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Research Article
Cytochrome c Expression by Andaliman (*Zanthoxylum acanthopodium*) on Cervical Cancer Histology

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Abstract

Background and Objective: Andaliman is a wild plant in Indonesia and it has been used for centuries as traditional medicine. This study aimed to evaluate the effect of methanol extract of andaliman on apoptosis cancer cells via cytochrome c protein. **Materials and Methods:** The rats are divided into 5 groups. K: Control, K_+: Cancer model rats, P_1: A dose of 100 mg/b.wt./day of andaliman, P_2: A dose of 200 mg/b.wt./day and P_3: A dose of 400 mg/kg/b.wt./day for 30 days. The rats were dissected, then the cervical tissue was prepared on paraffin blocks, given Immunohistochemistry staining with cytochrome c antibody. **Results:** There was a significant difference in body and cervical weight ($p<0.01$). The histology also showed a significant difference between each treatment ($p<0.01$) in cytochrome c. The highest cytochrome c expression was at P_2 and the lowest was at K_-. **Conclusion:** Andaliman methanol extract can thus be developed into a cervical cancer drug candidate because it can reduce the positive index of cytochrome c in cervical histology.

Key words: Andaliman, cancer, cervical, cytochrome c, herbal plant, immunohistochemistry, zanthoxylum

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cervical cancer is a type of cancer with quite a high incidence in Indonesia. The previous studies showed the high incidence of cervical cancer occurring in women is due to low awareness[1,2]. If the woman knows of early detection with a pap smear, cervical cancer can be prevented and reduce the rate of death in women[3].

The death rate of cancer is higher in developing countries than in developed countries. This difference is reflected in mitigating risk factors and successful detection and treatment, as well as the availability of such treatment. Control of cervical cancer is one of the world's priorities in health. Several strategies for prevention and treatment have been carried out to improve public health[4].

Cervical cancer is the most deadly cancer after breast cancer in Southeast Asia including Indonesia[5,6]. This cancer is most feared by women and it is the second-largest cancer incidence in Indonesia[7]. The mortality rate for cervical cancer is 17 per 100,000 population and 7.7 per 100,000 population in Indonesia[5]. Therefore it is hoped that there will be an appropriate drug for the treatment of cervical cancer[8].

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 tumour of cervical intraepithelial neoplasia[9]. Tumours that arise on the endocervix are more likely to become adenocarcinomas. Factors that lead to the development of persistent infection and malignant transformation are unhealthy habits, tobacco (smoking) consumption, prolonged use of contraceptives and is directly related to cancer-causing viruses[9]. One of the most researched strategies for developing chemotherapy drugs in cancer is the apoptosis pathway by using cytotoxic anticancer agents, usually derived from herbs or chemicals[10,11].

The ingredients derived from traditional medicinal plants can control complex phenomena such as changes in gene expression and induction of apoptosis[12]. Plant-derived products such as flavonoids[13] and antioxidants can be an alternative approach to inducing apoptosis in cancer cells. Cytochrome c in the intrinsic pathway indicates the Apoptosis process as Protease Activating Factor-1 (APAF-1). This forms apoptosomes, which is the downstream trigger of the caspase 9 or 3 signalling cascade, which is defined as the primary process of cell death by apoptosis[14]. This pathway can be targeted for chemotherapy or treatment using medicinal plants that contain anticancer activity.

Zanthoxylum acanthopodium (locally known as andaliman) is a wild plant in North Sumatra in Indonesia. It has been used for centuries as a traditional medicine[11,15,16]. This plant has anti-inflammatory and antioxidant activity against the growth of mycelium fungi and in vitro anti-tumour activity[1,17,18]. The antioxidants from this plant reduce the levels of Malondialdehyde (MDA) in the blood and increase HSP-70[19,20].

Besides, this plant is also safe for the liver and kidneys in pre-eclampsia or hypertensive patients[1,20,21]. This plant has a co-chemotherapy effect for breast cancer (T47D cancer cells) and shows changes in the accumulation of T47D cancer cells that occur in the G0-G1 cycle from *Zanthoxylum acanthopodium* induction[14,22].

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

MATERIALS AND METHODS

Study area: This study used 30 Wistar rats from the Animal House of Biology Laboratory, the University of Sumatera Utara (USU), Medan, Indonesia. 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 Sumatera Utara, Indonesia from December, 2019-August, 2020.

Cytochrome c detection used a monoclonal mouse anti-cytochrome c antibody (ready to use) 7H8.2C12 (Medaysis Enable Innovation Company).

Preparation of *Zanthoxylum acanthopodium* extract methanol (ZAM): The andaliman fruit (*Zanthoxylum acanthopodium*) comes from the Bukit Gibeon Sibisa Parapat area, District of North Sumatra. *Zanthoxylum acanthopodium* is cleaned off the soil or dust that sticks to the fruit.

The fruit extract is manufactured in the following 3 steps:

- **Drying of the crude drug:** The fruit of andaliman is cleaned and drained dry, then mashed in a blender
- **Manufacture of andaliman extract:** The fruit of andaliman is macerated with methanol 96% for ±1 night. It is then percolated until a clear liquid is obtained. The concentrated liquid is then evaporated until the powder extracts are obtained

- **Manufacture of pharmaceutical suspension:** Given that the extract of andaliman partly does not dissolve in water, a homogeneous mixture is obtained by using a suspending agent CMC 1.5% as much as 1.0% or 1 mL in 150 mL of distilled water. The drugs are washed with solvent methanol 96% and then transferred to a closed container and left in a cool place protected from light for 2 days

Animal studies: This study used 30 rats (*Rattus norvegicus*) with 180-200 g in weight, which is taken and maintained in the Animal House Laboratory, University of Sumatera Utara. The rats are acclimatized to laboratory conditions for 4 weeks before the study and given standardized rat pellets and abundant water. The rats are made in the animal model of cancer by inducing benzopyrene 50 mg/b.wt. in cervix tissue and let cancer grow until three months later[23].

Study design: The rats are divided into five groups. Group K_- is the control group, Group K_+ is the model of cancer rats, group P_1 is the model of cancer rats with a dose of 100 mg/b.wt. of ZAM, group P_2 is of cancer rats with a dose of 200 mg/b.wt. of ZAM and the group P_3 is a model of cancer rats with a dose of 400 mg/b.wt. of ZAM during 30 days administration[23]. The rats are dissected on day 30 after the administration of ZAM. The cervical tissues are then prepared on paraffin blocks and given immunohistochemistry staining.

Immunohistochemistry staining of cytochrome c: Paraffin cervical tissue was cut using a microtome with a thickness of 4-6 microns. 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 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: 10-25% stained, 2: 25-50% stained, 3: 50-75% stained and 4: >75% stained cells. The intensity of staining was categorized into 1: Weak, 2: Moderate intensity and 3: Strong[24].

Data analysis: The data were analyzed by the ANOVA test and non-parametric data from the Kruskal Wallis test in SPSS 22 program. Asterisks indicate the level of statistical significance (*$p<0.05$, **$p<0.01$).

RESULTS

Body and cervical weight after given ZAM: The result in Table 1 showed that the mean values of cervical weight in K_- (0.37±0.06 g) and K_+ (1.61±0.16 g), P_1 (1.08±0.07 g), P_2 (0.78±0.18 g) and P_3 (0.38±0.03) with a significant difference. Insignificant difference ($p> 0.05$) on day 1 before injection of benzopyrene 50 mg/b.wt. in cervical tissue but after injection of benzopyrene, there was a significant difference between group K_- and K_+ ($p<0.05$, $F = 0.048$. The injection of benzopyrene 50 mg/b.wt. and given ZAM in cervical tissue affects body weight and cervical weight significantly in rats. Based on Table 1, it is known that ZAM can affect cervical cancer weight but does not affect cancer rat body weight.

Expression of cytochrome c on cervical tissue histology: The histological results showed a significant difference between each treatment ($p<0.01$) (Table 2). It 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 (Fig. 1a). Squamous epithelium provides diagnostic information

Table 1: Body and cervical weight after given ZAM

	Body weight (BW)		
Treatments	Before (g)	After (g)	Cervical weight (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**
P_1	199.83±9.94	277.16±9.95	1.08±0.07*
P_2	198.83±25.89	276.67±9.93	0.78±0.18*
P_3	201.50±27.77	275.83±8.81	0.38±0.13**

K_-: Control, K_+: Rats model of cancer P_1: Rats model of cancer with a dose of 100 mg/b.wt. of ZAM, P_2: Rats model of cancer with a dose of 200 mg/b.wt. of ZAM, P_3: Rats model of cancer with a dose of 400 mg/b.wt. of ZAM (*$p<0.05$, **$p<0.01$)

Table 2: Kruskal-Wallis and Mann-Whitney analysis of cytochrome c expression in cervical tissue

			Mann-Whitney				
Groups	Mean rank	Kruskal-Wallis	K_-	K_+	P_1	P_2	P_3
K_-	7.30			0.050	0.017*	0.006*	0.006*
K_+	14.30				0.005*	0.004*	0.004*
P_1	18.80	0.000				0.007*	0.009*
P_2	22.60						0.015*
P_3	12.30						

K_-: Control, K_+: Rats model of cancer P_1: Rats model of cancer with a dose of 100 mg/b.wt. of ZAM, P_2: Rats model of cancer with a dose of 200 mg/b.wt. of ZAM, P_3: Rats model of cancer with a dose of 400 mg/b.wt. of ZAM (*p<0.05)

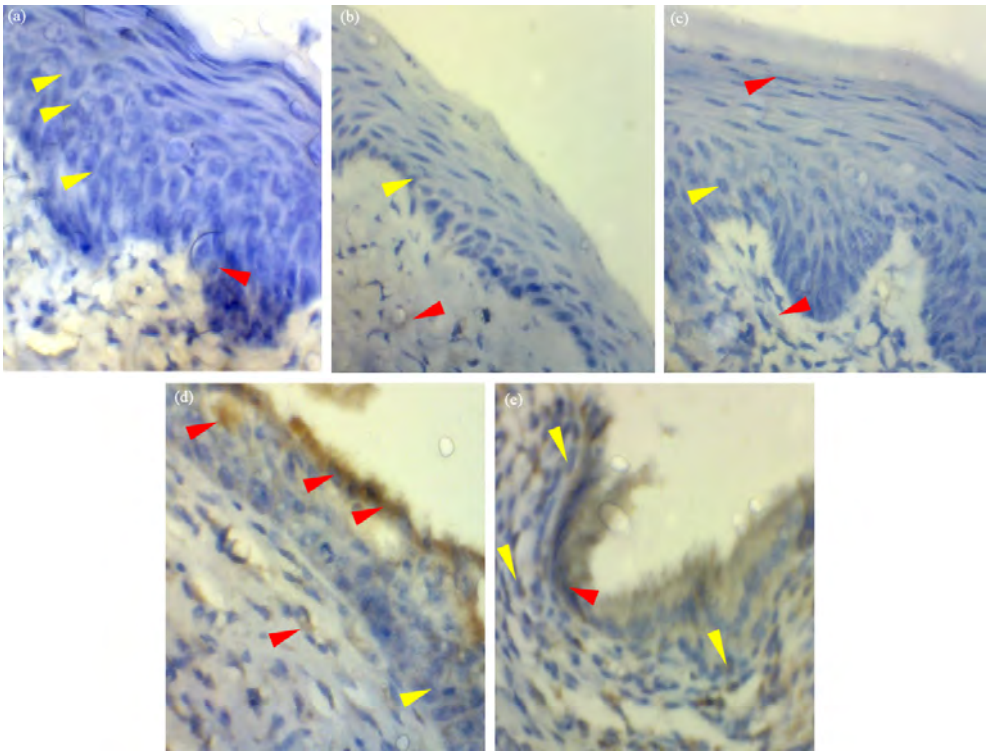

Fig. 1(a-e): Expression of cytochrome c on cervical tissue histology, (a) Control (K_-), (b) Rats model of cancer (K_+), (c) Rats model of cancer with a dose of 100 mg/b.wt. of ZAM (P_1), (d) rats model of cancer with a dose of 200 mg/b.wt. of ZAM, (P_2) and (e) Rats model of cancer with a dose of 400 mg/b.wt. of ZAM (P_3)
Yellow arrows: Negative expression and Red arrows: Positive expression

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 irregular cell, the ratio of the cell nucleus to the cytoplasm, many variations in the shape of the nucleus (Fig. 1b). Overexpression of cytochrome c on the cervix in the red arrow can cause this protein to leave the mitochondria after changes in electrochemical potentiation in the membrane. A response to deadly stimuli such as hypoxia, oxidative stress and DNA damage can activate this pathway. This pathway involves mitochondria because it contains pro-apoptotic factors such as cytochrome c and AIF (apoptosis-inducing factors). Both are dangerous substrates and are stored in mitochondria. Although ZAM contains anti-inflammatory or anti-cancer properties, the overdose of ZAM on cells can also increase apoptosis.

The P_1 dose started to show cytochrome c expression which is indicated by the arrows (Fig. 1c). The highest cytochrome c expression was at P_2 (Fig. 1d) and the lowest was at K_+ (Table 2). The cervical histology at P_3 began to resemble in the control group (Fig. 1e). The expression of cytochrome c (marked brown) was in the ZAM treatment on

cervical histology. These proteins will bind, inhibit proteins, cell cycle development, modulate cell division and high intrinsic signal transduction pathways of apoptotic signalling. So that ZAM administration showed a significant difference in cervical tissue after benzopyrene injection.

DISCUSSION

The injection of benzopyrene and given ZAM in cervical tissue affects body weight and cervical weight significantly in rats. ZAM can affect cervical cancer weight but does not affect cancer rat body weight. ZAM administration also showed a significant difference in cervical tissue after benzopyrene injection. ZAM can inhibit the expression of cytochrome c in cervical cells because it has high antioxidants, reduces MDA is anti-inflammatory and increases HSP-70[15,19]. The n-hexane fraction of *Zanthoxylum acanthopodium* contains bio-active compounds and is effective as an anti-cancer, inhibits apoptosis[25] and downregulates Cyclin D1 expression[26]. The ethanol extract of the fruit from this plant have higher anti-radical activity compared to the acetone and hexane extracts[27]. The ethanolic extract of *Zanthoxylum acanthopodium* decreased the expression of COX-2, MMP-9, TNF-α and blocked IL-6, COX-2, TNF-α, MMP-9, iNOS and mRNA expression[28]. The increase in cytochrome c at the P_2 dose was thought to be due to the presence of alkaloids in ZAM. The molecular mechanisms used by various alkaloids during induction of cell death is not uniform[29]. The DNA-targeting action of the alkaloids correlates with their cytotoxic activity[30]. Alkaloids affecting the function of the Bcl-XL and cytochrome c protein are the main mechanisms that can be directly involved in the action of these alkaloids on mitochondria[29,30]. It was shown that these alkaloids cause activation of pro-caspase-8 involved in receptor-dependent apoptosis in cancer cells[29]. Mitochondria act as intracellular machinery for amplification of apoptosis signalling after binding to apoptosis-inducing ligands with appropriate receptors on the target cell surface and a caspase cascade[30]. 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.

Apoptosis disrupts 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)[31,32], DNA damage that spurs the expression of a protein known as p53[29] and increases intracellular Ca^{2+} ions through signal transduction[25]. Based on histology and positive index (Fig. 1 and Table 2). Our results indicated that andaliman there was potential for reducing the cytochrome c expression in a histological change of cervical rats, and safe natural material and the form of it, so we can be recommended by adding this material to the candidate of cancer therapy molecularly, and because reduced the damaged of cervical histology in tissues rats and so this herb may be developed to drug-human cancer via apoptosis pathway.

Conclusion

We demonstrated that the injection of benzopyrene 50 mg/b.wt. and given *Zanthoxylum acanthopodium* extract Methanol (ZAM) in cervical tissue affects body weight and cervical weight significantly in rats ($p<0.01$). Cytochrome c protein exposures in rats after being given *Zanthoxylum acanthopodium* extract Methanol (ZAM). This plant can be developed into a cervical cancer drug candidate.

SIGNIFICANCE STATEMENT

This study discovers the possible effect of ZAM that can be beneficial for decreased histological changes in cancer rates. This study will help researchers to uncover that this herb may be beneficial for reduced of cytochrome c in cancer, molecularly, because cytochrome c expression is also a protein of cancer and affected the apoptosis of damaged cells in the cervical. Thus, a new theory on these herbal may be arrived at.

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