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Research

Effect of Administrating ~~on~~ *Zanthoxylum acanthopodium* methanol extract on CDK4 expression to cervical cancer

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ABSTRACT:
Cervical cancer is a disease from the Human papillomavirus (HPV) through transmission, virus persistence, clone development until infecting the cells in the cervical. This study ~~was~~ is to determine CDK4 expression in cervical cancer cells after being given *Zanthoxylum acanthopodium* methanol extract (ZAM) and the histological description of cervical cancer cells. This study consisted of 5 treatment groups. K-: control group, 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, and P3: rats model of cancer with a dose of 400 mg/BW of ZAM. The cervical tissue was prepared ~~for~~ on paraffin blocks and given Immunohistochemistry staining. Results showed that the expression of CDK4 are reduced in the ZAM treatment at doses of 200 and 400mg/KgBW ($P<0.05$) in cervical histology, but in doses of 100mg/kg BW, ~~M~~ many brown marks are still visible on the cervical tissue. These proteins will bind, inhibit proteins, cell cycle development, modulate cell division, and signal transduction pathways of apoptotic signaling. The injection of benzopyrene and given ZAM in cervical tissue affect hematological values. ZAM affects and improves cervical histology after benzopyrene injection. The extract from this plant can be developed into a cervical cancer drug candidate.

KEYWORDS: Andaliman, benzopyrene, CDK4, Cervical cancer, *Zanthoxylum acanthopodium*

INTRODUCTION:

Cervical cancer is the deadliest cancer after breast cancer in Southeast Asia. The burden of cervical cancer is high in developing countries and this disease is common in women in several developing countries[1]. The incidence and mortality rates for cervical cancer are 17 per 100.000 population and 7.7 per 100.000 population in Indonesia respectively[2]. Women also ~~had~~ have 70% inadequate knowledge and 30% ~~had~~ have moderate knowledge of cervical cancer, and there was a significant relationship between variables such as age and gender in the analysis of knowledge about cancer[3,4]. The incidence of cancer is also influenced by the low knowledge of women about cervical cancer (Simanullang, 2018) and if done early detection such as pap smears can reduce mortality in women (Simanullang, 2020).

Cervical prevention and control strategies such as vaccination programs, screening, and treatment with standard of care up to the molecular level. HPV (Human papillomavirus) infection is cancer that attacks several types of epithelium, such as the anus, cervix, and oropharynx[5]. [Cervical cancer arises ~~through~~ from the HPV virus through transmission, virus persistence, clone development constantly infects cells to precancerous[6,7]. ?] *... ← ?(this sentence some words missing here?)* Women's work, the number of children, regular menstruation, perineal hygiene practice, gynecological consultation, including cervical risk factors[8,9]*... ← ?(this sentence is not complete to make sense?please elaborate)* Cervical cancer treatments that are currently known to the public are chemotherapy, radiation therapy, along with surgical treatment[10].

CDK4 is an inhibitory protein in endocrine therapy in cancer and is also an inhibitor of MAPK (Mitogen-activated protein kinase)[11]. CDK4 also plays a role in uncontrolled cellular proliferation and activation of cyclin-dependant~~ent~~ kinases (CDKs) to promote cell cycle development[11,12]. This protein keeps cells from resting or triggers cells to apoptosis, CDK4 is able to prevent cell growth from becoming malignant cancer also occurs due to disruption in the cell cycle (G1, S, G2, and M) resulting in unlimited proliferation and the inability of cells to apoptosis[13,14]. The degree of damage or mutations in CDK4 in ovarian cancer tissue can also indicate an early occurrence in ovarian carcinogenesis[15]. HPV overexpression or abnormal expression of multiple positive regulators of the cell cycle has been closely associated with apoptosis. Expression of cyclins and cdks involved in oncogenic transformations including carcinoma, ovary, and gastrointestinal tract which shows a correlation between overexpression of cell cycle proteins[16,17]. Apoptosis occurs ~~by~~ due to many factors, such as suppressive oncogene products, which determine proliferative activity and ultimately survival. The relationship of the upregulation of CDK4 with apoptosis is involved in the cycle regulating molecules in apoptosis[17].

Zanthoxylum has been used in various systems of traditional medicine for its antioxidant, anti-inflammatory, hepatoprotective and other medicinal activities. *Zanthoxylum acanthopodium*

(*andaliman*) is a wild plant in North Sumatra in Indonesia. It has been used for centuries as traditional medicine[18]. This plant has anti-inflammatory and antioxidant activity against the growth of mycelium fungi and in vitro antitumor activity[19]. The antioxidants from this plant reduce the levels of malondialdehyde (MDA) in the blood and increase HSP-70[20]. Besides, this plant is also safe in the liver and kidneys for treatment of ~~in~~ preeclampsia or hypertension[20-22]. 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[23,24].

The purpose of this study ~~was~~ is to determine CDK4 expression in cervical cancer cells after being 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.

MATERIAL AND METHODS:

Preparation of *Zanthoxylum acanthopodium* extract methanol (ZAM)

The fruit used (andaliman) ~~used~~ comes from the Bukit Gibeon Sibisa Parapat, District of Sumatera Utara. *Zanthoxylum acanthopodium* is cleaned from the soil or dust that sticks to the fruit, then dried for 3 days at room temperature and mashed in a fine blender without branches (only the fruits). ~~After~~ ~~t~~ The manufacture of the extract of andaliman is done in ~~with~~ three steps: (1) Drying of the crude drug: the fruit of andaliman is cleaned, and drained dry, then mashed with the blender. (2) The manufacture of Andaliman extract: powder, fruit of Andaliman is macerated with methanol 96% for ± 1 night. The results of the maceration and botanicals are percolated ~~ion~~ until clear liquid is obtained ~~clear liquid~~. The results of the percolation are concentrated(?) with the evaporator until concentrated extracts are obtained. ~~the extracts are concentrated~~. (3) The manufacture of pharmaceutical suspension: given that the extract of andaliman used partly do not dissolve in water, a suspending agent CMC 1.5 % as much as 1.0% or 1 ml in 150 ml of distilled water is used ~~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 then washed with solvent methanol 96%, and then transferred in a closed container and left into a cool place and protected from light for 2 days.

Animal Handling:

This study used 25 Wistar pregnant rats from the Animal House of Biology Laboratory, the University of Sumatera Utara (USU). This research project was conducted from May 2018 to September 2020. This study used 25 *Ratus norvergicus* (180-200g), rats which 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 then ~~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. *?←don't make sense the sentence...)*

Study design

This study consisted of 5 treatment 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 [20,25]. Rats dissected on day 30 after administration of ZAM, for cervical (what?) was taken, and then the cervical tissue was prepared ~~for~~ on paraffin blocks and given Immunohistochemistry staining. The blood sample was sent to the Medan City Health Laboratory for the examination of the hematological value based on manufactured standards in hematology.

Immunohistochemistry staining of CDK4

CDK4 detection used a monoclonal mouse CDK4 (cyclin-dependent kinase 4) (EP180, Zhongshan; Working solution), 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 CDK4 antibodies, respectively, at 37 °C then washed again with PBS before applying avidin–biotin peroxidase. Lastly, all sections were visualized using a chromogen DAB working solution followed by counterstained with Meyer's hematoxylin[26].

Data analysis:

Research data using SPSS software version 23 using the Anova test at 5% level then continued with the Post Hoc-Duncan test. If the data are not normally distributed and / or the variance is not homogeneous, then we use the *Kruskal Wallis* test and then proceed with the *Mann-whiney* test.

RESULTS:

Table 1. Hematological Value of rats blood

Parameters	Treatments				
	K-	K+	P1	P2	P3
Leukocytes (10^9/L)	7.68 ± 1.21	9.74 ± 2.01	4.54 ±2.77*	6.52 ± 2.98	6.12 ± 2.04
Erythrocytes(10^9/L)	7.79 ± 1.20	6.56 ± 2.78	9.31 ± 2.89	8.12 ± 2.12	8.14 ± 2.12
Hemoglobin (g/dL)	15.01 ± 5.22	10.10 ± 3.21	16.10 ± 3.23	14.9 ± 2.11	15.2 ± 2.45
Hematocrit (%)	44.8 ± 3.21	33.2 ± 12.19	50.30 ± 11.20	45.1 ± 4.34	46.2 ± 3.66

Thrombocytes (10^3/UL)	772 ± 11.42	853 ± 44.79	887 ± 87.20	1117 ± 78.90*	1150 ± 87.92*
Neutrophils (10^9/L)	18 ± 1.09	12 ± 3.21	18 ± 2.99	14 ± 3.412	17 ± 1.89
Lymphocytes (10^9/L)	78 ± 2.08	82 ± 2.66	62 ± 21.20	68 ± 22.10	69 ± 3.98
Monocytes (10^9/L)	2 ± 0.04	48 ± 2.21**	19 ± 2.87*	10 ± 2.17*	11 ± 2.12*
Eosinophils (10^9/L)	2 ± 0.12	0.82 ± 0.09*	1 ± 0.02	2 ± 0.08	3 ± 0.03
Basophils (10^9/L)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

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 a significant difference in leukocyte levels in the blood of rats, where the P1 value F = 0.04, P <0.05 compared to the K+ group. An insignificant difference (P> 0.05) was found in the levels of erythrocytes, hemoglobin, neutrophils, lymphocytes, eosinophils, and basophils. Basophil levels were not detected in all groups. The thrombocytes values differed significantly in the P2 (F = 0.03, P <0.05) and P3 (F = 0.045, P <0.05) groups compared to the K + group. There was an increase from K- to P3 in the thrombocytes value and an abnormal increase in thrombocytes. Significant differences were also found in the control group monocytes with the K + group (cervical cancer rats) with values of F = 0.002, (P <0.01), P2 (F = 0.04, P <0.05) and P3 (F = 0.03, P <0.05). compared with the K + group. Eosinophil levels were also significantly different in the K- and K + groups (F = 0.043, P <0.05) but not significantly on the ZAM administration.

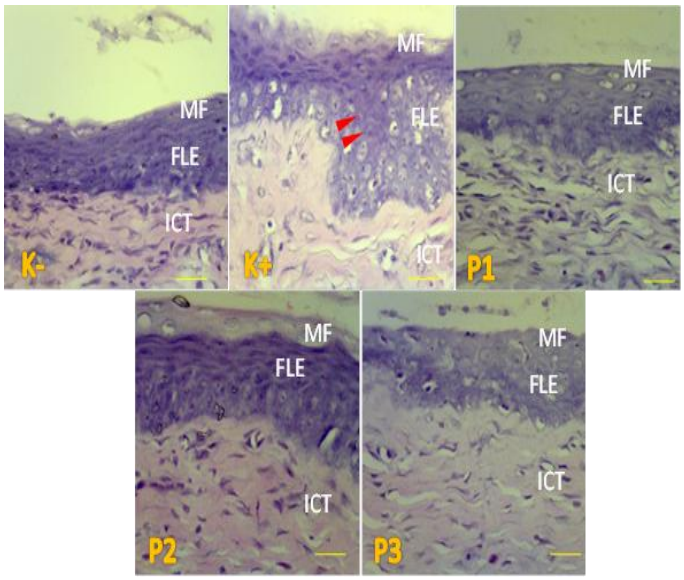

Fig. 1: Histology of cervical tissue induced by benzopyrene in several treatments with Haematoxylin Eosin (HE) staining (400x magnification), *K-*: Control, *K+:* rats model of cancer, *P1*: rats model of cancer with a dose of 100mg/BW of ZAM, *P2*: rats model of cancer with a dose of 200 mg/BW of ZAM, *P3*: rats model of cancer with a dose of 400 mg/BW of ZAM. MF:Mucous folds,FLE: Flattened layered epithelium,ICT:Interstitial connective tissue. Arrow red: Insertion of cancer tissue, Yellow line:200 µm.

Figure 1 showed normal mucosal folds and flattened epithelial portions and interstitial connective tissue. Cervical tissue is usually covered by squamous epithelium or flattened layered epithelium. The mucosal lining of the cervix is composed of glands that produce mucus. The flattened layered epithelium undergoes changes in histology (Figure 1), it can increase in size, cell maturation, and release of epithelial cells. K- has a normal tissue structure than K+. The K+ is a cancerous tissue after injection by benzopyrene, marked with a red arrow as the insertion of cancer tissue. The mucosal folds are irregular, and the interstitial connective tissue stretches, the shape of the cervix improves again when given doses of P2 and P3 of ZAM. Andaliman effect is evident in the histology of Figure 1, ZAM can reduce the insertion of cancerous tissue in the cervix.

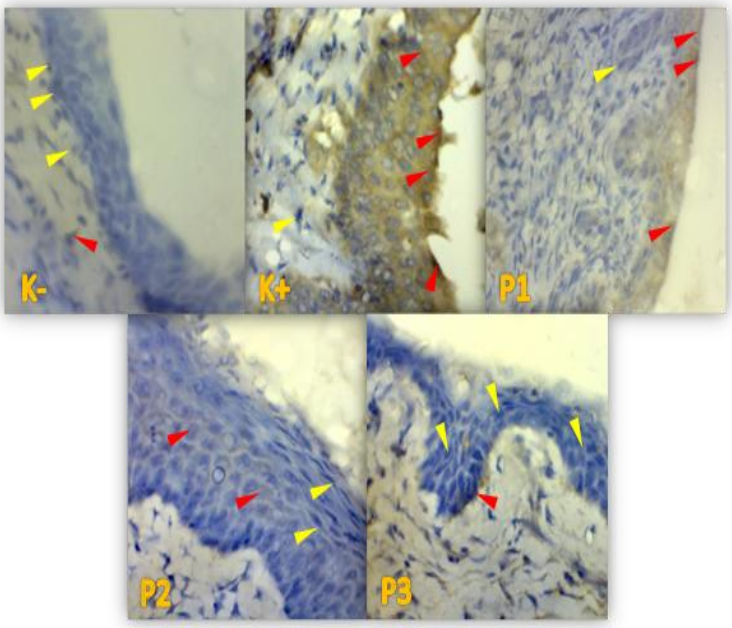

Fig. 2: Histology of cervical cells on CDK4 expression induced by benzopyrene. *K-:* Control, *K+:* rats model of cancer P1: rats model of cancer with a dose of 100mg/BW of ZAM, *P2*: rats model of cancer with a dose of 200 mg/BW of ZAM, *P3*: rats model of cancer with a dose of 400 mg/BW of ZAM. Yellow arrows: Negative expression, Red arrows: Positive expression.

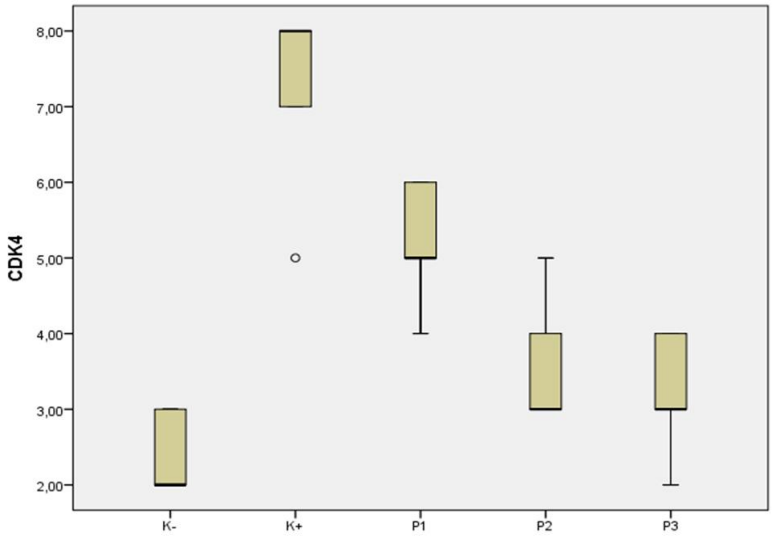

Fig. 3: Box plot of CDK4 expression data in cervical tissue induced by benzopyrene. *K-:* Control, *K+:* rats model of cancer, *P1*: rats model of cancer with a dose of 100mg/BW of ZAM, *P2*: rats model of cancer with a dose of 200 mg/BW of ZAM, *P3*: rats model of cancer with a dose of 400 mg/BW of ZAM

Figure 2 showed the CDK4 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 cell, the ratio of the cell nucleus to the cytoplasm, many variations in the shape of the nucleus. The highest CDK4 expression was at K+ and the lowest was at K- (Table 2). The expression of CDK4 (marked brown) reduced in the ZAM treatment at doses of 200 and 400mg/KgBW (P2 and P3) in cervical histology, but in P1, many brown marks are still visible on the cervical tissue.

These proteins will bind, inhibit proteins, cell cycle development, modulate cell division, and signal transduction pathways of apoptotic. The majority of CDK4 c in P2 and P3 have more negative expressions than positive expressions in histology. The histology showed a significant difference with Mann-Whitney test between each treatment (P <0.05) in Table 2. The cervical tissue histology of the normal (K-) and P3 groups has an almost normal cell structure. P2 and P3 doses are good doses in cervical tissue because these doses can reduce the overexpression of CDK4. Insignificant data (P> 0.05) were found in the control and P3 groups (Table 2 and Figure 3). So that ZAM administration showed a significant difference in cervical tissue after benzopyrene injection.

Table 2. Kruskal Wallis and Mann-whitney analysis of CDK4 expression in cervical tissue

Groups	n	Mean Rank	Kruskal-Wallis	Mann-Whitney				
				K-	K+	P1	P2	P3
K-	5	4.70			0.007*	0.008*	0.032*	0.118
K+	5	22.30				0.041*	0.008*	0.008*
P1	5	17.90	0.001				0.031*	0.016*
P2	5	11.00						0.690
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).

DISCUSSION:

Injection of benzopyrene after three months into rats resulted in the insertion (manifestation?) of cancer cells in the K+ group (Figure 1). Carcinogenic substances are substances that trigger the development of normal cells into cancer cells such as benzopyrene. This has been shown to cause tumors in each experimental animal model through the path of food, respiration, or skin surface contact[27]. The carcinogenic initiation process of benzopyrene can occur in tissue parts far from the point of origin of exposure[28,29].

Injection of benzopyrene in rats affects ~~to~~ the hematological the total erythrocyte count, leukocyte count, and leukocyte differentiation as a whole, and is an important indicator of tumorigenesis or cancer in rats[22]. Increased blood thrombocytes were due to the long treatment time. The administration of ZAM affects the level of blood hematology, especially thrombocytes, monocytes, and leukocytes. Neutrophils are an integral part of the immune system. These phagocytes are usually found in the bloodstream. However, during the acute phase of inflammation, neutrophils leave blood vessels and migrate to the site of inflammation and persist for one or two days before undergoing spontaneous apoptosis[30]. Monocytes are easy because they have an oval, notched or horseshoe-shaped nucleus. They leave blood circulating and

migrate to the tissues where they mature into macrophages and are involved in another foreign phagocytosis[30].

Cervical cancer is a malignant tumor /carcinoma that grows in the cervix. Cervical cancer is caused by several types of high-risk human papillomavirus (HPV), such as HPV16 and HPV18, which have oncogenes E6 and E7. ~~b~~ Both of these gene expressions are prerequisites for cancer development and defense of the malignant phenotype. The elimination of these two oncogenes ~~is~~ are considered for application in molecular therapy of cervical cancer[30]. The flattened epithelium undergoes histological changes (Figure 1), there can be an increase in size, cell maturation, and the release of epithelial cells. K- has a normal network structure than K+.

The abnormal shape of the cervix changes when given doses of P2 and P3 from ZAM. Andaliman effect shown on histology Figure 1, ZAM can reduce the entry of cancerous tissue in the cervix. Cervical tissue has complex histology of cervical tissue against a background, squamous epithelium containing the cell nucleus and cytoplasm, and normal stroma, mucosal folds, epithelium, and connective tissue (Figure 2). Epithelial tissue provides diagnostic information as well as the state of body metabolism and is related to the state of normal cells or abnormal cancer cells. Andaliman has quercetin glycosides (flavonoid group) such as the *Cosmos caudatus* plant which can regulate the cancer cell cycle by binding to several targets[31].Andaliman is also like the Haramonting plant which has antioxidant activity to treat various diseases, prevent DNA damage and improve tissue histology[32].

The bio-active molecules from the Zanthoxylum or as a complementary treatment for a sequential treatment of Zanthoxylum with chemotherapeutic drugs might be a new therapeutic for the anti-cancer treatment (Sing et al. 2015). The previews study also reported that the hydroethanolic stem bark extract of the Zanthoxylum tetrasermum has shown good inhibition on lipid peroxidation and proliferation which indicated the antitumor activity of the plant extract in in vivo model against N-methyl-N-nitrosourea (MNU) induced breast carcinoma in mice and it is believed very strongly that in cancer of the cervix is also an inhibitor of proliferation because it has the same process (Narayanasami and Ragavan. 2014), CDK4/6 inhibitors have a proven clinical benefit in breast cancer (Kalu and Johnson. 2017),

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The histology of cancer cell tissue shows cell abnormalities as indicated by nuclear enlargement, uncontrolled development of structures, the irregular shape of the mucosal layer, and many variations in the shape of the nucleus. The highest expression of CDK4 was found in K + and the

lowest was in K- (Figure 3 and Table 2). ZAM can inhibit CDK4 expression in cervical cells because it has high antioxidants, reduces MDA, anti-inflammatory, and increases HSP-70[20,34]. The n-hexane fraction of *Zanthoxylum acanthopodium* contains bioactive compounds and is effective as an anticancer, inhibits apoptosis, and reduces the expression of Cyclin D1[35]. The ethanol extract of the fruit from this plant has higher anti-radical activity compared to acetone and hexane extracts[35].

CDK4 / 6 associates with D-type cyclins and mediation through the stages of G1 when the cell is preparing for DNA synthesis. CDK4 were related to the pathogenesis of cervical cancer and these proteins are suggested as candidates of new pathological tumor markers for cervical cancer (Song et al. 2012). CDK4/6 and cyclin D1 are highly expressed and are mostly localized in the nucleus with some localized in the cytoplasm of cervical cancer cell lines, then the role of CDK4/6 on cervical cancer is an inhibitor of growth cancer cells and can be considerate as an important factor in human cervical cancer pathogenesis (Xiong et al. 2019).

Based on the toxicity test, in addition to having high antioxidants, this plant also has low toxicity[36]. Natural products are unique due to their chemical complexity, diversity, and related biological activity to be cytotoxic anticancer in cervical cancer cells[37]. CDK4 is very important in cells because CDK4 keeps cells from resting or triggers cells to apoptosis, preventing cell growth into malignant cancer[13].

This research can provide information to the public and the Indonesian government about the benefits of this endemic fruit as an anticancer so that it can be cultivated in every yard. Because ZAM can act as an anticancer candidate for molecular strategies.

CONCLUSION

In conclusion, ~~W~~ we demonstrated that the injection of benzopyrene and given *Zanthoxylum acanthopodium* extract methanol (ZAM) in cervical tissue affect hematological value and this herb affects CDK4 expression in cancer cervical histology. This plant can be developed into a cervical cancer drug candidate.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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7. Xiong Y. et al. 2019. Biomed and Pharmachotherapy, 112Ribociclib, a selective cyclin D kinase 4/6 inhibitor, inhibits proliferation and induces apoptosis of human cervical cancer *in vitro* and *in vivo*

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