Apoptosis of cervical cancer cells

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Apoptosis of cervical cancer cells via cytochrome c expression given by *Zanthoxylum acanthopodium* methanol extract

INTRODUCTION

Cervical cancer is a type of cancer which sufferers are quite high in Indonesia. The death rate from cancer is higher in developing countries than in developed countries. These differences reflect differences in risk factors and successful detection treatment, as well as the availability of treatment. As a world health priority, cervical cancer control is carried out to improve public health, resources, and to address prevention and treatment strategies (1). In Southeast Asia, cervical cancer is the most common cancer after breast cancer (2,3). In Indonesia, cervical cancer is the second most common cancer among women (4). The mortality rate for each cervical cancer patient is 17 per 100,000 population and 7.7 per 100,000 population in Indonesia (3). Cervical cancer patients in Indonesia are generally on a higher scale, but most women report high cancer symptoms and side effects of treatment. Therefore it is expected that there will be an appropriate drug or treatment (5).

Cervical cancer occurs due to human papillomavirus (HPV) infection with identified HPV DNA as transient can disappear spontaneously, and can persist and develop into a malignant condition of cervical intraepithelial neoplasia (6). Tumors that arise on the endocervix are more likely to become adenocarcinomas. Factors leading to the development of persistent infection and malignant transformation are smoking, long-term use of oral contraceptives, high parity, and co-infection with the human immunodeficiency virus (6). Preventing apoptotic events by malignant cells is a hallmark of cancer and the induction of apoptosis by cytotoxic anticancer agents is usually one strategy for the development of chemotherapy drugs for cancer treatment (7). The ingredients derived from traditional medicinal plants can control complex phenomena such as changes in gene expression and

induction of apoptosis (8). Plant-derived products such as flavonoids and antioxidants can be an alternative approach in inducing apoptosis in cancer cells. Cytochrome c in the intrinsic pathway indicates the apoptosis process as protease activating factor-1 (APAF-1) which forms apoptosomes, which is the downstream trigger of the caspase 9 or 3 signaling cascade, which is defined as the primary process of cell death by apoptosis (9). This pathway can be targeted for chemotherapy or medicinal plants that contain anticancer activity.

Zanthoxylum acanthopodium is a wild plant in North Sumatra in Indonesia. It has been used for centuries as traditional medicine (10). This plant has anti-inflammatory and antioxidant activity against the growth of mycelium fungi and in vitro antitumor activity (11). The antioxidants from this plant reduce the levels of malondialdehyde (MDA) in the blood and increase HSP-70 (12). Besides, this plant is also safe in the liver and kidneys in preeclampsia or hypertension (12-14). This plant has an inhibitory effect on the growth of T47D breast cancer cells and has the potential to be co-chemotherapy for breast cancer because the results of flow cytometry analysis show that changes in cell accumulation occur in the G0 - G1 cycle from *Zanthoxylum acanthopodium* induction (9,15).

The purpose of this study was to determine apoptosis in cervical cancer cells via cytochrome c (mitochondrial pathway) after given *Zanthoxylum acanthopodium* and the histological description of cervical cancer cells. So it can be seen that these plants can be developed into candidates for cervical cancer drugs in the future.

MATERIALS AND METHODS

Prepare of *Zanthoxylum acanthopodium* extract methanol (ZAM)

The fruit andaliman used comes from the Bukit Gibeon Sibisa Parapat, District of North Sumatra. Andaliman is washed thoroughly; then the wind is dried for 3 days at room temperature, then blended until smooth. After that, the manufacture of the extract of

andaliman with three steps: (1) Drying of the crude drug: the fruit of andaliman cleaned, and drained dry, thenmashed with the blender. (2) The manufacture of Andaliman extract: powder, fruit of Andaliman macerated with methanol 96% for ± 1 night. The results of the maceration and botanicals percolation until obtained clear liquid. The results of the percolation concentrated with the evaporator until obtained the extracts are concentrated. (3)The manufacture of pharmaceutical suspension: given the extract of Andaliman is used partly do not dissolve in water, then to get a homogeneous mixture used a suspending agent CMC 1,5 % as much as 1.0% or 1 ml in 150 ml of distilled water. The dregs are washed with solvent methanol 96%, and then transferred in a closed container and left into a cool place protected from light for 2 days.

Animal

The study was conducted at the Biology Laboratory of the University of North Sumatra, the Pathology and Anatomy Laboratory of the Faculty of Medicine, University of North Sumatera, Indonesia. This study used 30 *Rattus norvergicus* (180-200g), rats were taken and maintained in the Animal House Laboratory, University of Sumatera Utara. The rats were acclimatized to laboratory conditions for 4 weeks before the study and the rats were given standardized rat pellets and abundant water. The rats made in the animal model of cancer by inducing benzopyrene 50 mg/BW in cervical and let growing cancer until three months later.

Study design

The rats were divided into five groups. Group K- were a control group, Group K+ were rats model of cancer, group P1 were rats model of cancer with a dose of 100mg/BW of ZAM, group P2 were rats model of cancer with a dose of 200 mg/BW of ZAM, and the group

P3 were rats model of cancer with a dose of 400 mg/BW of ZAM during 30 days administration (16). Rats dissected on day 30 after administration of ZAM, for cervical was taken, and then the cervical was prepared for paraffin blocks and Immunohistochemistry staining.

Immunohistochemistry staining of Cytochrome c

Cytochrome c detection used a monoclonal mouse anti-cytochrome C antibody (ready to use) 7H8.2C12 (Medaysis Enable Innovation Company), formulation in PBS pH 7.4, containing BSA and ≤0.09% sodium aide (NaN3). The cervical tissue was embedded by paraffin and cut into four microns of thickness using microtome. For pre-treatment, the tissue was heated in citrate buffer at pH 6.0 and 350 W. After washing with PBS, the tissue was incubated with cytochrome C antibodies, respectively, at 37 °C then washed again with PBS before applying avidin–biotin peroxidase. 3,3-Diaminobenzidine (DAB) hydrochloride was used for chromogenic visualisation reaction and then stained with haematoxylin Mayer. The cervical tissue on the slide was stained with hematoxylin, then the score was calculated as a positive result multiplied by the staining intensity, 0: less than 10% of cells were stained, 1: for 10 - 25% stained, 2: for 25 - 50% stained, 3: for 50 - 75% stained, and 4: for more than 75% stained cells. The intensity of staining was categorized into 1: weak, 2: moderate intensity, and 3: strong (17).

TUNEL Assay

To detect the integrity of placental DNA, the paraffin-embedded part was stained with TUNEL techniques with detection kit (Promega, Cat # G7130; USA). The cervical tissue was embedded by paraffin and cut into four microns of thickness using microtome. The cervical

tissue in the slide was immersed in fresh xylene for 5 min. The slides were rehydrated with multilevel ethanol and washed with NaCl 0.85% and PBS for 5 min. After rehydration, incubation was conducted for 15 min at room temperature with proteinase K (20 µg/ml). The final labelling reaction was carried out by adding the reaction mixture of rTdT to the slides in a humid room (37 ℃ for 1 h). The reaction of rTdT enzyme was ended by immersing the slide in a buffer at room temperature. The slides were washed 5 min with PBS. The endogenous peroxidase was blocked by 0.3% hydrogen peroxide to PBS. A streptavidin-HRP solution was added to the tissue and incubated at room temperature. A chromogenic substrate DAB was added to the slide. All slides were dehydrated with graded ethanol, and cleaned in 100% xylene for 5 min each with three times.

Data analysis

The data were analyzed by Anova test and non-parametric data form Kruskal Wallis test in SPSS 22 program. Asterisks indicate the level of statistical significance (*P<0.05, **P<0.01).

RESULTS

Table 1. Body and Cervical Weight after given ZAM

Treatment	Body Weight (BW)		Cervical Weight (g)
	Before (g)	After (g)	
K-	200.50 ± 7.00	245.80 ± 16.77	0.37 ± 0.06
K+	207.33 ± 10.52	266.00 ± 10.52*	1.61 ± 0.16**
P1	199.83 ± 9.94	277.16 ± 9.95	1.08 ± 0.07*
P2	198.83 ± 25.89	276.67 ± 9.93	0.78 ± 0.18*
P3	201.50 ± 27.77	275.83 ± 8.81	0.38 ± 0.13**

K-: Control, K+: rats model of cancer P1: rats model of cancer with a dose of 100mg/BW of ZAM, P2: rats model of cancer with a dose of 200 mg/BW of ZAM, P3: rats model of cancer with a dose of 400 mg/BW of ZAM (*P<0.05, **P<0.01).

Table 1 showed that body and cervical weight in cancer model rats rats. Insignificant difference (P> 0.05) on day 1 before injection of benzopyrene 50 mg/BW in cervical, but after injection of benzopyrene, there was a significant difference between group K- and K+

(P<0.05, F=0.048). Table 1 also showed that cervical weight in K- and K+ (P <0.01, F=0.005), P1 (P<0.05, F=0.048),P2 (P<0.05, F=0.048) and P3 (P<0.01, F=0.005) compared with K+. The injection of benzopyrene 50 mg/BW in cervical affects body weight and cervical weight in rats.

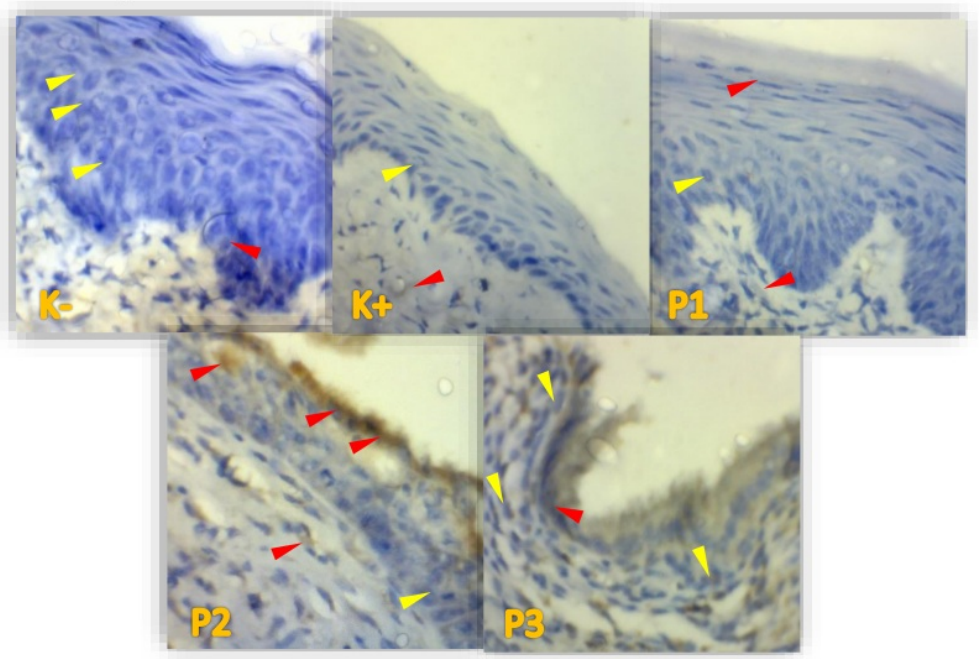

Figure 1. Expression of cytochrome c on cervical tissue histology**.** K-: Control, K+: rats model of cancer P1: rats model of cancer with a dose of 100mg/BW of ZAM, P2: rats model of cancer with a dose of 200 mg/BW of ZAM, P3: rats model of cancer with a dose of 400 mg/BW of ZAM. Yellow arrows: Negative expression, Red arrows: Positive expression.

Table 2. Kruskal Wallis and Mann-whitney analysis of cytochrome C expression in cervical tissue

Groups	n	Mean Rank	Kruskal -Wallis	Mann-Whitney				
				K-	K+	P1	P2	P3
K-	5	4.70			0.050	0.017*	0.006*	0.006*
K+	5	22.30				0.005*	0.004*	0.004*
P1	5	17.90	0.000				0.007*	0.009*
P2	5	11.00						0.015*
P3	5	9.10						

K-: Control, K+: rats model of cancer P1: rats model of cancer with a dose of 100mg/BW of ZAM, P2: rats model of cancer with a dose of 200 mg/BW of ZAM, P3: rats model of cancer with a dose of 400 mg/BW of ZAM (*P<0.05).

Figure 1 showed the cytochrome C expression of rats cervical histology after injection of benzopyrene and administration of ZAM at different doses. K- showed the complex histology of cervical tissue against a background, squamous epithelium containing the cell nucleus and cytoplasm and stroma. Squamous epithelium provides diagnostic information relating to the state of the cells normal or abnormal. K+ denotes cell abnormality indicated by enlargement of the nucleus, uncontrolled development of the structure, the shape of the cell irregular, the ratio of the cell nucleus to the cytoplasm, many variations in the shape of the nucleus. The highest cytochrome c expression was at K+ and the lowest was at K- (table 2). The expression of cytochrome c (marked brown) was in the ZAM treatment at doses of 200 and 400mg/KgBW (P2 and P3) in cervical histology. These proteins will bind, inhibit proteins, cell cycle development, modulate cell division, and high intrinsic signal transduction pathways of apoptotic signaling. The majority of cytochrome c in P2 and P3 have more positive expressions than negative expressions. The histology showed a significant difference between each treatment ($P < 0.05$) in table 2. So that ZAM administration showed a significant difference in cervical tissue after benzopyrene injection.

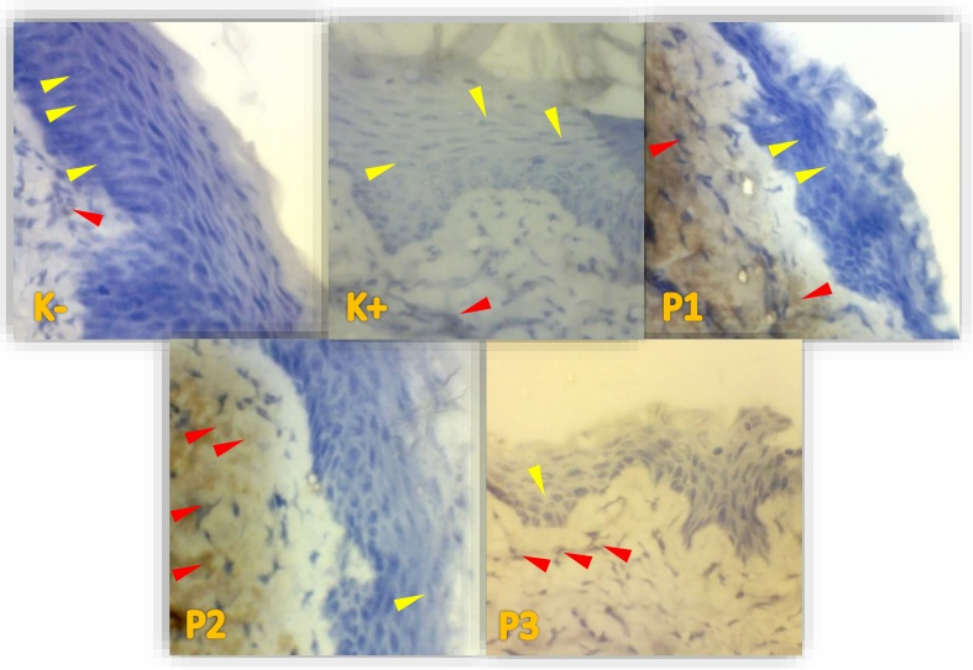

Figure 2. Expression of TUNEL on cervical tissue histology**.** K-: Control, K+: rats model of cancer P1: rats model of cancer with a dose of 100mg/BW of ZAM, P2: rats model of cancer with a dose of 200 mg/BW of ZAM, P3: rats model of cancer with a dose of 400 mg/BW of ZAM. Yellow arrows: Negative expression, Red arrows: Positive expression.

Table 3. Kruskal Wallis and Mann-whitney analysis of TUNEL expression on cervical tissue

Groups	n	Mean Rank	Kruskal-Wallis	Mann-Whitney				
				K-	K+	P1	P2	P3
K-	5	4.70			0.513	0.156	0.005*	0.005*
K+	5	22.30				0.309	0.006*	0.006*
P1	5	17.90	0.001				0.009*	0.019*
P2	5	11.00						0.072*
P3	5	9.10						

K-: Control, K+: rats model of cancer P1: rats model of cancer with a dose of 100mg/BW of ZAM, P2: rats model of cancer with a dose of 200 mg/BW of ZAM, P3: rats model of cancer with a dose of 400 mg/BW of ZAM (*P<0.05).

Figure 2 showed the apoptosis of rats cervical histology after injection of benzopyrene and administration of ZAM at different doses. The normal (K-) group showed healthy and normal histology of cervical tissue but very different after benzopyrene injection (K+). Statistical data (table 3), K+ has the highest apoptosis than other treatments. TUNEL can be used to detect enzymes involved in the destruction of the cell nucleus. Figure 2 shows the histology of the cervix with the irregular shape of the cell nucleus into bubbles called apoptotic bodies. The environment in the cell nucleus appears cut off and karyorrhexis. Cells become circular (red arrow) because the structure of the protein that makes up the cytoskeleton is digested by the specific peptidase enzyme (caspase), which has been activated in the cell. The histology showed a significant difference between each treatment (P <0.05) in table 3. So that ZAM administration showed a significant difference in cervical tissue after benzopyrene injection.

DISCUSSION

The injection of benzopyrene 50 mg/BW and given ZAM in cervical affects body weight and cervical weight significantly in rats (Table 1). An increase in weight and cervical weight due to the rats were given standardized rat pellets and abundant water every day. The injection of benzopyrene 50 mg/BW in 3 months without treatment also caused inflammation and became a tumor.

The majority of cytochromes in P2 and P3 have more positive expressions than negative ones (Figure 1). The histological results showed a significant difference between each treatment ($P < 0.05$) in Table 2 and 3. ZAM can inhibit the expression of cytochrome c in cervical cells because it has high antioxidants, reduces MDA, anti-inflammatory, and increases HSP-70 (10,12). The n-hexane fraction of *Zanthoxylum acanthopodium* contains bioactive compounds and is effective as an anticancer, inhibits apoptosis, and downregulates Cyclin D1 expression (18). *Zanthoxylum acanthopodium* has antiradical activity andaliman extracted with ethanol is higher than hexane and acetone extracts (19). The ethanolic extract of *Zanthoxylum acanthopodium* decreased the expression of TNF-α, COX-2, and MMP-9 proteins in macrophages and blocked TNF-α, IL-6, Inos, COX-2, and MMP-9 mRNA expression (20). Based on the toxicity test, besides having high antioxidants, this plant also has low toxicity (21). So that ZAM administration shows a significant difference in cervical tissue after benzopyrene injection.

Overexpression of cytochrome c on the cervix in Figure 1 (red arrow) can cause this protein to leave the mitochondria after changes in electrochemical potentiation in the membrane. This pathway is usually activated in response to other lethal stimuli such as DNA damage, oxidative stress, and hypoxia. Mitochondria contain proapoptotic factors such as cytochrome c and AIF (apoptosis-inducing factors). Both are dangerous substrates and are

stored in mitochondria (22,23). Although ZAM contains anti-inflammatory or anti-cancer properties, the overdose of ZAM on cells can also increase apoptosis.

The normal (K-) group showed apoptosis and normal histology of cervical tissue but very different after benzopyrene injection (K+) in figure 2. Apoptosis causes disruption of oxidation-phosphorylation and electron transport due to radiation and the presence of certain second messengers such as ceramides, changes in cell redox potential and derivatives of Reactive Oxygen Species (ROS) (24,25), DNA damage that spurs the expression of a protein known as p53 (26) and increases intracellular Ca2 + ions through signal transduction (22). Based on histology and positive index (Figure 2 and table 3), this herb can be developed into a cervical cancer drug candidate.

Conclusion

In conclusion, we demonstrated that the injection of benzopyrene 50 mg/BW and given *Zanthoxylum acanthopodium* extract methanol (ZAM) in cervical affects body weight and cervical weight significantly in rats. Cytochrome c protein exposures in rats cervical affect number of apoptosis cells after given *Zanthoxylum acanthopodium* extract methanol (ZAM). This plant can be developed into a cervical cancer drug candidate.

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