000 µg/ml had 133.00 %; 15.625 µg/ml had 0.00 %) and in ethanol destilate water the apoptotic rate was lower (2000 µg/ml had 42.50 %; 15.625 µg/ml had 0.05 %)

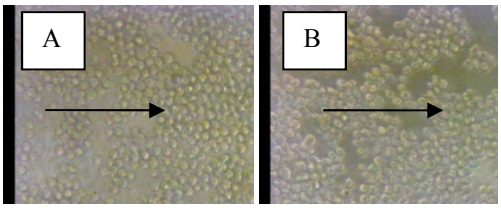

FIG. 1: Raji cells are treated with partition of leaves *Annona muricata Linn*. *Left:* (A) Before treatment and, *Right:* (B) After treatment.

Another results showed (**Figure 2A and B and Table 2**) that Raji cells are treated with fractionation of *Annona muricata Linn* leaf in n-hexan the apoptotic rate was higher (2000 µg/ml have 103.20 %; 15.625 µg/ml had 7.17 %) and in ethanol distillate water the apoptotic rate was lower (2000 µg/ml had 40.24 %; 15.625 µg/ml had 6.06 %).

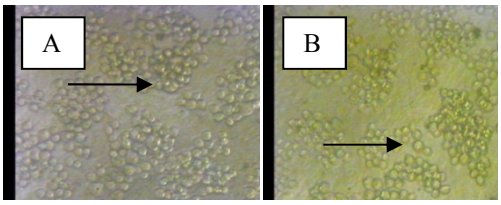

FIG. 2: Raji cells are treated with fractionation of leaves *Annona muricata Linn*. *Left:* (A) Before treatment and, *Right*: (B) After treatment.

The third results in **Figure 2** showed that Raji cells are treated with fractionation of *Annona muricata Linn* leaf so the p53 expression before treatment was lower while the expression after treatment was higher. The results in **Table 3** showed that Raji cells are treated with fractionation of *Annona muricata Linn* leaf in ethyl acetate the p53 strong expression was higher (55 %) and in n-Hexan the p53 strong expression was lower (51 %).

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The third results in **Figure 3** showed that Raji cells are treated with fractionation of *Annona muricata Linn* leaf so the hsp70 expression before treatment was higher while the expression after treatment was lower.

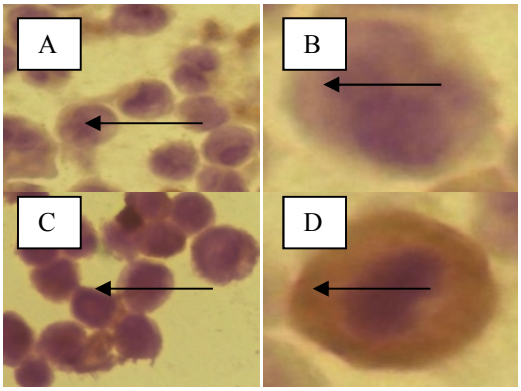

FIG. 2: Raji cells are treated with fractionation of leaf *Annona muricata Linn* so the p53 expression (bluish) before treatment was lower (A: X100 and B: X400) while the expression (brownish) after treatment was higher (C: X100 and D: X400)

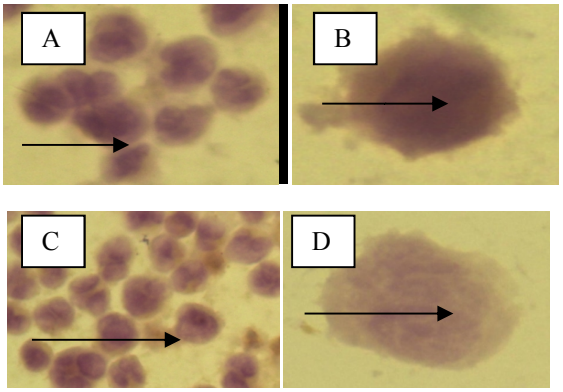

FIG. 3: Raji cells are treated with fractionation of leaves *Annona muricata Linn* so the hsp70 expression (brownish) before treatment was higher (A: X100 and B: X400) while the expression (bluish) after treatment was lower (C: X100 and D: X400).

The results in **Table 4** showed that Raji cells are treated with fractionation of *Annona muricata Linn* leaf in ethyl acetate the hsp70 strong expression was higher (57 %) and in ethanol water the hsp70 strong expression was lower (44 %).

TABLE 1: Raji cells are treated with partition of leaf *Annona muricata Linn* in ethyl acetate the apoptotic rate was higher (2000 µg/ml had 133.00 %; 15.625 µg/ml had 0.00 %) and in ethanol distillate water the apoptotic rate was lower (2000 µg/ml had 42.50 %; 15.625 µg/ml had 0, 05 %).

No.	Concentration	Cell Death Rate (%) In Solvent			
		Ethyl Acetate	n-Hexan	Chloroform	Ethanol Water
1	2000.000 µg/ml	133.00	103.20	81,92	42,50
2	15,625 µg/ml	0.00	7.17	9,27	0,05

TABLE 2: Raji cells are treated with fractionation of leaves *Annona muricata Linn* in n-hexan the apoptotic rate was higher (2000 µg/ml have 103.20%; 15.625 µg/ml had 7.17 %) and in ethanol distillate water the apoptotic rate was lower (2000 µg/ml had 40.24%; 15.625 µg/ml had 6.06%).

No.	Concentration	Cell Death Rate (%) In Solvent			
		n-Hexan	Chloroform	Ethyl Acetate	Ethanol Water
1	2000.000 µg/ml	103.20	92.37	87,55	40,24
2	15,625 µg/ml	7.17	9.27	25,48	6.06

TABLE 3: Immunohistochemistry staining using p53 antibody anti p53 protein in Raji cells that are treated with the active compounds of fractionation *Annona muricata Linn* leaf. The expression of p53 was 55 % strong in ethyl acetate solvent and 38 % strong in n-Hexan solvent.

No	In Solvent	p53			Total
		Tenuous	Midts	Strong	
1	Ethyl Acetate	8 (09 %)	31 (36 %)	47 (55 %)	86 (100 %)
2	Chloroform	7 (12 %)	19 (33 %)	31 (55 %)	57 (100 %)
3	Ethanol Water	9 (20 %)	11 (25 %)	24 (55 %)	44 (100 %)
4	n- Hexane	15 (19 %)	23 (30 %)	38 (51 %)	76 (100 %)
	Mean	(15 %)	(31 %)	(54 %)	

TABLE 4: Immunohistochemistry staining using hsp70 antibody anti hsp70 protein in Raji cells that are treated with the active compounds of fractionation *Annona muricata Linn* leaf. The expression of hsp70 was 57% strong in ethyl acetate solvent and 44 % strong in ethanol water solvent.

No	In Solvent	Hsp70			Total
		Tenuous	Midts	Strong	
1	Ethyl Acetate	5 (15 %)	9 (27 %)	19 (57 %)	33 (100 %)
2	Chloroform	11 (16 %)	18 (27 %)	37 (56 %)	66 (100 %)
3	n- Hexane	7 (15 %)	14 (31 %)	23 (52 %)	44 (100 %)
4	Ethanol water	20 (21%)	31 (33 %)	41 (44 %)	92 (100 %)
	Mean	(18 %)	(30 %)	(52 %)	

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Discussion

The usual approach to cancer prevention are by: prevention of interaction with cancer causing agent, increasing defense mechanism againt cancer and lifestyle modification.[23, 24] A number of herb have been found to inhibit proliferation, supress angiogenesis, retard metastases and enhence chemotherapy, induce apoptosis both in vitro and in vivo.[25, 26] Those studies have shown the possibility of compounds that selectivity kill cancer cells without damaging normal cells. The compound was derived from *Pandanus conoideus* Lam that tested for cancer therapy. The study above was canducted at the level of cellular.[2, 4, 27] Currenly the treatment of cancer focused on how to enhance apoptosis considering has been proven that occur immortalization on cancer.[28] The refugees of apoptosis can occur through multiple pathway. One point that has a close link with cancer through the induction apoptosis is much play by p53 proteins.[29]

The expression of p53 protein is a form of cell respone to a stressor.[29] Decrease expression of the p53 protein can cause a decrease in cell apoptosis mechanism. A protein p53 are known as a cancer inhibitor and proteins that plays an important role in apoptosis regulation. A hallmark of cancer is a decline in apoptosis and increase proliferation of the cells. Fivety five of human cancer lose in p53 function of due to gene mutations and proteins misfolding and finally causes degradation of ubiquitine chaperones.[30, 31, 32, 33] The recovery of p53 function could potentially trigger a mass apoptosis that effectively kill cancer cells

To turn stress gene it is essential in order to respond to stress. Stress (either physical or chemical) also activate or pressing many gene, including the various housekeeping gene. A proteins stress, who temp by stress gene, is critical role in biogenesis physiological proteins.[34] Heat shock proteins continously and thoroughly expressed in the most normal tissue and cancer.[16] and implication for the interaction of many protein folding, such as united states translocation and prevent improper aggregation and degradation. Increased expression of hsp70 responsible for the misfolding of proteins implicated in the fascicle that lead and constribute to the pathogenesis of pain including cancer incidence.[17, 18, 19] Molecules chaperone e.g. hsp70 also play a role in groves the target protein that active in mitochondria, in this the protein folding for the benefit of the activity at mitochondria. Chaperone mitichondria a protein heat shock 70 to faciliting transport protein that goes through membrane mitochondria and do folding in a matrix. The reaction is conducted by two complex $_m$hsp70 different: 1. The form of wich is the form of $_m$Hsp70-ADP Bound complex wich are at the inner membrane mitochondria and complex import consisted of $_m$Hsp70, a fastener tim44 in its membrane and $_m$Hsp20-30.[35] 2. The form of ATP-bound of $_m$Hsp70 is the form of complex who was insite the matrix mitochondria and folding complex consisted of $_m$Hsp70, $_m$Hsp40 homologue (mdj1), and $_m$Hsp20-30.[35, 36, 37]

Acetogenin in the *Annonaceae* is composed of mono-unsaturated acids. As anticancer acetogenin can serve as immunosupressive, pesticide, antiprotozoal and antimicrobial. Acetogenin inhibit mitochondrial to produce ATP, resulting in the production of energy in cancer cells and stop cancer cells growth for eventually die.[38] Acetogenin also very selectived, only attacks cancer cells. These compound do not attck other normal cells in the body.[38] Some derivated, in different type of structures and some isomers showed significant to kill cancer cell line, for example, to fight against prostate cancer. The play mode of action is acetogenin inhibitor of NADH: Oxireductase Uniquinone, the enzyme complex important in oxidative phosphorelation in the mitochondria and inhibits NADH: Oxidase Uniquinone the plasma membrane of the cancer cells.[39]

Conclusion

The higher the dose of *Annona muricata Linn*, the higher the p53 expression, thereby activates apoptosis process. Higher dose of *Annona muricata Linn* also leads to lower hsp70 expression, indicating stable Raji cells homeostasis.

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Conflict of interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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