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Original article

Histological changes of cervical tumours following *Zanthoxylum acanthopodium* DC treatment, and its impact on cytokine expression

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ABSTRACT

Cervical cancer is the second most lethal cancer in Indonesia, behind breast cancer. One of the reasons cancer cells are difficult to treat is that the immune system is sometimes unable to recognise them as foreign. Cytokinin therapy is carried out so that the immune system can strengthen its response to cancer cells, with the aim of slowing or stopping the development of malignant cells. *Zanthoxylum acanthopodium* DC, also known as andaliman, is an Indonesian herb and a member of the Rutaceae family. It is rich in antioxidants and has anti-inflammatory and anti-cancer properties. The current study aimed to investigate the histological changes and changes in the expression of cytokines, such as IL-10, IL1β, VEGFR1, and TGFβ1, associated with andaliman treatment. Sample tissues and serums extracted from cervical cancer rat models were used. Rats were divided into five groups: a control group (C−), cancer model group (C+), cancer with a dose of *Z. acanthopodium* methanolic extract (ZAM) 100 mg/body weight (BW) ZAM (ZAM100), cancer with a dose of ZAM 200 mg/BW ZAM (ZAM200), and cancer with a dose of ZAM 400 mg/BW ZAM (ZAM400). Treatment lasted for 1 month. Blood samples were prepared for ELISA analysis, and cervical tissue was stained for immunohistochemistry using antibodies against IL-10, IL-1β, VEGFR1, and TGFβ1. Administration of ZAM had no significant effect on rat body weight and cervical organs ($p > 0.05$). However, it impacted haematological parameters in rats with cervical cancer ($p < 0.05$). Elevated malondialdehyde levels may be linked to superoxide dismutase deficiency in tumour tissue. ZAM significantly decreased the expression of IL1β, TGFβ1, and VEGFR1 ($p < 0.01$), while it increased the expression of IL-10. Therefore, ZAM may be a potential target for molecular cytokine therapy for cervical cancer.

1. Introduction

Cervical cancer is the second most frequent cancer globally, behind breast cancer, and it is particularly prevalent in developing nations, such as Indonesia (Simanullang, 2018). According to the International Agency for Research on Cancer (IARC), breast cancer affects 40 out of every 100,000 women, while cervical cancer affects 26 out of every 100,000. Human papillomavirus infection (HPV) and cervical lesions are caused by a combination of intrinsic and extrinsic causes (Simanullang and Sitopu, 2020). In this context, the involvement of immunoregulatory mechanisms is crucial (Simanullang, 2018; Simanullang et al., 2021a).

Cytokines are inflammatory response products that play a crucial role in the immune system response. The interleukin-1 (IL-1) family of proteins, including interleukin-10 (IL-10) and IL-1β, plays

Abbreviations: C−, Control; C+, Cancer rats; DAB, 3,3-Diaminobenzidine; IARC, International Agency for Research on Cancer; IL-10, Interleukin-10; IL1β, Interleukin 1-beta; IFN, Interferon; MDA, Malondialdehyde; NGAL, Neutrophil gelatinase associated lipocalin; SOD, Superoxide dismutase; SGOT, Serum Glumatic Oxaloacetic Transamine; SGPT, Serum Glutamic Pyruvic Transaminase; TGFβ1, Transforming growth factor-beta; VEGF, Vascular endothelial growth factor; VEGFR1, Vascular endothelial growth factor receptor-1; ZAM100, Cancer rats + 100mg/Kg BW of ZAM; ZAM200, Cancer rats + 200mg/Kg BW of ZAM; ZAM400, Cancer rats + 400mg/Kg BW of ZAM.

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a critical role in the innate immune system (Garlanda et al., 2013). The IL-1β genotype can be functionally changed to contribute to the aetiology of cervical cancer. IL-1β has been found to regulate gene expression, cytokine production, cellular adhesion and migration, angiogenesis, and immunological responses, among other things (Zhang et al., 2020). IL-10, also known as an immunoregulatory cytokine, is a potent anti-inflammatory cytokine found in cancer cells (Oft, 2019). Increased IL-10 expression in normal and abnormal cervixes has been observed to correlate with the severity of squamous intraepithelial lesions (Batchu et al., 2021). IL-10 has the primary biological role of restricting and terminating the inflammatory response, as well as suppressing tumour development (Oft, 2019; Batchu et al., 2021). Overexpression of IL-10 in the tumour microenvironment can result in the immunological rejection of cancer (Batchu et al., 2021).

Transforming growth factor beta (TGFβ) is a multifunctional cytokine that plays a role in cell proliferation and differentiation, angiogenesis, immunosuppression, cell motility, apoptosis, wound healing, embryonic development, and cancer pathogenesis, among other things (Park et al., 2021). Because of its ability to interrupt the cell cycle and induce apoptosis, it operates as a tumour suppressor gene during the early stages of carcinogenesis (Dash et al., 2020). Cervical cancer has been connected to TGFβ1, one of several cytokines that regulate cell development, maturation, and differentiation (Park et al., 2021). Vascular endothelial growth factor (VEGF) is a protein released by tumour cells that stimulates the creation of blood vessels in some cancers (Nascimento et al., 2021). Vascular endothelial growth factor receptor-1 (VEGFR), one of the three receptor tyrosine kinases for VEGF, is a key regulator of cancer angiogenesis (Nascimento et al., 2021). Although VEGFRs were previously thought to only be expressed on endothelial cells (ECs), new research has shown that VEGFR-1 is present in malignant, non-EC-type cancer cells (Ceci et al., 2020; Nascimento et al., 2021) and has the ability to enhance mitogen-activated protein kinase (MAPK) signalling, migration, and invasion. As carcinoma cells produce VEGFR-1 ligands, this effect may be autocrine-regulated (Ceci et al., 2020).

Andaliman (*Zanthoxylum acanthopodium* DC), from the genus *Zanthoxylum*, is a member of the Rutaceae family. In Indonesia, this spice grows wild in the dense forests of Toba Samosir, North Tapanuli, and Dairi in the Northern Sumatran region (Djati and Christina, 2019). Alkaloids, glycosides, tannins, phenols, and flavonoids are antioxidants found in andaliman that have the ability to act as natural preservatives, anti-inflammatories, and antibacterial agents (Wijaya et al.,2019). Previous studies have shown that andaliman is a potent antioxidant, anti-inflammatory, and anticancer agent against cervical cancer cell (HeLa) lines and MCF-7 cell lines (Arsita et al., 2019; Simanullang et al., 2021a; Syari et al., 2019). Aside from its applications in cervical cancer (Simanullang et al., 2021b), andaliman can improve the histology of placental tissue, hypertensive kidneys and livers in rats, human trophoblast Hes1 and Notch1 genes, and diabetic burns (Situmorang et al., 2019a; Situmorang et al., 2019b; Situmorang et al., 2019c; Situmorang et al., 2021a; Situmorang et al., 2021b; Manurung et al., 2021). Members of the *Zanthoxylum* family can be used as anti-cancer therapeutic agents (Okagu et al., 2021). This study aimed to investigate the histological changes associated with andaliman treatment, as well as the associated expression changes of cytokines, such as IL-10, IL1β, VEGFR1, and TGFβ1 in sample tissues and serums from cervical cancer rat models, so that andaliman may be developed into cervical cancer drugs in the future using cytokine therapy.

2. Material and methods

2.1. Materials

The *Z. acanthopodium* used in this study originated in Dairi, North Sumatra Province. The voucher was identified and authorised by Dr Nursahara Pasaribu, MSc at the University of Sumatera Utara, Medan, Indonesia and was stored at the Medanense Botanical Herbarium (Registration number 5399/MEDA/2020). The antibodies used for the ELISA kit were the IL-1β polyclonal antibody, catalogue #13-7112-81; IL-10 monoclonal antibody (JES3-9D7), catalogue #16-7108-81; TGF β-1 monoclonal antibody, catalogue #MA1-169 (B11-4C3); VEGFR1 (soluble) polyclonal antibody, catalogue # 36-1100, (eBioscience Inc, San Diego, USA); IL-10 polyclonal antibody, catalogue # BS-20373R; IL-1β polyclonal antibody; VEGFR1 polyclonal antibody, catalogue # PA1-21731; and TGFβ1 polyclonal antibody, catalogue # BS-0086R (Thermo Fisher, Waltham, Massachusetts). Additionally, a phosphate buffer solution (PBS) of 50% glycerol and 1% bovine serum albumin (BSA) was used as the storage buffer solution (Catalogue # BS-0812R).

2.2. Preparation of the Z. acanthopodium methanolic extract (ZAM)

Z. acanthopodium fruits were cleaned of any soil or dust that had adhered to the fruit. Three steps were used to create the fruit extract. First, the andaliman fruit was cleaned and drained until dry, then mashed in a blender. Second, the ZAM extract was made by macerating the fruit in a 96% methanol solvent overnight. It was then percolated until it was clear. The concentrated liquid was then evaporated to obtain the powder extracts. Finally, as the extract does not dissolve completely in water, a homogeneous mixture was obtained by mixing 1–1.5% (1 mL) of the suspending agent CMC in 150 mL of distilled water. The dregs were washed with the 96% methanol solvent before being transferred to a closed container and stored in a cool, dark place for two days. Methanol extract was used as it was able to identify 20 chemical compounds in the fruit. Sixteen of the chemical compounds were derived from terpenes and terpenoids. The other four compounds were identified structurally as aliphatic derivatives (neoherculin, ethyl linoleate, ethanol,2-(3,3-dimethylcyclohexylidene), and 9,12-Octadecadienoyl chloride; (Sibero et al., 2020).

2.3. Experimental Animals

The research was conducted at the Utara's Biology Laboratory and the Pathology and Anatomy Laboratory, Laboratory of the Faculty of Medicine, University of Sumatra, from January to October 2021. Rats were divided into five groups, a control group (Group C−), cancer model group (Group C+), cancer rats who received a dose of 100 mg/body weight (BW) of ZAM (Group ZAM100), cancer rats who received a dose of 200 mg/BW of ZAM (Group ZAM200), and cancer rats who received a dose of 400 mg/BW of ZAM (Group ZAM400). Treatments were administered for 30 days. The doses used were determined based on a toxicity test and previous research (Situmorang et al., 2020). The rats were dissected after the 30th day of ZAM administration. Blood samples were then prepared for ELISA analysis, and immunohistochemical staining of cervical tissues was carried out. The Ethics Committee for Handling Experimental Animals, Faculty of Mathematics and Natural Sciences, USU, approved this research (Ethical Clearance: No. 0262/KEPH-FMIPA/2021).

2.4. Rat model of cervical cancer

The Animal House Laboratory at the University of Sumatera Utara provided 30 rats (*Rattus norvergicus*) weighing 180–200 g for this investigation. The number of rats in each study group was 6. Food pellets and water were provided so the rats could acclimatise to the laboratory conditions for four weeks prior to the trial. Rats in Group C− were given normal food and drinking water ad libitum. Groups C+, ZAM100, ZAM200, and ZAM400 received cervical injections of 50 mg benzopyrene diluted with corn oil in order for tumour formation to occur (Sanchala et al., 2018). Tumours were observed in the rats after 3 months. Identification of tumour presence was carried out from preparations made via the Pap smear method and by cervical dissection that were then observed in paraffin blocks. ZAM was administered after 3 months to the ZAM100, ZAM200 and ZAM400 groups for 30 days. After 1 month of oral ZAM administration, an anaesthetic combination of 300 mg/kg BW of ketamine and 15–30 mg/kg BW of xylazine was administered. The cervix was immediately dissected and was either fixed with 10% formalin for histopathological study or was washed with cold PBS, smeared with tissue paper to remove excess PBS, weighed, and stored at −80 °C for immunohistochemistry analysis.

2.5. Measurement of haematology parameters

The blood samples were collected from the heart with a sterile syringe. To avoid haemolysis, exposure to water was avoided. A total of 0.5 mL was placed in a microcentrifuge tube with 10 μL/1 mL of EDTA anticoagulant. The Medan City Health Laboratory performed the haematology examinations. Each sample was run three times according to the manufacturer's instructions.

2.6. Measurement of superoxide dismutase (SOD), malondialdehyde (MDA), and neutrophil gelatinase-associated lipocalin (NGAL)

SOD: The blood samples were collected from the heart and then placed into test tubes and deposited for 30 min at room temperature. The samples were then centrifuged at 1500 rpm for 30 min. Following centrifugation, 100 μL of serum was placed into an Eppendorf tube via micropipette. Preparation of the reagents and samples followed the standards set by the manufacturer's instructions. Once the 96-well microplate was prepared, the standard solution in the Eppendorf tubes was transferred to the wells. A 0.1 mL sample diluent buffer was added as a control on the microplate, while 0.1 mL of the rat blood serum was added to each empty well. The microplate was duplicated in the lid and incubated at 37 °C for 90 min. The microplate cover was opened, and the contents of the wells drained and turned over to tissue paper so that no liquid remained on the side of the microplate. Following that, 0.1 mL of the biotin SOD antibody solution was added to each well. The microplate was then closed and incubated at 37 °C for a further 60 min. Following incubation, 0.1 mL of streptavidin–biotin complex (SABC) solution was added to each well. The microplate was then covered and incubated at 37 °C for 30 min. The wells were then washed 5 times using a wash buffer before 90 μL of TMB was added to each well. The microplate was then closed and incubated at 37 °C for 25–30 min until the colour changed to blue, at which point 0.1 mL of tetramethylbenzidine (TMB) stop solution was added, and the optical density (OD) absorbance read at 450 nm on an ELISA reader.

MDA: A standard reagent for duplication was added to each well (50 μL/well), while the blood samples were added to the other wells (50 μL/well). Following that, 50 μL of the biotinylated detection ab working solution was added to each well. That plate was then covered and incubated for 45 min at 37 °C. Following incubation, 350 μL of wash buffer was added to each well. The buffer was left to soak for 1–2 min. The solution was then aspirated from each well, which were then pat dry on absorbent paper. The washing was repeated 3 times, after which 100 μL of HRP conjugate working solution was added to each well. The plate was again covered and incubated for 30 min at 37 °C. After incubation, 90 μL of substrate reagent was added to each well. The plate was then covered with a new cover plate and incubated for 15 min at 37 °C. Following the final incubation, the plate was protected from light, and each well had 50 μL of stop solution added. The OD absorbance was read at 450 nm on an ELISA reader.

NGAL: The blood samples were collected from the heart and placed in test tubes that were immediately centrifuged at 4230 rpm for 5 min. Following the removal of the supernatant, the samples were divided into 3 cup samples which held up to 0.5 ccs. The samples were then frozen and stored at −20 °C until measurement. Measurement was done using a commercial NGAL. The OD absorbance was read at 450 nm on an ELISA reader.

2.7. Elisa

Enzo Life Sciences' ELISA test was used to perform quantitative cytokine analysis. In this study, the IL-1β (rabbit) ELISA kit, the IL-10 (rat) ELISA kit, the TGFβ1 (rat), and the VEGFR1 (rabbit) ELISA kits were used. Each sample was run three times according to the manufacturer's instructions. The blood sample in each 1.5 cc tube sample was then inserted into the EDTA tube for blood plasma to be taken. The samples were then put into microcentrifuge tubes without EDTA to collect the serum. The blood samples were then centrifuged to separate the blood plasma and blood serum from the blood cells. The wash solution was then prepared. The plate was washed with the wash solution 4 times using the ELISA tool. Following washing, 100 μL of the diluted enzyme to each well. The plate was then covered with aluminium foil (in the dark) and incubated for 30 ± 2 min. Then, 100 μL of TMB Substrate Solution (chromogen-substrate solution) was added to each well, and the plate was incubated at room temperature in the dark for 10 min. Following the final incubation. 100 μL of stop solution was added to each well. All wells were then entered into the ELISA at an absorbance wave of 450 nm.

2.8. Immunohistochemistry

Immunohistochemical analysis was conducted to investigate the histological changes to the expression of several cytokines such

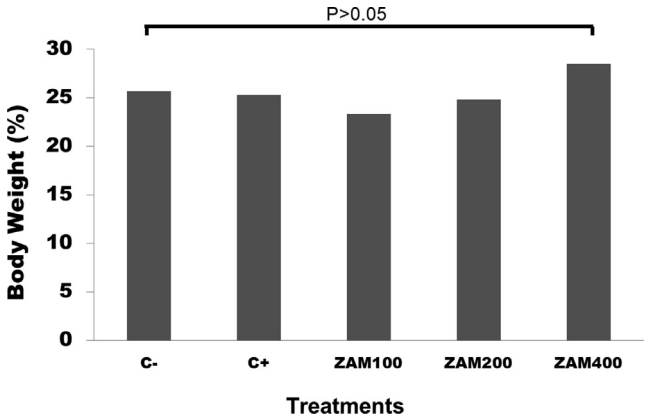

Fig. 1. Effect of *Z. acanthopodium* methanol extracts on Body Weight Percentage of rats. C−: Control, C+: Cancer rats without treatments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. (P > 0.05 = Non-signifikan).

Table 1
Effect of Z. acanthopodium methanolic extract on Hematology parameters of rats.

Hematology parameters	Unit	Groups (Mean ± SD)				
		C−	C+	ZAM100	ZAM200	ZAM400
Hemoglobin	g/dL	14.82 ± 0.88	26.87 ± 2.12$^{\#}$	20.32 ± 1.34	19.78 ± 2.21*	15.22 ± 1.97*
Erythrocytes	x10^6/μL	7.52 ± 0.44	8.99 ± 0.76$^{\#}$	8.67 ± 0.98	7.69 ± 1.03	7.53 ± 1.54
Leukocytes	x 10^3/μL	7.60 ± 0.51	11.23 ± 1.92 $^{\#}$	10.72 ± 1.22	9.76 ± 1.22	8.02 ± 1.82*
Haematocrit	%	41.8 ± 1.77	45.98 ± 3.87	44.77 ± 6.91	43.99 ± 11.13	41.2 ± 7.22
Neutrophils	x 10^3/μL	1.203 ± 0.12	2.90 ± 0.67	2.21 ± 0.43	1.94 ± 0.87	1.301 ± 0.67
Lymphocytes	x 10^3/μL	5.871 ± 1.43	7.891 ± 1.87$^{\#}$	7.541 ± 1.22	6.54 ± 1.97	5.92 ± 1.03*
Eosinophils	x 10^3/μL	0.045 ± 0.01	0.098 ± 0.02 $^{\#}$	0.085 ± 0.02	0.072 ± 0.01	0.054 ± 0.01*
Monocytes	x 10^3/μL	0.496 ± 0.09	0.872 ± 0.03 $^{\#}$	0.761 ± 0.02	0.674 ± 0.02	0.573 ± 0.02*
SGOT	u/L	112. 18 ± 7.21	211.56 ± 12.01$^{\#}$	170.77 ± 11.22	157.65 ± 19.88*	120.87 ± 19.21*
SGPT	u/L	48. 76 ± 5.43	153.87 ± 17.89$^{\#\#}$	140.12 ± 16.02	98.65 ± 9.56*	59.21 ± 8.97**
Creatinine	mg/dL	0.43 ± 0.02	0.76 ± 0.04$^{\#}$	0.73 ± 0.12	0.52 ± 0.11	0.45 ± 0.09*
Ureum	mg/dL	22.02 ± 1.02	35.22 ± 2.90	30.12 ± 2.05	22.14 ± 1.89	20.94 ± 0.99

C−: Control, C+: Cancer rats without teratments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. ($^{\#}$p < 0.05 compared C−, $^{\#\#}$p < 0.01 compared C−, *p < 0.05 compared C+, **p < 0.01 compared C+).

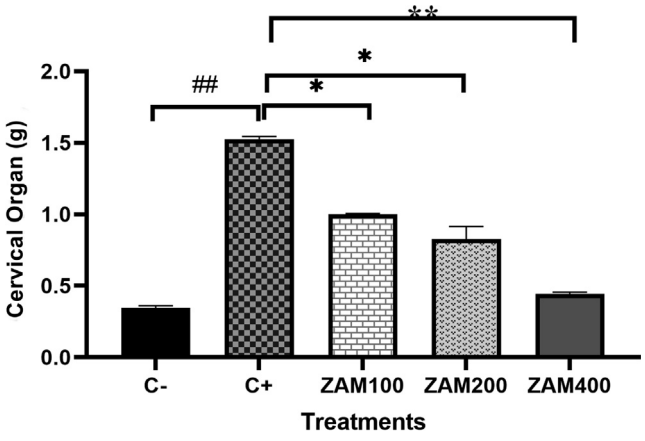

Fig. 2. Effect of *Z. acanthopodium* methanol extracts on cervical organs (g). C−: Control, C+: Cancer rats without treatments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. ($^{\#\#}$p < 0.01 compared C−, *p < 0.05 compared C+, **p < 0.01 compared C+).

as IL-10, IL-1β, VEGFR1, and TGFβ1, after andaliman administration. A microtome was used to cut paraffin-embedded cervical tissue with a thickness of 4–6 μm. The tissue was heated in a citrate buffer at pH 6.0 and 350 W for pre-treatment. After washing with PBS, the tissue was incubated with IL-10, IL-1β, VEGFR1, and TGFβ1 antibodies at 37 °C, then washed with PBS again before being treated with avidin–biotin peroxidase. For the chromogenic visualisation reaction, 3,3-Diaminobenzidine (DAB) hydrochloride was used, which was then stained with haematoxylin Mayer (Situmorang et al., 2021a). The cervical tissue on the slide was stained with haematoxylin, and the score was calculated by multiplying the positive result by the staining intensity: 0 indicated that<10% of the cells were stained, 1 indicated that 10–25% of the cells were stained, 2 indicated that 25–50% of the cells were stained, 3 indicated that 50–75% of the cells were stained, and 4 indicated that >75% of the cells were stained. The staining intensity was graded as 1 (weak), 2 (moderate), or 3 (strong).

2.9. Statistical analysis of data

The ANOVA test and the Kruskal-Wallis test (for non-parametric data) were used to analyse the data using the SPSS 22 programme.

3. Results

3.1. Effect of Z. acanthopodium methanolic extract on body weight and cervical organ weight

There was no significant statistical difference in body or cervical organ weight between benzopyrene-injected rats and the control

Table 2
Effect of *Z. acanthopodium* methanolic extract on level of Superoxide dismutase (SOD), Malondialdehyde (MDA) and Neutrophil gelatinase-associated lipocalin (NGAL) in Rats.

Parameters	Unit	Groups (Mean ± SD)				
		C−	C+	ZAM100	ZAM200	ZAM400
SOD	pg/mL	23.51 ± 0.051	12.66 ± 0.61$^{\#}$	25.22 ± 1.22*	20.12 ± 0.87*	14.66 ± 0.09
MDA	μM/L	6.77 ± 0.23	13.92 ± 2.11$^{\#}$	11.22 ± 1.45	9.34 ± 1.12*	7.01 ± 1.03*
NGAL	Ng/mL	0.156 ± 0.01	0.542 ± 0.02$^{\#}$	0.340 ± 0.02	0.211 ± 0.02	0.135 ± 0.01*

C−: Control, C+: Cancer rats without teratments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. ($^{\#}$p < 0.05 compared C−, *p < 0.05 compared C+).

Table 3
Statistical analysis of IL-1β expression on histological changes of cervical after *Z. acanthopodium methanolic* extract administration.

Groups	Mean ± SD	Kruskal-Wallis	p value (Mann-Whitney)				
			C-	C+	ZAM100	ZAM200	ZAM400
C-	12.32 ± 1.28			0.0023	0.0040	0.0032	0.0450
C+	42.22 ± 3.23$^{\#\#}$				0.0600	0.0400	0.0025
ZAM100	37.14 ± 2.23	0.00				0.0450	0.0450
ZAM200	29.21 ± 2.41*						0.0440
ZAM400	16.22 ± 1.98**						

C−: Control, C+: Cancer rats without teratments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. ($^{\#\#}$p < 0.01 compared C−, *p < 0.05 compared C+, **p < 0.01 compared C+).

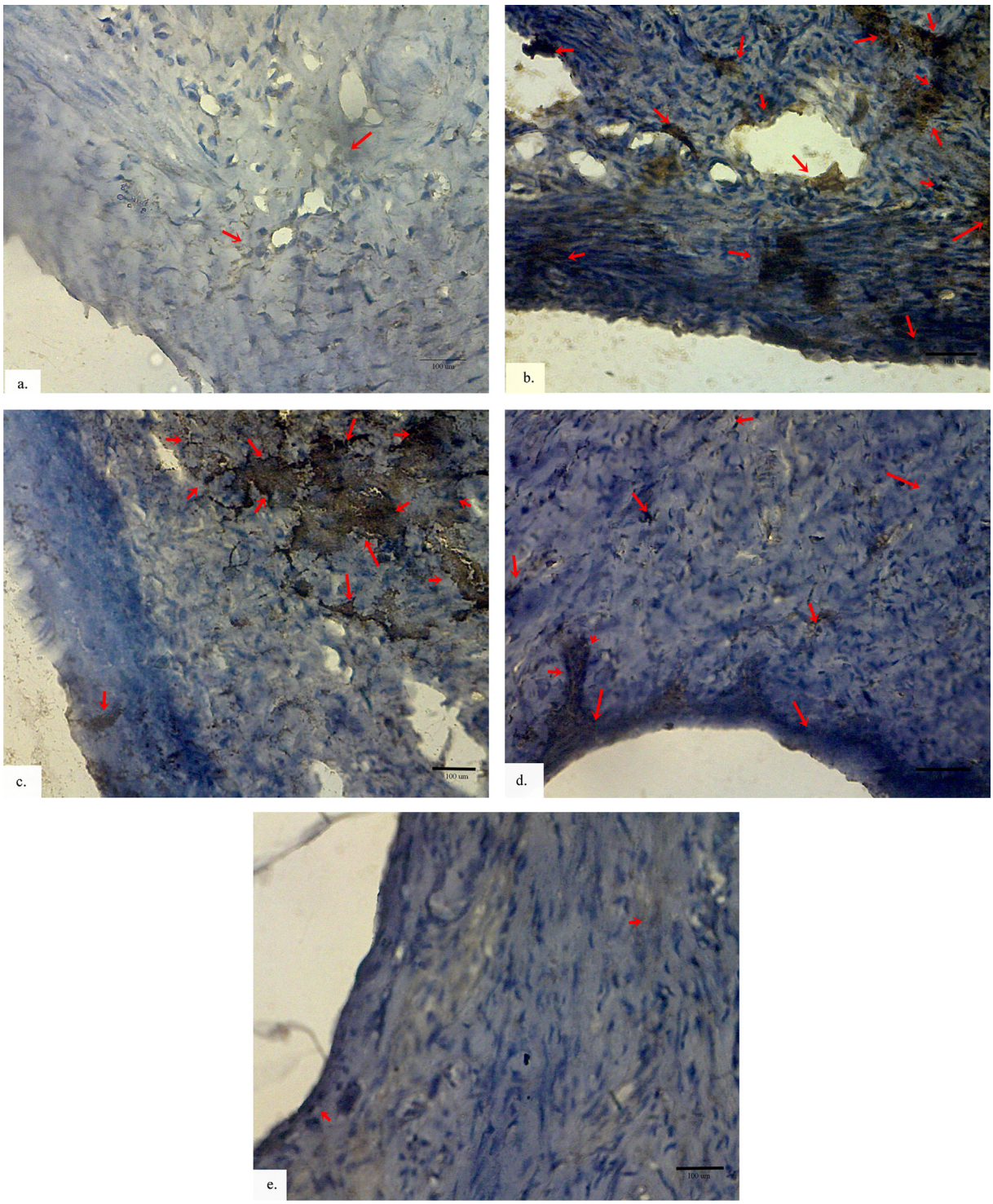

Fig. 3. Histological changes of cervical rats on IL1β expression by *Z. acanthopodium* methanol extracts. a. Control, b. Cancer rats without treatments, c. Cancer rats + 100 mg/Kg BW of ZAM, d. Cancer rats + 200 mg/Kg BW of ZAM, e.Cancer rats + 400 mg/Kg BW of ZAM. red arrows: positive IL1β (400×).

group (P > 0.05). As shown in Fig. 1, the administration of ZAM at a dose of 100–400 mg/Kg BW had no effect on the body weight of the rats. At the time of cervical organ weighing for all groups, there was a significant difference in cervical organ weight with the control group (C−) compared C+ (p = 0.01; Fig. 2). There were also significant differences in the groups given 100 mg/kg BW ZAM (p < 0.05, p = 0.045), 200 mg/kg BW (p < 0.05, p = 0.040), and 400 mg/kg BW (p < 0.01, p = 0.002) compared to the C+ group,

3.2. Effect of Z. acanthopodium methanolic extract on the haematology parameters of rats

The administration of ZAM at a dose of 100–400 mg/kg BW altered the haematological parameters in rats, as shown in Table 1. The administration of ZAM at a dose of 100 mg/kg BW showed an insignificant difference in all haematology parameters compared to the C+ group. The administration of ZAM at a dose of 200 mg/kg

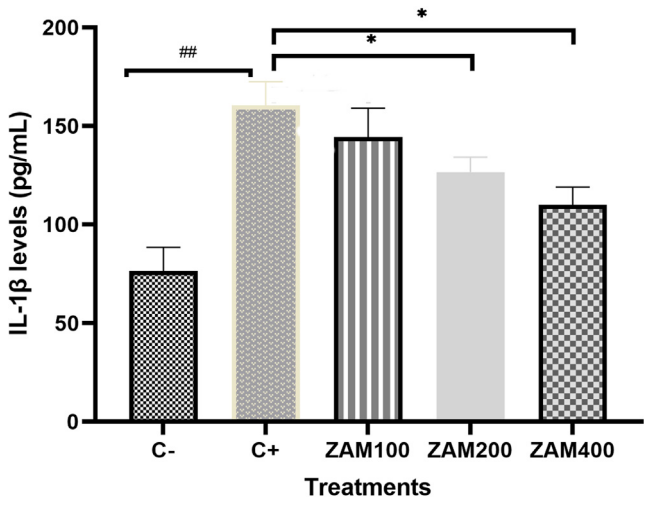

Fig. 4. Effect of *Z. acanthopodium* methanol extracts in IL-1β levels on rats serum. C−: Control, C+: Cancer rats without treatments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM (. ($^{##}p < 0.01$ compared C−, $^{*}p < 0.05$ compared C+,).

BW showed a significant difference in haemoglobin, SGOT, and SGPT values compared to the C+ group. The administration of the 400 mg/kg BW dose demonstrated significant differences in the values of haemoglobin, leukocytes, lymphocytes, eosinophils, monocytes, SGOT, SGPT and creatinine when compared to the C+ group. The difference in the values of neutrophils, haematocrit, and ureum was not significant ($p > 0.05$) across all groups. The administration of ZAM at a dose of 100–400 mg/kg BW, therefore, impacted the haematological parameters of rats, excluding levels of neutrophils, haematocrit, and ureum, as shown in the haematological data in Table 1. Neutrophil, haematocrit, and ureum levels were classified as normal in all groups.

3.3. Effect of Z. acanthopodium methanolic extract on levels of superoxide dismutase (SOD), malondialdehyde (MDA) and neutrophil gelatinase-associated lipocalin (NGAL) in rats

There were significant differences in SOD, MDA, and NGAL levels in cancer model rats ($p < 0.05$, $p = 0.040$). SOD levels in rats given ZAM were significantly different at doses of 100 and 200 mg/kg BW compared to the C+ group; this difference decreased with increasing ZAM dosage Table 2. MDA levels showed a significant decrease at doses of 200 and 400 mg/kg BW ZAM compared to the C+ group. The decrease in MDA levels increased with the increase in the ZAM dose. NGAL levels were only significantly different at a dose of 400 mg/kg BW ZAM Table 2. Antioxidants found in andaliman can lower levels of MDA and serum NGAL, thereby increasing SOD activity. By increasing SOD activity through ZAM administration, it is possible to protect cells from oxidant disorders and oxidative stress, which can lead to a variety of diseases, including cancer. In cervical cancer, increased lipid peroxide due to antioxidant deficiency is associated with increased levels of MDA and circulating NGAL and decreased SOD activity Table 2.

3.4. Histological changes of cervical organs after Z. acanthopodium methanolic extract administration relating to IL-1β expression

Kruskal-Wallis tests indicated a significant difference in IL-1 β expression in all groups (Table 3). Following the Mann-Whitney test, it was found that there was a significant difference ($p < 0.01$, $p = 0.006$) in the expression of IL-1β when compared to the C+ group. At the lowest ZAM dose (100 mg/kg BW), this difference was not significant. However, at doses of 200 and 400 mg/kg BW, there were significant differences ($p < 0.05$; $p < 0.01$) compared to the C+ group. The C+ group had the highest levels of IL-1β expression, while the C− group and 400 mg/kg BW ZAM groups had the lowest.

The presence of positive IL-1β expression was identified in the nucleus and cytoplasm by brownish-black staining, as shown by the red arrow in Fig. 3. The most crucial information for the diagnosis of cervical cancer is in the nucleus and cytoplasm of the cell, while the background and stroma are eliminated. Small cancer cells that could only be detected using a microscope had spread to adjacent lymph nodes. Enlargement of the cell nucleus (Fig. 3b and c), uncontrolled cell development, uneven cell shape, a significant ratio of the cell nucleus to the cytoplasm, and various variations in the form of the nucleus are all signs of cell abnormalities. When ZAM was administered (Fig. 3d and e), the expression of IL-β1 began to drop, with the nucleus appearing black under staining. The cell shape began to become irregular, the nucleus to cytoplasm ratio began to balance, and the histology of the cervical tissue began to improve similar to the C− group. This is consistent with serum IL-1β values obtained using the ELISA reader (Fig. 4). There was a significant difference in serum values between the C− and C + groups ($p < 0.01$). At a dose of 100 mg/kg BW, serum IL-1β expression decreased, but not significantly ($p > 0.05$) compared to the C+ group. In serum with doses of ZAM 200 and 400 mg/kg BW, the suppression of IL-1β expression was observed ($p < 0.05$). As seen in Table 3, Fig. 3, and Fig. 4, ZAM treatment suppressed the production of IL-1β, which promoted cancer cell growth in rats.

3.5. Histological changes of cervical organs after Z. acanthopodium methanolic extract administration relating to IL-10 expression

Kruskal-Wallis tests showed a significant difference in IL-10 expression in all groups ($p < 0.00$; Table 4). Based on the Mann-Whitney follow-up test, it was found that there was a significant difference ($p < 0.01$, $p = 0.003$) in the expression of IL-10 when compared to the C+ group. At the lowest ZAM dose (100 mg/kg

Table 4
Statistical analysis of IL-10 expression on histological changes of cervical after *Z. acanthopodium methanolic* extract administration.

Groups	Mean ± SD	Kruskal-Wallis	p value (Mann-Whitney)				
			C-	C+	ZAM100	ZAM200	ZAM400
C-	72.18 ± 1.44			0.0025	0.045	0.042	0.050
C+	16.10 ± 2.10##				0.062	0.060	0.045
ZAM100	20.66 ± 2.41	0.00				0.055	0.035
ZAM200	23.88 ± 3.21*						0.020
ZAM400	48.19 ± 2.88**						

C−: Control, C+: Cancer rats without teratments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. ($^{##}p < 0.01$ compared C−, $^{*}p < 0.05$ compared C+, $^{**}p < 0.01$ compared C+).

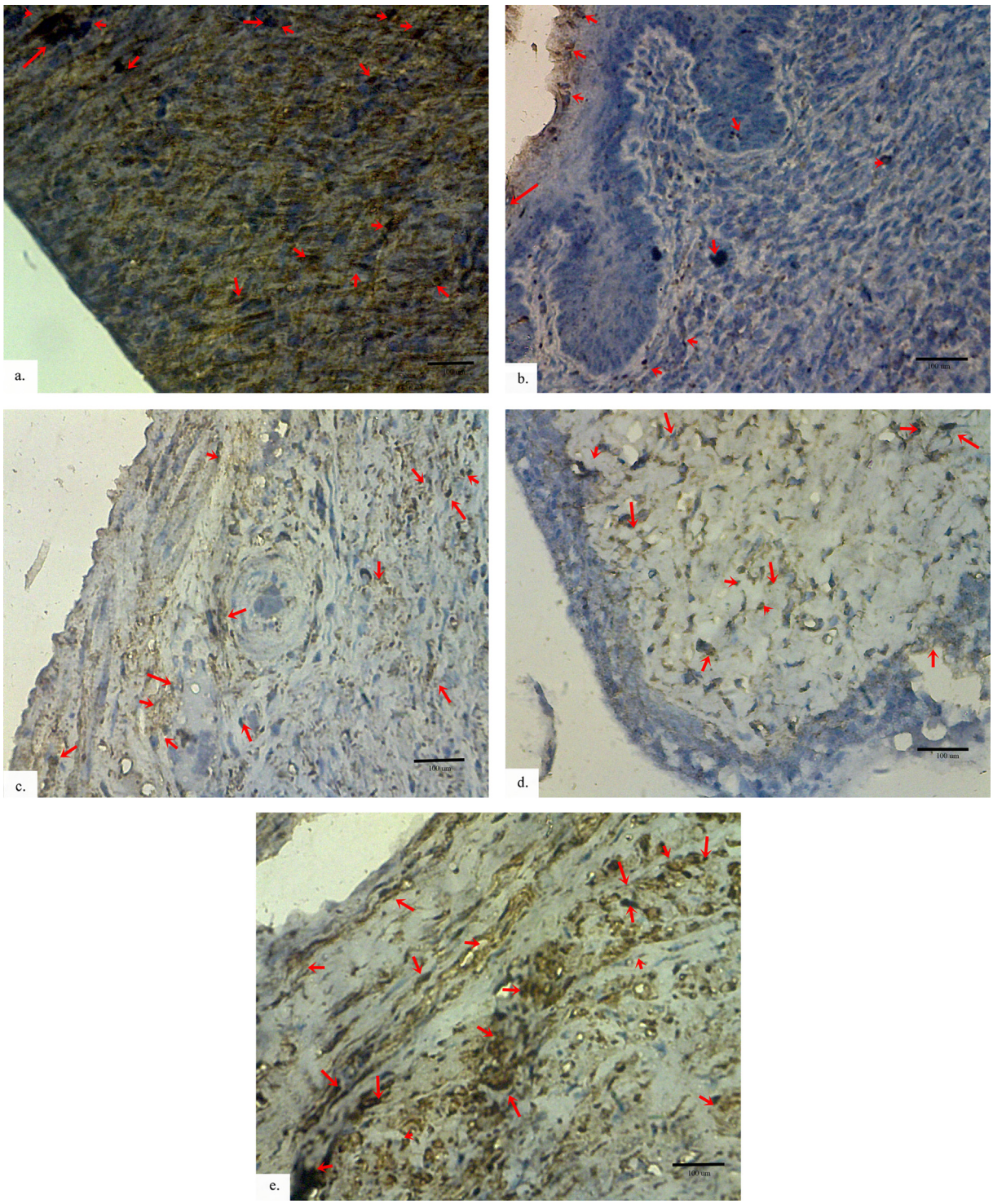

Fig. 5. Histological changes of cervical rats on IL-10 expression by *Z. acanthopodium* methanol extracts. a. Control, b. Cancer rats without treatments, c. Cancer rats + 100 mg/Kg BW of ZAM, d. Cancer rats + 200 mg/Kg BW of ZAM, e.Cancer rats + 400 mg/Kg BW of ZAM. red arrows: positive IL-10 (400×).

BW), this difference was not significant. However, at doses of 200 and 400 mg/kg BW, there was a significant difference ($p < 0.05$, $p < 0.01$) compared to the C+ group. The ZAM400 group had the highest IL-10 expression, while the C−+ group had the lowest.

IL-10 expression is characterised by black or brown nuclei under staining (Fig. 5a). This expression was observed to be increased in the C− group following immunohistochemical examination (Fig. 5). Because IL-10 activity is suppressed in a hypoxic cell environment, the number of nuclei stained black by immunohistochemical dyes were reduced in the cancer model rats. There was a significant reduction in cervical squamous cell cancer following ZAM treatment, which increased as doses increased. Enlargement of the cell nucleus indicates cell abnormalities (Fig. 5b and c). There were no significant changes in the histological classification of cervical tumours after ZAM treatment (Fig. 5c–e), which was corroborated with the results from the ELISA reader (Fig. 6). IL-10 expression in the C+ group was lower ($p < 0.05$, $p = 0.040$) compared to the C− group. Administration of all ZAM doses signif-

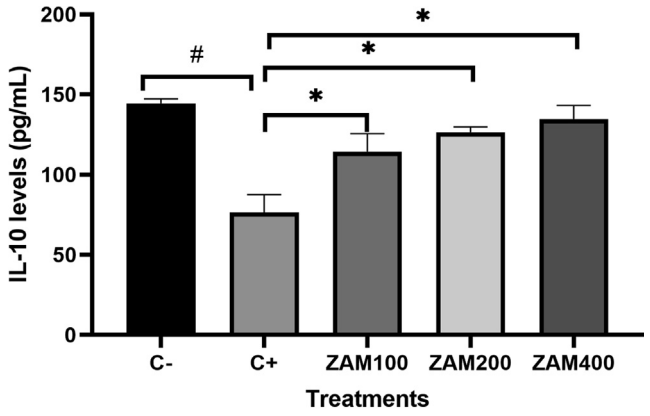

Fig. 6. Effect of *Z. acanthopodium* methanol extracts in IL-10 levels on rats serum. C−: Control, C+: Cancer rats without treatments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM ($^{\#}p < 0.05$ compared C−, $^{*}p < 0.05$ compared C+).

icantly increased IL-10 expression ($p < 0.05$). As shown in Table 4, Fig. 5, and Fig. 6, administration of ZAM increased the expression of IL-10 in cervical cancer rats, thereby suppressing the growth of cervical cancer.

3.6. Histological changes of cervical after Z. acanthopodium methanolic extract administration relating to TGFβ1 expression

As shown in Table 5, the Kruskal-Wallis test demonstrated a significant difference in TGFβ1 expression in all groups ($p < 0.00$). The average expression level across all non-control groups was significantly different from that of group C− ($p < 0.01$, $p = 0.003$). The administration of ZAM at a dose of 100–400 mg/kg BW led to a significant difference in TGFβ1 expression levels ($p < 0.01$) when compared to group C+. The highest TGFβ1 expression was found in the untreated cancer group (C+), while the lowest was found in the control group (C−). Undifferentiated cells in the C+ group were confined to the lower layer of the epithelium and developed mitotic features. Cellular changes confined to the lower epithelium were characterised by epithelial thickening and increased TGFβ1 expression (Fig. 7b). TGFβ1 expression in cancer tissue decreased as the dose of ZAM increased. ZAM (Fig. 7c–e) administration at various doses reduced the number of nuclei stained brown by immunohistochemistry, indicating a positive index of TGFβ1 expression in cancer tissue. Carcinomas that spread uncontrollably in the untreated group were slowed in the ZAM groups and no longer developed into the epithelium. The findings of this histology were consistent with the findings of the ELISA reader analysis of serum TGFβ1 expression in cancer model rats (Fig. 8). TGFβ1 serum expression was higher in the cancer rats ($p < 0.05$, $p = 0.040$) than in the control group. However, this difference was not significant when ZAM doses of 100 and 200 mg/kg BW were administered. The highest dose of ZAM (400 mg/kg BW) did significantly suppress TGFβ1 expression ($p < 0.05$). As shown in Table 5, Fig. 7, and Fig. 8, administration of ZAM, specifically at a dose of 400 mg/kg BW, suppressed TGFβ1 serum expression and histology in cervical cancer rats.

3.7. Histological changes of cervical organs after Z. acanthopodium methanolic extract administration relating to VEGFR1 expression

The Kruskal-Wallis test and the Mann-Whitney follow-up test both showed significant differences in VEGFR1 expression across all groups. Based on the mean values, there was a significant difference in the expression of VEGFR1 between groups C+ and C ($p < 0.05$, $p = 0.040$). While, compared to the C+ group, this difference was not significant at the lowest ZAM dose (100 mg/kg BW), it was significant at doses of 200 and 400 mg/kg BW ($p < 0.05$) Table 6. The C+ group had the highest level of VEGFR1 expression, while the C− group and the ZAM400 group had the lowest. Histological examination of the C+ group revealed that the carcinoma had spread to the pelvic wall; there was no clear space between the tumour and the pelvic wall, and the core was irregular (Fig. 9b). This was in stark contrast to the histology of the C− group, where cervical tissue still contained normal cells (Fig. 9a). At the lowest ZAM dose (Fig. 9c), lesions were larger than in the control group; however, VEGFR1 expression had begun to decrease. The reduction in VEGFR1 expression at ZAM doses of 200 and 400 mg/kg BW demonstrated that ZAM could reduce VEGFR1 expression, as the empty space between the tumours was reduced, the carcinoma stopped expanding, and the nucleus began to take on a normal shape (Fig. 9d–e). This was confirmed by comparing the VEGFR1 serum of cancer model rats (C+) with group C− using an ELISA reader ($p < 0.05$; Fig. 10). At a dose of 100 mg/kg BW ZAM, there was no significant difference. At doses of 200 and 400 mg/kg BW ZAM, improved cervical histology was observed, alongside decreased expression levels of VEGFR1 ($p < 0.05$). As shown in Table 6, Fig. 9, and Fig. 10, ZAM administration decreased VEGFR1 serum expression and improved histology in cervical cancer rats.

4. Discussion

At doses ranging from 100 to 400 mg/kg BW, ZAM demonstrated no significant effect on the body weight of cervical cancer rats. While body weight may be a significant factor in cancer survivors, it was not significant in cervical cancer model rats (Clarke et al., 2018). This could be due to the small size of the cervical cancer tumours, little fat, or overactivity of rats (Clarke et al., 2018). However, the cervical tumours did impact the cervical organ weight in both the control and ZAM groups. Tumour cells can grow indefinitely and undergo excessive angiogenesis. When this happens in specific areas of a tumour, it results in a lack of nutrients, including oxygen. In this state, the tumour cells enter a resting phase in which oxygen is depleted, causing the cells to become

Table 5
Statistical analysis of TGFβ1 expression on histological changes of cervical after *Z. acanthopodium methanolic* extract administration.

Groups	Mean ± SD	Kruskal-Wallis	p value (Mann-Whitney)				
			C-	C+	ZAM100	ZAM200	ZAM400
C-	10.30 ± 1.77			0.045	0.040	0.040	0.070
C+	73.89 ± 4.03$^{\#\#}$				0.080	0.060	0.040
ZAM100	30.61 ± 3.43**	0.00				0.070	0.035
ZAM200	25.22 ± 2.6**						0.020
ZAM400	19.28 ± 2.28**						

C−: Control, C+: Cancer rats without teratments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM ($^{\#\#}p < 0.01$ compared C−, $^{**}p < 0.01$ versus C+).

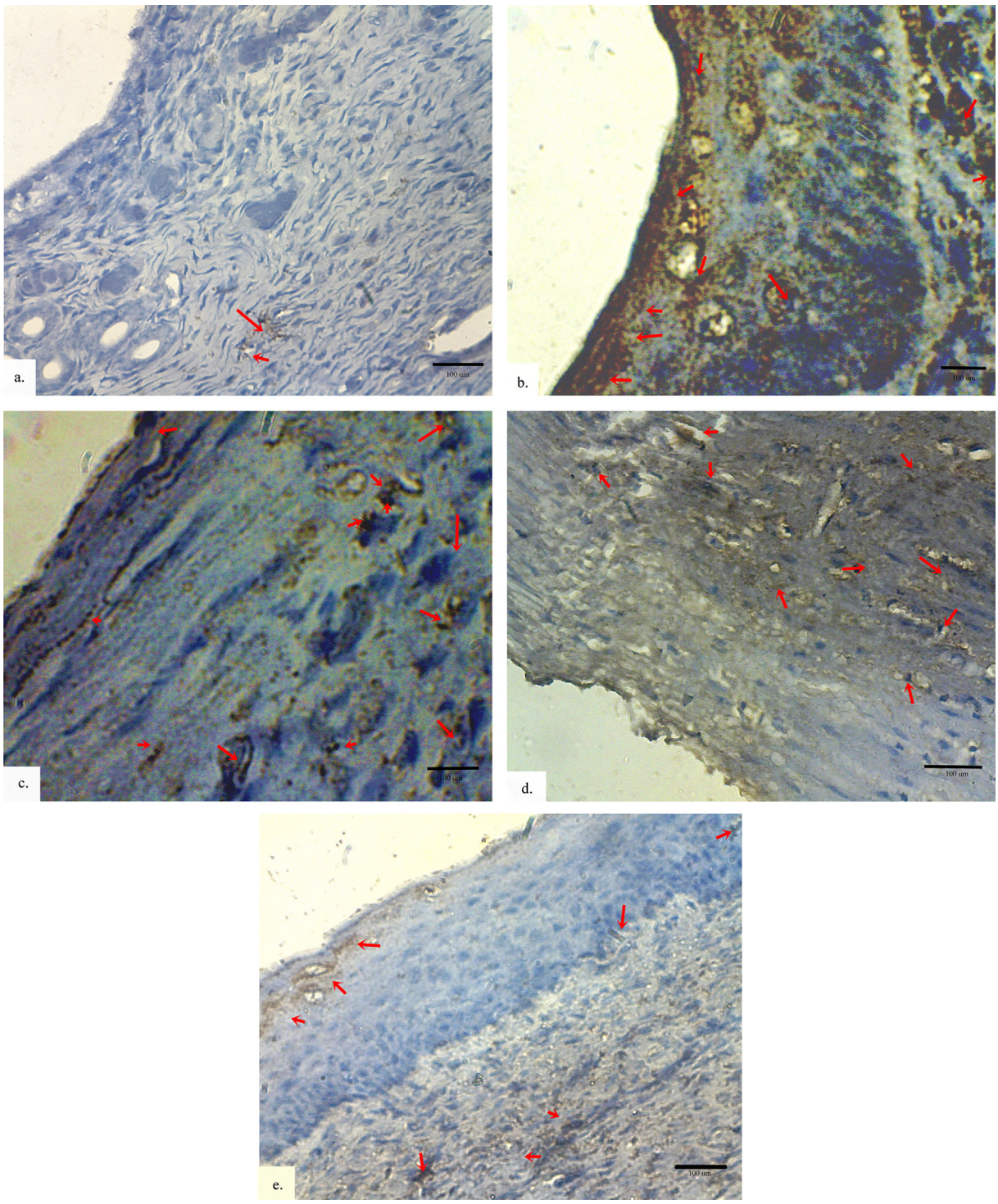

Fig. 7. Histological changes of cervical rats on TGFβ1 expression by *Z. acanthopodium* methanol extracts. a. Control, b. Cancer rats without treatments, c. Cancer rats + 100 mg/Kg BW of ZAM, d. Cancer rats + 200 mg/Kg BW of ZAM, e.Cancer rats + 400 mg/Kg BW of ZAM. red arrows: positive TGFβ1 (400×).

hypoxic or even anoxic, which, in turn, results in cellular necrosis (Clarke et al., 2018). The presence of abnormal cells that continue to multiply and form cancerous tissue can cause an increase in organ weight, especially in organs where a tumour is growing. This disrupts the body's metabolic system.

With the exception of neutrophils, haematocrit, and urea, the administration of ZAM at doses of 100–400 mg/kg BW impacts a range of haematological values in cervical cancer model rats. Only patients with eosinophil cell infiltration in the tumour have been found to have metastases (Thakur et al., 2015). Table 1 demonstrates that the changes in the numbers of haemoglobin, leukocytes, lymphocytes, eosinophils, and monocytes in cervical cancer are very reasonable due to the cervix having an endocervical mucosal layer containing mucus-producing columnar epithelium in a thick lamina propria that is connected to the vagina (McCracken et al., 2021). In this area, stratified columnar epithelium gives way to the stratified squamous epithelium (McCracken et al., 2021). The middle layer of the cervix is deeper,

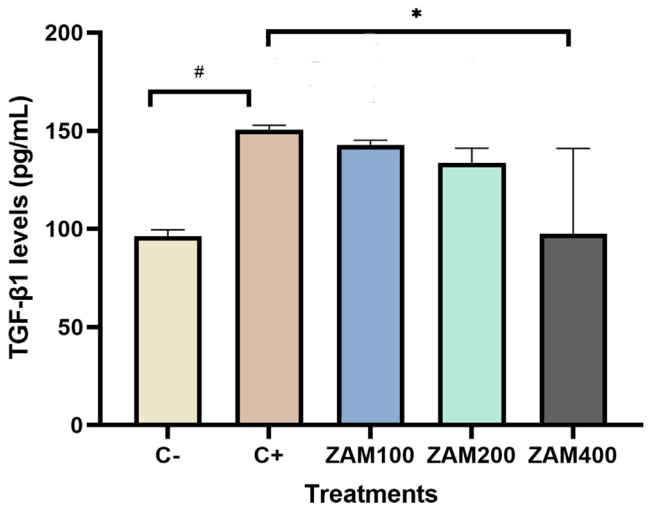

Fig. 8. Effect of *Z. acanthopodium* methanol extracts in TGFβ1 levels on rats serum. C−: Control, C+: Cancer rats without treatments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM ($^{\#}p < 0.05$ compared C−, $^{*}p < 0.05$ compared C+).

having less smooth muscle and denser connective tissue. This middle layer contains a large number of lymphocytes and leukocytes that help to strengthen the body's defence against microorganisms and abnormal cells, such as cancer cells (Thakur et al., 2015). Because ZAM contains strong antioxidants that can counteract free radicals, it can be used to control the state of these cells (Situmorang et al., 2020).

ZAM was more effective at managing the expression levels of SOD at doses of 100 and 200 mg/kg BW, doses of 200 and 400 mg/kg BW for MDA levels, and doses of 400 mg/kg BW for NGAL levels in cancer model rats. The increased lipid peroxide activity, due to antioxidant deficiency, was associated with increased levels of MDA and circulating NGAL and decreased SOD activity (Sherif et al., 2018; Thakur et al., 2015). Elevated MDA levels in tumour tissue may be associated with SOD deficiency. If the elevated levels persist, superoxide anions accumulate, which are highly radical and capable of penetrating membranes, causing negative effects in areas where a tumour is not physically present (Sherif. et al., 2018). Antioxidants found in andaliman can lower levels of MDA and serum NGAL, thereby increasing SOD activity. By increasing SOD activity through ZAM administration, it is possible to protect cells from oxidant disorders and oxidative stress, which can lead to a variety of diseases, including cancer.

ZAM administration can reduce the expression of IL-1β, which promotes the proliferation of cancer cells in rats. In cervical tissue cells, cells of the innate immune system recognise foreign structures not found in the host via receptors that signal the expression of inflammatory cytokines (Conesa-Zamora, 2013; Zhang et al., 2020). Because these immune cells do not always perform at their optimal level, they often require external help, such as the provision of more antioxidants. ZAM, an antioxidant found in plasma and erythrocyte membranes, can modify responses that affect second messengers and arachidonic acid cascade products, both of which have a significant impact on cell proliferation (George and Abrahamse, 2020). Biological reactions, such as oxidative stress caused by a lack of antioxidants in cells, can harm cellular components and result in a variety of diseases (Gulcin, 2020). The formation and progression of cancer is caused by DNA damage. Antioxidants and cancer have a close relationship, with the use of antioxidants a widely accepted therapeutic approach in the treatment of many cancers (George and Abrahamse, 2020). The primary mechanism underlying many tumour-killing chemotherapy and radiation agents is not an increase in antioxidants but rather an increase in free radicals that cause irreversible tissue damage (George and Abrahamse, 2020). Appropriate antioxidant inhibitors and/or free radical-producing compounds can be an effective cancer treatment strategy (Gulcin, 2020). In addition to antioxidants, the herb genus *Zanthoxylum* is known to possess anti-inflammatory, analgesic, antinociceptive, antioxidant, antibiotic, hepatoprotective, anti-plasmodial, cytotoxic, antiproliferative, anthelmintic, larvicidal, antiviral, and anti-cancer properties (Wijaya et al., 2019; Situmorang et al., 2020).

Administering ZAM to cervical cancer model rats increased the expression of IL-10, which suppressed the growth of cancer. In cancer patients, ZAM administration has been shown to protect against benzopyrene-induced oxidative stress. In this regard, cancer may cause a decrease in IL-10 by reducing benzopyrene-induced oxidative stress. Increased reactive oxygen species (ROS) production and oxidative stress have been linked to apoptosis. Therefore, apoptosis plays a role in cancer pathogenesis and aetiology (Situmorang and Ilyas, 2018). Phytochemicals can function as antioxidants or modulators of carcinogenic and cancer-prevention processes (Wijaya et al., 2019; George and Abrahamse, 2020). Antioxidants in ZAM stimulate IL-10, which inhibits or eliminates the inflammatory response and regulates the development and differentiation of abnormal cells. IL-10 is a cytokine secreted widely by monocytes that has pleiotropic effects on the immune system and on inflammation (Batchu et al., 2021). The activity and effector function of T cells, monocytes, and macrophages can be inhibited by IL-10 (Couper et al., 2008). In the presence of ZAM, IL-10 acts as the primary anti-inflammatory in the natural and adaptive immune response, preventing an excessive inflammatory response by inactivating macrophages and local and systemic inflammatory mediators (Couper et al., 2008). The body naturally produces large amounts of these cytokines, making them typically easily detectable in serum. However, if abnormal cells proliferate in large numbers, the activity of IL-10 is disrupted, necessitating the provision of antioxidants from external sources.

ZAM administration, particularly at a dose of 400 mg/kg BW, can suppress TGFβ1 serum expression and histology in cervical cancer model rats. TGFβ1 dysregulation is a major driver of tumour development, being involved in processes such as angiogenesis,

Table 6
Statistical analysis of VEGFR1 expression on histological changes of cervical after *Z. acanthopodium methanolic* extract administration.

Groups	Mean ± SD	Kruskal-Wallis	p value (Mann-Whitney)				
			C-	C+	ZAM100	ZAM200	ZAM400
C-	21.03 ± 2.23			0.040	0.045	0.072	0.080
C+	51.01 ± 4.26$^{\#}$				0.060	0.055	0.045
ZAM100	35.16 ± 3.28	0.00				0.055	0.045
ZAM200	26.51 ± 3.47*						0.050
ZAM400	20.11 ± 2.46*						

C−: Control, C+: Cancer rats without teratments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM. ($^{\#}p < 0.05$ compared C−, $^{*}p < 0.05$ compared C+).

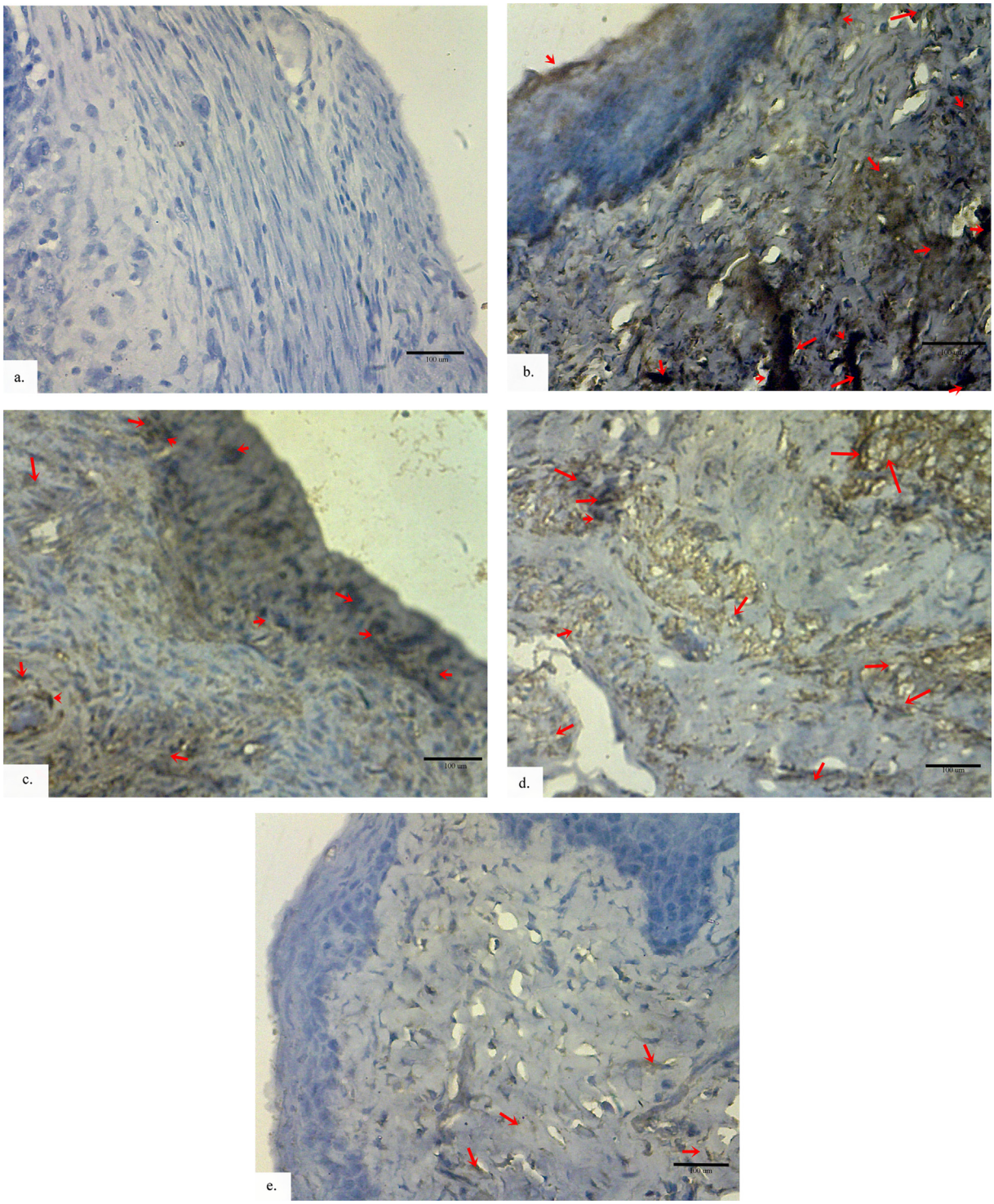

Fig. 9. Histological changes of cervical rats on VEGFR1 expression by *Z. acanthopodium* methanol extracts. a. Control, b. Cancer rats without treatments, c. Cancer rats + 100 mg/Kg BW of ZAM, d. Cancer rats + 200 mg/Kg BW of ZAM, e.Cancer rats + 400 mg/Kg BW of ZAM. red arrows: positive VEGFR1 (400×).

tissue invasion, metastasis, and immune suppression (Liu et al., 2021). TGF can maintain tissue homeostasis and prevent pre-cancerous tumours from progressing to malignancy by regulating cellular proliferation, differentiation, survival, adhesion, and the cellular microenvironment. However, TGF signalling promotes tumour growth and invasion (Park et al., 2021; Hua et al., 2020). In this study, ZAM was administered in order to suppress tissue invasion, metastasis, and immunity. TGFβ1 enforces homeostasis and suppresses tumour development in normal cells, either directly through cell autonomic tumour suppressor effects or indirectly through the suppression of inflammation and stromal-derived mitogens. When abnormal cells become cancerous, these cytokines lose their tumour suppressive abilities (Hua et al., 2020). Cancer cells use TGFβ1 to their advantage, using them to initiate immune evasion, produce growth factors, differentiate into invasive phenotypes, and establish and expand metastatic colonies (Hua et al., 2020). As a result, administering antioxidants is critical for restoring the typical cellular function of TGFβ1.

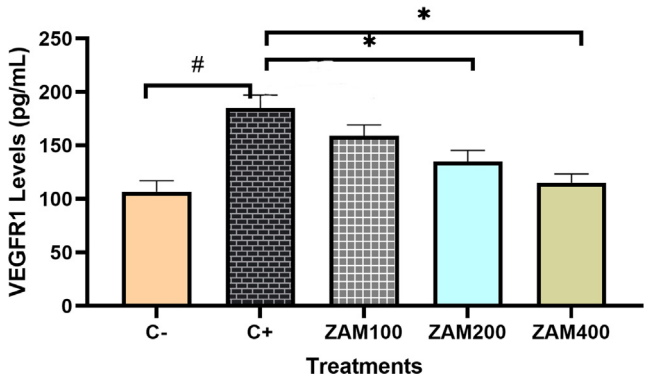

Fig. 10. Effect of *Z. acanthopodium* methanol extracts in VEGFR1 levels on rats serum C−: Control, C+: Cancer rats without treatments, ZAM100: Cancer rats + 100 mg/Kg BW of ZAM, ZAM200: Cancer rats + 200 mg/Kg BW of ZAM, ZAM400: Cancer rats + 400 mg/Kg BW of ZAM ($^{\#}p < 0.05$ compared C−, $^{*}p < 0.05$ compared C+).)

ZAM administration can suppress VEGFR1 serum expression and improve histology. In cancer cells, high VEGFR1 expression can be caused by lipid peroxidation due to a lack of antioxidants. Lipid peroxidation is crucial in the regulation of cell division. Low doses of ROS, caused by a lack of antioxidants, can stimulate cell proliferation while also inducing cytotoxicity and cell death. ZAM can improve histology in cervical cancer model rats due to the antioxidants found in andaliman, such as alkaloids, glycosides, tannins, phenols, and flavonoids, which have anti-inflammatory and anti-cancer properties (Situmorang et al., 2020; Wijaya et al., 2019).

The use of antioxidants during cancer treatment has been shown to reduce toxic side effects. Antioxidant interventions stem from the fact that plants, such as the herbal andaliman, contain antioxidants and are able to treat cancers with few side effects (Situmorang and Ilyas, 2018; Wijaya et al., 2019). The ability of such herbs to protect against DNA damage can be demonstrated by measuring the level of DNA damage with and without treatment. The estimated background DNA oxidation level in cells varies by more than threefold depending on the method used. A reduction in oxidative damage after supplementation with antioxidants from herbs has been previously demonstrated using a biomarker assay for DNA oxidation (Gulcin, 2020). In the current study, ZAM administration reduced the expression of IL1β, TGFβ1, and VEGFR1 in serum and cervical cancer histology, suggesting that it could be used as a molecular cytokine therapy for the treatment of cervical cancer. ZAM can also assist IL-10 in inhibiting the proliferation of differentiated abnormal cells. Although ZAM may be an effective molecular cytokine therapy for cervical cancer, cancer therapy success is dependent on a variety of factors, including clinical stage, tumour histological type and differentiation, cellular immune response, and apoptosis. Furthermore, factors external to the tumour, such as chemoradiation, immunotherapy, micronutrients and antioxidants consumed, and genetic susceptibility, all impact treatment success (Ming et al., 2021).

5. Conclusion

ZAM administration had no effect on the body weight of cervical cancer rats. Increased circulating MDA and NGAL in cervical cancer were associated with decreased SOD activity after ZAM administration. Elevated MDA levels in tumour tissue may be associated with SOD deficiency. Following ZAM administration, a significant decrease in the expression of IL1β, TGFβ1, and VEGFR1 in serum and cervical cancer histology was observed. ZAM was also found to assist IL-10 in inhibiting the proliferation of differentiated abnormal cells. Therefore, ZAM may be a potential, effective molecular cytokine therapy for the treatment of cervical cancer.

6. Contribution authors

The author declares that all authors participated in this research and publication.

Rostime Hermayerni Simanullang: Preparation of materials and methods, carry out research, Write paper and analyze data

Putri Cahaya situmorang: Histological analysis, reviewing the manuscript and statistical analysis

Meriani Herlina: Doing research, and analyzing statistical data and designing research

Noradina: Designing research, reviewing data and analyzing research results

Bernita Silalahi: Doing research and preparation of materials and methods

Sarida Surya Manurung: Doing research and analyzing research results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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