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Apoptosis of Colorectal Cancer Cell on Sprague-Dawley Rats Induced with 1,2 *Dimethylhidrazine* and *Phyllanthus niruri* Linn Extrac

Endang Sawitri#

Laboratory of Physiology, Medical Faculty, Mulawarman University, Jl. Krayan Kampus Gunung Kelua Samarinda

Email: ensa68@yahoo.com

Abstract *- Apoptosis cell is one of the main biomolecular predictors to determine kind of treatment given to patient with colorectal cancer and to predict the end result. Phyllanthus niruri Linn (P. niruri L) acts as antineoplastic but its potency on the process of cancer cell apoptosis has not been revealed yet. The objective of the research was to evaluate the apoptosis index of rats with colorectal cancer treated with and without P. niruri L. extract. This research used The Randomized Posttest-Only Control Group Design. As many as 30 male Sprague-Dawley rats were induced with 1,2 DMH 30 mg/kgBW once in every week. Four rats were sacrificed at week 9th, 11th and 13th to be observed the development of colorectal cancer. Induction was then stopped and other 18 rats were randomly located into two groups. The first group was positive control (K+) group consisted of 9 rats without P. niruri extract. The second group (X) was consisted of 9 rats with the application of P.niruri L. extract 13.5 mg/kg per day orally. All rats were terminated on week 19th, tumor lesion was proceed for Histopathology preparations and stained with Kit TUNEL-IHC (Apo-BrdU-IHC™ BioVision Cat #K403) to identify the apoptosis cell. Data were analyzed using unpaired t-test with significant level of p<0.05. The result showed that the average of apoptosis index of X treatment was 2.37 ± 0.48 higher than K+ treatment namely 1.45 ± 0.41 with a highly significance difference (p=0.000). Phyllanthus niruri L extract increased the apoptosis of colorectal cancer of Sprague-Dawley rats induced with 1,2 Dimethylhidrazine.*

Keywords— *Phyllanthus niruri Linn,* colorectal cancer, apoptosis, extract

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I. INTRODUCTION

The incidence of colorectal cancer still marks high number until today. More than one million people per year worldwide are diagnosed with colorectal cancer and this malignancy is the fourth leading cancer causes death (Courtney et al., 2013). *American Cancer Society* stated that in 2008 there were 1,2 million cases of colorectal cancer worldwide (Society American Cancer, 2011). It was estimated that about 134.000 new cases with 55.000 deaths per year and this colorectal cancer caused almost 15% deaths due to cancer (Kumar et al., 2012). The number of colon and rectum cancer incidence as well as the deaths number in Indonesia are also still high. Colorectal cancer is the third largest kind of cancer in Indonesia with a total of 1,8 cases/100.000 population and this number is predicted to increase along with lifestyle change of Indonesia people (Dharmais Hospital, 2009). This cancer mostly affects younga ge people with the same incidence comparison on male and female (Sjamsuhidajat, 2002).

1,2 *Dimethylhidrazine* (DMH) is a potential carcinogen for colon and rectum causing mutations on the cells located at the bottom of the crypts. During migration to the lumen surface, cells experiencing mutations will spread along the crypts axis. Colon tissue tries to protect them by triggering the death of the cells in those areas to eliminate cells that could potentially mutate. There might be mutated cells keep survive which at the end they become transformed cells and then migrate into crypt lumen and lead to uncontrolled proliferation, cells accumulation and finally form tumor. Tumor development at colon and rectum associates with cell growth dysregulation and gradual apoptosis inhibitory as in this condition causes tremendous consequence in tumor genesis. This phenomenon indicates that apoptosis is an adaptive responds against the damage triggered by 1,2 DMH

and tumor will grow very quick if cells loss apoptosis ability (Watson, 2006; Samantha et al., 2008; Yang et al., 2009).

Apoptosis is a specific process leading to cell death in a specific mechanism through activation of intracellular pathways that causes cellular change. A process on neoplastic transformation, progression and metastatic involves a normal apoptosis pathways change (Reed, 2003; Watson, 2004). It seems that in the early neoplastic progression, tumor cells become more sensitive to agents that induce apoptosis. Meanwhile, progressive cells will resistance to apoptosis stimulation. Therefore, the majority of chemotherapy agents or radiation uses apoptosis pathways to induce death of cancer cells. The changes on the balance between proliferation and apoptosis become the principal of neoplastic changes. Multiple genetic defect accumulation interfering normal growth control, differentiation and apoptosis pathways becomes the principle on the development of colorectal cancer (Koornstra et al., 2003; Watson, 2006). Detection on apoptosis body which expressed by cancer cells can be done by *Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling* (TUNEL) method at colorectal cancer histological tissue slice (Lifshitz et al, 2001; Koornstra et al., 2003).

The application of traditional medicinal plants gains more popularity along with *Back to Nature* movement including in Indonesia. Indonesia is the second largest country worldwide with the medicinal plants after Brazil thus Indonesia has big potential sources for the development. However, the disadvantages of medicinal herbs in Indonesia are lack of raw material standards and still have few clinical tests that promote the efficacy of those medicinal herbs (Adimoelja, 2002; Mangan, 2005). One of traditional medicinal herbals used in Indonesia is *Phyllantus niruri Linn* (*P. niruri L.*) which included in *Euphorbiaceae* family. This plant is also known as *meniran*, *meniran ijo, memeniran* (Sunda) and *gosau madungi* (Malacca). *Meniran* is widely grown in Indonesia, easy in the preparation, cheap and has minimum side effects (Sulaksana and Jayusman, 2004). *Phyllanthus niruri Linn* riches of chemical contents including of lignans (phyllanthin, hypophyllanthin, nirantin, isolintetralin, nirphylin, phylnirurin, hynikinin, lintetralin and phylantostatin), flavonoids (quercetin, quercitrin, isoquercitrin, astragilin, rhamnopynoside, routine and nirurin), alkaloids, tannins, saponins, triterpenoids, resins, steroids, potassium, vitamin C, fatty acids and phenolic acid (Taylor, 2003; Bagalkotkkar et al., 2006; Markom et al., 2007).

The potency of *P. niruri L* on enhancing immune system modulation has been proven both in experimental animals or humans. Immunology test on mice proves that *P. niruri L* extract works as immune stimulator (Ma'at S, 1996). Clinical test on human also proves that *P. niruri L* extract works as immune modulator (Ma'at, 2010) . The role of *P. niruri L* extract on inhibiting the growth of cancer through apoptosis induction has not been widely explored. In-vitro test on cancer cell line proves that *P. niruri* L extract applied into mamma carcinoma cells culture together with C3H mice mononuclear can reduce the viability of those cancer cells (Chodidjah, 2004). Extract of *P. niruri* L can inhibit the growth of HCT116 and HT29 of human color cancer cell line that measured by using MTT *cytotoxicity assay* method (Sawitri, 2009).

Based on the problem identification as described above, this research was conducted to study the effect of *P.niruri* L extract on the growth and development of colorectal cancer on *Sprague-Dawley* rats induced with 1,2 DMH by evaluating the number of apoptosis cells which expressed to calculate the difference of colorectal cancer apoptosis index between rats given by herbal and not.

II. Material and Method

This study was an experimental research set with *The Randomized Posttest-Only Control Group Design.* Samples were randomly selected from affordable population consisted of 30 of healthy male *Sprague-Dawley* rats raised at Integrated Research and Testing Laboratory Unit IV (Pre-Clinic) UGM, aged of 6-7 weeks with 170-220 grams weight. Samples were induced subcutaneously with 1,2 DMH 30mg/kg at the first day of every week (this procedure followed Dani *et al.*, 2007). At week of 9^{th}, 11^{th} and 13^{th}, four induced rats were sacrificed at every week to be observed the development of colorectal cancer. At week 13^{th}, cancer was found on the four rats so induction was stopped. Other 18 rats were randomly located into two groups. The first group was positive control (K+) group consisted of 9 rats without *P.niruri* extract. The second group (X) was consisted of 9 rats with the application of *P.niruri L.* extract.

The extract used at this experiment was made from the whole part of fresh *P. niruri L* plant which extracted using maceration method with alcohol solvent 70% (ethanol). After passing several processes, ethanol 70% was evaporated and formed solid powder as the extraction result. The administration of extract to rats must be in solution form. *P. niruri L* extract powder was weighed as needed and then diluted with sterile aquades and mixed with shaker until reached homogenous solution. *P. niruri L* extract was given orally to rats with 13,5 mg/kg of BW per day for 30 days.

At week 19^{th}, all rats were terminated; colorectal tissues with tumor lesion were excised and processed in paraffin block, cut into 4 micron thickness and then made for histopathology preparation. The next process was identification of apoptosis cell using TUNEL method. Histopathology preparate was processed and stained with Kit TUNEL-IHC (*Apo-BrdU-IHC™ BioVision Cat* #K403). The evaluation of apoptosis cell was carried out by counting the total cells in 10 crypts column and counting the tumor cells with brown color using TUNEL staining (positive-TUNEL) at 5 visual fields under 400x magnification of light microscope. This method followed Kim and Park (2003). Counting was performed by 2 persons namely the researcher and Patholog with clinical agreement 95%. Apoptosis index was defined as the percentage of positive TUNEL cell per crypt column divided by the average cell per crypt column which counted at 10 crypts with 2 times repetitions and calculated the average.

The entire processes of tissue processing and reading were carried out at Laboratory of Pathology Anatomy of Medical Faculty, UGM/RSUP dr Sardjito Yogyakarta. Data showed normal distribution, therefore data analysis included of univariate analysis and bivariate analysis. Univariate analysis was carried out to determine the average of each group which

illustrated into Boxplot graph. Bivariate analysis was carried out using unpaired t-test to determine the difference result due to treatments given to both groups. Significance level was $p<0.05$ and confidence interval was 95%.

III. RESULT AND DISCUSSION

Apoptosis index in this research was counted from the number of colorectal cancer cells which positively expressed TUNNEL dots divided by total cells number per crypt column. TUNEL staining method was used as it was very sensitive to apoptosis cells so it could easily distinguish cells experiencing apoptosis or not. The result of univariate analysis of apoptosis index of the two experimental groups is shown in Figure 1.

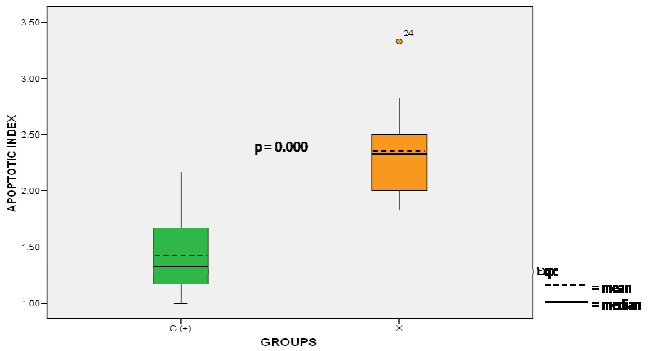

Figure 1. Boxplot graph of apoptosis index of K+ and X group.

Figure 1 shows the apoptosis index average of K+ (induced with 1,2 DMH subcoutan) was 1.45 ± 0.41. The apoptosis index of X group which induced with same carcinogens and *P. niruri L* extract was higher compared with K+ group namely 2.37 ± 0.48.

Hypotheses test to prove the application of *P. niruri L* could increase the apoptosis index was carried using unpaired t-test. The analysis result showed that there was significant different on the cells number which expressed TUNEL positive between K+ and X at p=0.000. TUNEL apoptosis cell expression on colorectal cancer cell can be seen on Figure 2 and 3.

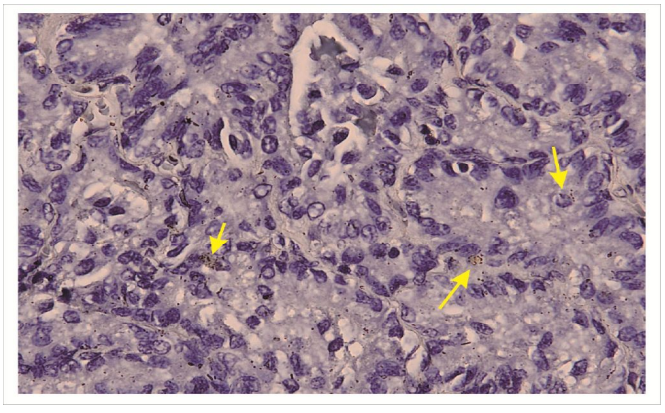

Figure 2. Apoptosis cell on K+ group. Brown color of apoptosis body with TUNEL staining (yellow arrow) between colorectal cancer cells (TUNEL staining, 400x magnification.

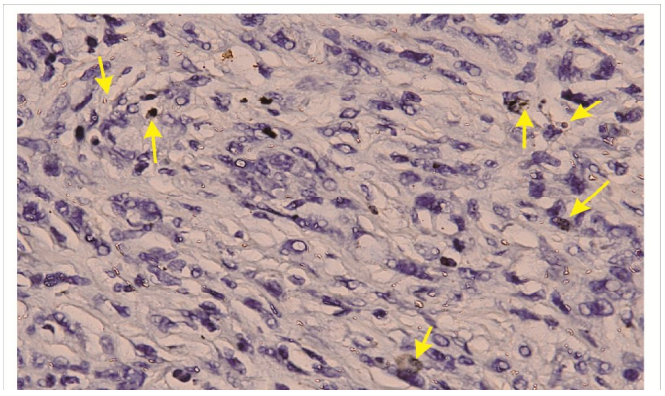

Figure 3. Apoptosis cell on X group. More brown color of apoptosis body with TUNEL staining (yellow arrow) between colorectal cancer cells (TUNEL staining, 400x magnification).

Apoptosis is also called as programmed cell death and an important physiological process of cell death which occurred without the release of intracellular matrix. The ability to undergo apoptosis is an important mechanism to control cells population and to eliminate cells experiencing DNA damage which cannot be repaired. This reducing ability or the presence of apoptosis dysregulation caused cells retention with the damage of DNA which in turn increased the risk of mutation including those with carcinogenic characteristics and considered as an important process in colorectal carcinogenesis (Crighton and Ryan, 2004; Subowo, 2004; Greystoke et al., 2007; Rousselot and Garnero, 2007; Hector et al., 2009).

This research result proved that apoptosis index on rats with *P.niruri* L extract significantly increased compared with those without extract application. Thus it confirmed that hypothesis was accepted. This indicated that the application of *P. niruri* L extract could increase apoptosis and apoptosis index could be used as a therapy response indicator towards colorectal cancer. *P. niruri* L extract could induce apoptosis through activating caspase-3 and producing TNF-α and inhibiting IL-8 expression and COX-2 on hepatocellular carcinoma cell HepG2 (Sureban et al., 2006).

Detection of the apoptosis body can be done on the histological tissue by TUNEL method. This method was applied based on the assumption that genomic DNA was fragmented on the death cell and fragment production was consistent with the number of cells which experienced apoptosis. Detection of fault DNA strand through the addition of nucleotide including *deoxyuridine* was based on DNA fragmentation catalyzed with *deoxynucleotidyl* transferase with the presence of dUTP as the substrate. Specific painting was reacted with the addition of *anti-digoxigenin* antibodies that brings peroxidase as conjugate reporter (Lifshitz et al., 2001). Morphological characteristic of apoptosis cell includes of plasma cell swelling, intact cell membrane integrity, reduced cell volume and triphosphate (dUTP) labeled with biotin at the tip of DNA fragment (Koornstra et al., 2003). The application of TUNEL method still could protect cell organ to undergo chromatin condensation and nucleus fragmentation to form apoptosis body. The apoptosis process mechanism depends on the

activation of caspase cascade (Huerta et al., 2006; Krysko et al., 2008).

Based on the previous research and result, the researcher found that *P. niruri* L extract could improve immunology status and inhibit the development of colon cancer on experimental animals induced with 1,2 DMH. The improvement of immunology status was marked with the increasing of lymphocytes infiltration with the increasing of perforin secreted by NK and CTL cytotoxic lymphocytes. The increasing of immune competent cells improves the immunology status of the animals thus the antitumor cellular immune responses works better and ensures the occurrence of apoptosis on colon cancer cell (Sawitri *et al*., 2013).

The ability of cytotoxic lymphocytes to initiate the apoptosis process on the transformed cells and kill the cellular target depends on two strategies of signal transduction that are principally different and require cell contact between effector and target (Yip et al., 2000; Trapani and Smyth. 2002). The strategy was apoptosis mechanism which occurred through extrinsic or intrinsic path. The identification of apoptosis path will give opportunity to understand the homeostatic control of normal colorectal tissue and reveal new target in colorectal cancer therapy (Johnstone et al., 2002; Fleischer et al., 2006).

The apoptosis mechanism of target cells/cancer cells by NK and CTL cells was through two transduction signal paths. The first path, effector cells expresses TNF family ligan such as Fas/CD95 on the cell surface and used as weapon to kill the target cells or to destroy transformed cells which expresses suitable Fas receptor. Fas-ligan (FasL) is important in the process of eradicating tumor mediated with CTL. Meanwhile, TRAIL (*Tumour Necrosis Factor-related Apoptosis-inducing Ligand*) or Apo2-ligan is important to eliminate tumor mediated with NK cell (Yip et al., 2000; Screpanti et al., 2005; Grimm et al., 2010). The second path involves granzyme traffic mediated with perforin by releasing the content of cytotoxic granule (cytotoxicity exocytosis) which are destructive from CTL/NK to the target cells where they promotes death cell by breaking/hydrolyzing specific protein substrate including caspase (Yip et al., 2000; Metkar et al., 2002; Lieberman, 2003; Bolitho et al., 2007).

The series of complex molecular interaction indicate extrinsic apoptosis pathway mechanism. The bond between Fas death receptor with Fas ligan causes recruitment of FADD (*Fas associated death domain*) adaptor molecular and protease catalysator namely FLICE (*Fas associated death domain*). Death effector domain dimerization will cause FADD joins with procaspase 8 and forms protein complex named as DISC (*death-inducing signalling complex*) which activates proscaspase 8. This series of process will activate caspase 9 and then activate executor caspase i.e. caspase 3 and 7 leads to the death of cancer cells in apoptosis way (Reed, 2003; Elmore, 2007; Yang et al., 2009).

Apoptosis induced by perforin/granzyme (intrinsic pathway) is another pathway of immune system to eliminate transformation cells/tumor cell which conducted by NK cell and CTL effector cytotoxic lymphocyte. Besides of fully inducing apoptosis through activating apoptosis sistein protease (caspases) which mediated by granzyme B, this granule exoxitoxic pathway also activates death cell which not involve caspase which mediated by granzyme A to ensure the death of target cell (Lieberman, 2003; Bolitho et al., 2007; Elmore, 2007; Afonina et al., 2010). CTL cell should make contact with the closest cancer cell to both impinge upon each other resulting in the secretion of cytotoxic granule content. Cytotoxic granule is a complex macromolecular consisted of granzyme and perforin bind with serglycin (proteoglycan matrix) and calreticulin (a perforin inhibitor) will enter into immunological synapse which formed between effector cell and the target. After exoxitosis entering space between target and effector cell, perforin was separated from granule complex and undergo polymerization so they can work separately to deliver granzyme-serglycin complex into the cell target through endocytosis (Catalfamo and Henkart, 2003; Lieberman, 2003; Bolitho et al., 2007).

Intrinsic pathway is marked by the release of c cytochrome from mitochondria causing caspase activation. After it is activated, cell will undergo apoptosis. Bax oligomerization facilitates the opening of channel or porous in the outside of mitochondria thus protein inter membrane including of c cytochrome and other pro-apoptosis molecular will leave and enter into cytosol. Cytosolic c cytochrome mediates the development of *caspase–activating complex* named as apoptosome consisting of c cytochrome, *apoptosis protease activating factor*-1 (APAF-1) and *caspases-recruitment domains* (CARD) containing of procaspase-9. Apoptosome will activate caspase initiator (caspase 9) and recruites procapase-3 which then divided and activated by caspase-9. This activity activates effector caspase (cascade) series such as caspase-3 and caspase-7 that promotes quick cell death. The substrate target of caspase is iCAD (*inhibitor of DNase caspase activating deoxyribonuclease*). Other mediator that released by mitochondria is *Endonuclease-G* (Endo-G) and apoptosis inducing factor (AIF) that would be trans located into nucleus and caused chromatin condensation, swelling cell and nucleus-DNA fragmentation (Watson, 2006; Elmore, 2007; Pardo et al., 2009; Yang et al., 2009).

Therapy that is designed to stimulate apoptosis on the target cell gained more important role to treat colorectal cancer. Medicine that directly induces apoptosis can reduce toxicity risk and reduce possibility of drug resistance (Reed, 2003; Yang et al., 2009). It seems that strategy on inducing apoptosis will result in new safe therapy against cancer especially by using herbal (Johnstone et al., 2002; Fleischer et al., 2006). Previous researches studied the effect of immune modulator *P.niruri* L extract with the application of this plant's decoction on mice BALB/c (Ma'at S, 1996) orally and ethanol extract orally upon Hepatitis patient (Ma'at, 2010). The result showed that *P. niruri* L extract stimulated immune responses both cellular and humoral maximally by influencing the functions and activities of immune-competent cells. The potency of anti-tumor and anti-carcinogenic of *P. niruri* L extract had been evaluated on *mamma* mice C3H adenokarsinoma (Chodidjah, 2004), hepatocellular carcinoma of Wistar rats (Rajeshkumar and Kuttan, 2000), sarcoma BALB/c mice and ascites tumor of albino Swiss mice (Rajeshkumar et al., 2002).

This result proved that *P. niruri* L extract could inhibit the development of colorectal cancer which marked by the

increasing number of cancer cells experiencing apoptosis thus the apoptosis index in *Sprague-Dawley* rats increased. The decreasing number of colorectal tumor was due to cells experiencing apoptosis after the application of *P. niruri* L extract as the activity on molecular level. It was assumed that bioactive compound on *P. niruri* L extract initiated that activity. One of bioactive compound contained in *P. niruri* L extract is Lignan with antineoplastic characteristic (Giridharan et al., 2002; Wei et al., 2002) and flavonoid which worked as immune stimulator and antineoplastic (Erlund, 2004; Cragg and Newman, 2005; Lakhanpal and Rai 2007; Cherng et al., 2008). Lignan can inhibit tolemorase and c-myc activity thus limits cancer cell proliferation, bcl-2 and activates caspase 3 and caspase 8 that also induces apoptosis of cancer cell HepG2, EL-1 monocytes, HeLa and MCP7 cell lines (Giridharan et al., 2002). Flavanoid is also proved can inhibit proliferation and induces apoptosis on human colon cancer cell line of Caco-2, HT-29 (Kuntz et al., 1999) and SW480 (Murthy et al., 2012).

Interruption on apoptosis mechanism significantly influences cancer pathogenesis. Cancer cell might inhibit apoptosis by failing on expressing *mannose 6-phosphate receptor* (MPR) at the cell surface (Trapani and Smyth, 2002), excessively express serpin endogeneous such as granzyme B specific inhibitor of P19 (Lieberman, 2003) or expressing a large number of FasL at the cell surface, causes death of CTL and NK cell (Zimmermann and Green, 2001; Trapani and Smyth. 2002). Failure on the interaction between TCR and CTL with MHC complex class I on tumor causes failure on the signal transformation through cascade including of *phosphatidylinositol 3-kinase* (PI3K) pathway, a function needed by polarization and exocytic granule to release granule toxin (Catalfamo and Henkart, 2003; Bolitho et al., 2007). Anti-apoptosis Bcl-2 family can also inhibit the release of cytochrome c leading to the failure of apoptosis (Reed, 2003; Watson, 2006; Yang et al., 2009).

Many conditions may occur if apoptosis mechanism fails. Among others are: allows neoplastic cells to survive, gives chance for the accumulation of genetic disorders that regulate cell proliferation, interferes differentiation, promotes angiogenesis and increases cell motility and ability of malignant cell invasion during tumor progression. Defects on apoptosis mechanism also facilitate metastasis by allowing malignant epithelial cells keeps survive without being bound by extracellular matrix. That condition causes resistance to body immune system when immune competent cells such as CTL and NK cells rely heavily to the apoptosis machine integrity on fighting against tumor malignant. Therefore, apoptosis defects regulation is a fundamental aspect of a cancer biology (Koornstra et al., 2003; Watson, 2006; Elmore, 2007; Gross and Walden, 2008). The application of *P. niruri* L extract in this research proved that herbal material which marked by higher apoptosis index could overcome apoptosis mechanism disorder against tumor progression.

IV. CONLUSIONS

Based on the analysis as shown in the result and discussion, it could be concluded that the application of *P. niruri* L extract could increase the number of cells experiencing apoptosis thus the colorectal cancer apoptosis index was higher at *Sprague-Dawley* rats induced with 1,2 DMH and given by those herbal material.

SUGGESTION

Anti-neoplastic of *P. niruri* L should be more comprehensively understood so that it could give more fundamental principal for clinical test. Therefore, further research is necessary to be done to deeply understand anti-cancer activity of *P. niruri* L extract by evaluating others apoptosis indicators such as ligan activity and Fas receptor, granzyme activity, c cytochrome expression, activity of p53 and Bcl-2, activity of caspase initiator and executor.

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