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Antioxidative and Apoptotic Activities of *Vernonia amygdalina* Ethanolic Extract in the AOM/DSS Mice Model of Colon Cancer

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ABSTRACT
This study examined the efficacy and safety of *Vernonia amygdalina* ethanol extract (VAEE) therapy in treating *azoxymethane/dextran sulfate sodium* (AOM/DSS)-induced colon cancer in mice, aiming to uncover the underlying mechanisms. A colon cancer mice model was used to evaluate the effects of VAEE on antioxidant enzyme levels and apoptosis. VAEE treatment increased SOD and caspase-3 levels while reducing MDA and Bcl-2 levels. These results indicate the potential of VAEE as an anticancer agent, with several doses showing significant increases in SOD and caspase-3 levels while significantly reducing MDA and Bcl-2 levels compared to the negative control ($p<0.05$). A dose of 500 mg/kg BW appears to be a potential candidate anticancer agent. Further studies on the molecular mechanisms and long-term effects are needed to support clinical applications.
Keywords: Antioxidants, AOM/DSS, Apoptosis, VAEE, Colon Cancer

ABSTRAK
Studi ini menguji efektivitas dan keamanan terapi ekstrak etanol Vernonia amygdalina (EEDA) dalam mengobati kanker kolon yang disebabkan oleh azoxymethane/dextran sulfate sodium (AOM/DSS) pada mencit, yang bertujuan untuk mengungkap mekanisme yang mendasarinya. Model mencit kanker kolon digunakan untuk mengevaluasi dampak VAEE pada kadar enzim antioksidan dan apoptosis. Pengobatan dengan EEDA meningkatkan kadar SOD dan caspase-3 sekaligus mengurangi kadar MDA dan Bcl-2. Hasil ini menunjukkan potensi EEDA sebagai agen antikanker, dengan beberapa dosis menunjukkan peningkatan signifikan dalam kadar SOD dan caspase-3 sekaligus mengurangi kadar MDA dan Bcl-2 yang signifikan dibandingkan dengan kontrol negatif ($p<0,05$). Dosis 500 mg/kg BB tampak berpotensi sebagai kandidat agen antikanker. Penelitian lebih lanjut tentang mekanisme molekuler dan efek jangka panjangnya diperlukan untuk mendukung aplikasi klinis.
Kata kunci: *Antioksidan, AOM/DSS, Apoptosis, EEDA, Kanker Kolon*

1. Introduction

Colon cancer is the 3rd most commonly diagnosed cancer and the 2nd leading cause of death in the world after lung cancer. In Asia, the incidence and mortality rates from colon cancer are ranked highest, followed by Europe and then America according to GLOBOCAN data in 2020. In Indonesia, colon cancer ranked 3rd and 4th in ASEAN countries in 2008 with an incidence rate of 17.2% and a mortality rate of 12.9% per 100,000 population. Colon cancer is a tumour that originates from the colon tissue to the colon. High-fat consumption, low fiber consumption and exposure to free radicals are some of the risk factors for colon cancer. Protective effects can be obtained by consuming antioxidants found in vegetables and fruits [1].

Antioxidants are important in reducing oxidative stress and can reduce damage caused by free radicals. Endogenous antioxidants that can repair the effects of oxidative stress are SOD enzymes. In cancer cases, normalization of SOD levels contributes to the reversion of cancer cell phenotype [2]. Natural sources of antioxidants can be found in herbal plants is including African leaves. The flavonoid content in African leaves is a better source of antioxidants than vitamin C. African leaves are known to have high levels of antioxidants because they contain luteolin which is an active compound from the flavonoid group [3].Based on previous studies using mice, it can increase the levels of the SOD enzyme induced by isoproterenol, the higher the dose of VAEE given, the more it increases the SOD levels in mice [4]. MDA is an aldehyde compound that is the end product of lipid (fat) peroxidation in the body that is used as a biological biomarker that can describe the degree of oxidative stress. High levels of MDA indicate high oxidative stress. Based on previous studies, the use of African leaves in mice can reduce MDA levels in the liver, lungs, heart and blood [5]. Previous studies have also shown that VAEE can reduce ROS expression in the cytoprotective effect test of VAEE on H2O2-induced Vero cells [6]. Apoptosis is a form or mechanism of programmed cell death or suicide. Cancer and many other diseases such as AIDS, diabetes, and Parkinson's syndrome occur as a result of imbalance and aberrant apoptosis pathway mechanisms. In the process of carcinogenesis, flavonoids interfere with multiple signal transduction pathways and thereby limit proliferation, angiogenesis, and metastasis or increase apoptosis [7]. Caspase-3 is a pro-apoptotic protein that is part of the caspase executor group. Activation of caspase-3 is one of the key points in the transmission of apoptosis signals because caspase-3 cleavage activity produces various morphologies and various types of biochemical materials from apoptosis [8]. Caspase-3 is activated in the apoptosis process both by external (death ligand) and intrinsic (mitochondrial pathway) pathways. Caspase-3 activation is required because otherwise caspase activity will kill cells without discrimination [9]. Bcl-2 is a powerful anti-apoptotic protein and excessive expression of Bcl-2 protein increases cell survival. In cancer cells, mutations in the Bcl-2 gene can cause increased expression that can suppress the normal function of pro-apoptotic proteins. If this occurs in the protein, it can cause a decrease in regulation, so that cells lose the ability to regulate apoptosis which can trigger cancer [10].

Flavonoids are essential compounds that are abundant in plants including African leaves, and have many pharmacological activities that have been tested in vitro, in vivo and clinically in humans. Flavonoids are categorized into several subclasses, namely flavonols (quercetin and rutin), flavones (apigenin and luteolin), anthocyanins (cyanidin and malvidin), flavonones (naringenin and hesperitin), and isoflavonoids (genistin and genistein) [11]. African leaves (*Vernonia amygdalina* Delile.) are plants that are widely found in Africa and also in tropical areas such as Indonesia which are part of the Asteraceae family, this plant has been widely used traditionally for medicine. African growth in Indonesia is known as African leaves. The leaf part of the plant is known to have the largest chemical components and nutritional content [12]. African leaves contain high antioxidants and have many pharmacological effects, one of which is as an anti-inflammatory and anticancer. African leaves have been studied to have anticancer activity including; nasopharyngeal cancer, skin cancer cells, prostate cancer cells, breast cancer cells, pancreatic cancer cells and liver cancer cells [13]. Based on previous studies, VAEE (doses of 100mg, 300mg and 500mg) showed very effective activity as an anti-colon cancer agent. VAEE is known to be able to reduce pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) while increasing the anti-inflammatory cytokine IL-10. VAEE also shows strong antioxidant activity and has no adverse effects on blood, kidney or liver biomarkers, thus confirming that VAEE is not toxic. Analysis of protein-protein interactions also shows the role of VAEE in inhibiting inflammatory markers, including COX-2, IL-18, and NF-kB [14].

In this study, researchers used a colon cancer model in mice induced by AOM/DSS. AOM (Methyl-methylimino-oxidoazanium, CH3N=N(→O)CH3) is a procarcinogen that is metabolized by cytochrome p450, isoform CYP2E1, converting it to MAM, a highly reactive alkylating species that induces the addition of O6 methylguanine in DNA resulting in a G→A transition. After being excreted into the bile, this compound is absorbed by the colonic epithelium and induces mutagenesis [15]. DSS is a heparin-like polysaccharide that is soluble in drinking water and causes damage to the colonic epithelium, inducing colitis that mimics some features of IBD. Combining AOM and DSS provides a two-step tumor model of CAC [16]. To date, there are various mice models of colon cancer induced by carcinogens, but compared with those carcinogens, AOM is the most frequently used carcinogen to induce colon cancer in mice. Meanwhile, the combination of AOM and DSS colitogen is also widely regarded as a powerful method to induce colon cancer in mice [17].

2. Materials and Methods

2.1.1 Materials

V. amygdalina Delile leaves were collected from the Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia (coordinates: 3◦ 33′36.5″ N, 98◦ 39′12.5″ E) (plant already registered in herbarium Medanese number 1554/MEDA/2023) authorized by Dr. Etti Sartina Siregar, M. Si. *Azoxymethane*/AOM (Merck, Rahway, NJ, USA), *Dextran sulfate sodium*/DSS (Merck Rahway, NJ, USA), ethanol (BrataChem, Medan, Indonesia), ethyl acetate (BrataChem, Medan, Indonesia), n-hexane (BrataChem, Medan, Indonesia), methanol (BrataChem Medan, Indonesia), sodium carboxymethyl cellulose (Sigma-Aldrich, St. Louis, MI, USA), aluminum foil (BrataChem, Medan, Indonesia), sodium acetate (BrataChem, Medan, Indonesia), Acetonitrile (Sigma-Aldrich, St. Louis, MI, USA), dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MI, USA), paraformaldehyde (Sigma-Aldrich, St. Louis, MI, USA) and distilled water (BrataChem) were used in this study along with the following enzyme-linked immunosorbent assay (ELISA) kits procured from ABclonal, Wuhan, China: SOD ELISA kit, MDA ELISA kit, Caspase-3 ELISA kit and Bcl-2 ELISA kit. The animals used were mice weighing 20-30 grams obtained from the Faculty of Pharmacy, Universitas Sumatera Utara, and approved by the Animal Research Ethics Committees (AREC) Universitas Sumatera Utara with approval number 0438/KEPH-FMIPA/2024.

2.1.2 Hematoxylin-eosin staining

For histopathological analysis colon samples from colon cancer mice models were immersed in liquid paraffin at 60 to 70 °C for 2 hours. Once shaped, they were cooled to solidify and then sectioned into 5–7 µm thick slices using a microtome. These slices were mounted on slides and briefly heated on a surface set to 56–58 °C for about 10 seconds to ensure proper tissue adherence without wrinkles or folds. The slides were then stained with hematoxylin and eosin. Initially, the slides were treated with xylene for 12 minutes to remove the paraffin (deparaffinization). Dehydration was performed by sequentially immersing the slides in alcohol solutions of increasing concentration (70%, 80%, 90%, and absolute ethanol), each for 5 minutes, followed by rinsing with running water. The slides were stained with hematoxylin for 5 minutes, rinsed, and then stained with eosin. Subsequent dehydration involved a series of ethanol baths (70%, 80%, 90%, and absolute ethanol) for 10 minutes, followed by clearing in xylene for 12 minutes. The stained slides were examined under a microscope (Thermo, Germany) at 100× magnification.

2.1.3 AOM and DSS solution preparation

A stock solution was prepared by dissolving 25 mg of AOM powder in 2.5 mL of sterile water to achieve a concentration of 10 mg/mL. The solution was then homogenized using rigorous vortexing and stored at -20 °C. Additionally, 20 g of DSS powder (molecular weight 35–50 kDa) was dissolved in 1000 mL of sterile water to create a 2.5% DSS solution, which also required rigorous vortexing for complete homogenization.

2.1.4 Experimental design for AOM/DSS-induced colon cancer model

A total of 30 male mice were obtained from the animal facility of the Faculty of Pharmacy at Universitas Sumatera Utara. The experimental animals were divided into 6 groups, each consisting of 5 male mice. The groups included a normal group (N), a negative control group (C-), a positive control group (C+), and three treatment groups (P1, P2, P3). Group N served as the normal group. Group C- received 10 mg/kg AOM along with three cycles of 2,5% DSS. Group C+ received 10 mg/kg/day AOM, three cycles of 2,5% DSS, and 100 mg/kg of quercetin. Group P1 received 10 mg/kg/day AOM, three cycles of 2,5% DSS, and 100 mg/kg VAEE. Group P2 received 10 mg/kg/day AOM, three cycles of 2,5% DSS, and 300 mg/kg VAEE. Group P3 received 10 mg/kg/day AOM, three cycles of 2,5% DSS, and 500 mg/kg VAEE. All groups were fed the Rodent Lab Chow 5001 diet. The mice were euthanized at the end of the experimental period. The experimental design is illustrated in **Figure 1**.

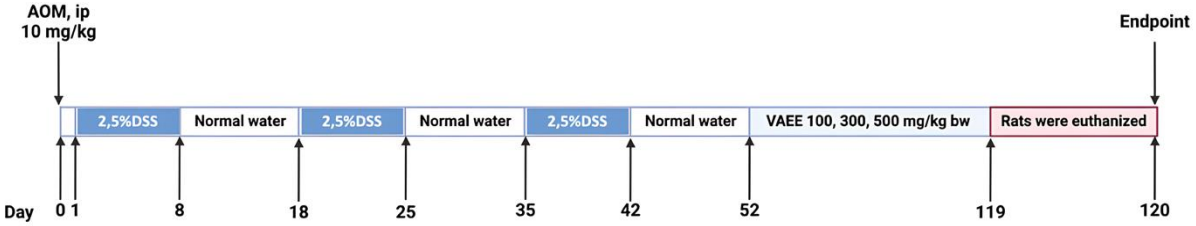

Figure 1. Experimental design

2.1.5 ELISA of SOD, MDA, Caspase-3, and Bcl-2

ELISA is a highly effective and efficient technique for measuring serum levels of SOD, MDA, Caspase-3, and Bcl-2. The quantification of these molecules was performed using specific ELISA kits, following the manufacturer's instructions (Bovine peroxisome proliferator-activated receptor gamma coactivator 1α ELISA kit, Cat. Abclonal, Wuhan, China). Media dilutions were tested alongside blank controls. Absorbance for each sample was measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA).

2.1.6 Statistical analysis

Protein expression was statistically analyzed using the Kruskal–Wallis and Mann–Whitney tests for non-parametric data. Graphs were created using GraphPad (San Diego, CA, USA), and the graphical abstract was designed with BioRender.

3. Result and Discussion

3.1.1 Histopathology of colon tissue

Colon cancer examination in AOM/DSS-induced mice using HE staining refers to the guidelines used, namely the STAR Protocols [18].

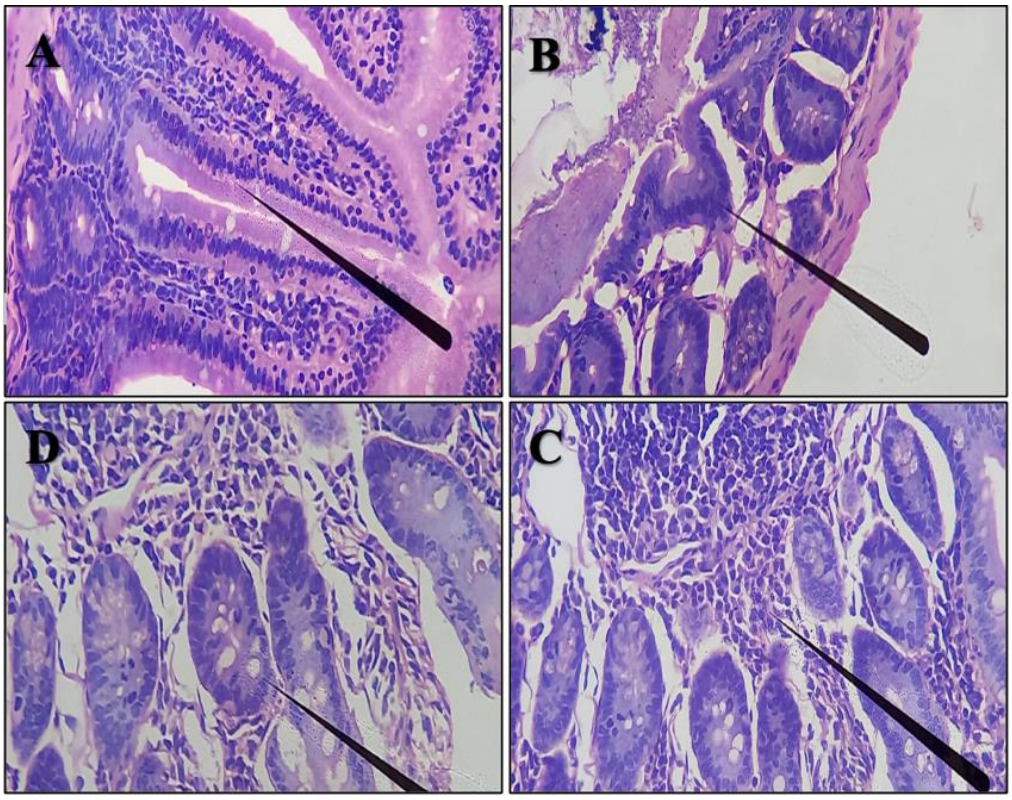

Figure 2. (a) normal cell; (b) adenocarcinoma cell; (c) inflamation cell; (d) dysplasia cell

Based on the results of histopathological examination with Hematoxylin-Eosin (HE) staining in colon cancer mice (Fig.2): (a) Histopathological picture shows normal cell tissue structure, without any signs of abnormalities or pathological changes. Colonic epithelial cells appear regular with round cell nuclei and homogeneous cytoplasm. (b) Cells show irregular proliferation with abnormal gland formation, which is characteristic of adenocarcinoma. Adenocarcinoma is a common form of colon cancer characterized by uncontrolled epithelial cell growth and glandular structure formation. These cells appear to undergo abnormal proliferation, with irregular glandular structures and an increased ratio of nuclei to cytoplasm. This indicates malignancy in the tissue. (c) Inflammatory cell infiltration is seen in certain areas. This infiltration indicates the body's immune response to tissue irritation or damage. The tissue around the epithelial cells shows signs of damage and inflammatory response, which often accompanies malignant conditions. Chronic inflammation is an important risk factor in the development of colon cancer. (d) Cells show morphological changes in the cell nucleus, including dysplasia, which is a condition in which the cell nucleus is enlarged and irregularly shaped, and may also show increased mitotic activity. Dysplasia is an early sign of neoplastic or precancerous changes that can progress to full-blown cancer if left untreated.

The use of AOM (*Azoxymethane*) and DSS (*Dextran Sodium Sulfate*) as inducers in mice animal models usually results in various stages of colon disease development, ranging from dysplasia to invasive cancer. Studies have shown that the combination of AOM and DSS can effectively induce colon cancer in mice models. AOM acts as a carcinogen, while DSS causes chronic inflammation that accelerates the carcinogenesis process [19]. Chronic inflammation has been identified as a major factor in the development of colon cancer. Inflammation causes DNA damage and promotes a microenvironment that supports cancer cell growth [20]. Histopathological characteristics such as adenocarcinoma and dysplasia are often found in colon cancer. Histopathological studies are essential for diagnosis and development of therapeutic strategies [21]. Studies have shown that the accumulation of Reactive Oxygen Species (ROS) can accelerate the development of colon cancer by damaging DNA and inducing mutations. ROS can also increase inflammation that contributes to carcinogenesis [22]. Hematoxylin and Eosin (HE) staining is the most commonly used histological staining technique to examine tissue structures under the microscope. HE staining produces a clear contrast between the cell nucleus and cytoplasm, allowing identification of cellular morphology and tissue structure. HE staining is very effective in detecting characteristic morphological changes in cancer cells.The use of HE staining in cancer diagnosis has long been recognized. Studies have shown that this technique remains the gold standard for histopathological examination [23]. Histopathological studies of colon cancer use HE staining extensively to diagnose and characterize adenocarcinoma and other dysplastic changes [21]. HE staining is a very useful diagnostic tool in detecting and analyzing colon cancer. Despite some limitations, this technique remains the standard method in histopathology because of its ability to provide clear and detailed morphological images of cancer tissue and cells. The results of the examination with HE staining show that AOM/DSS is effective in triggering pathological changes in mice colon tissue that are in accordance with the characteristics of human colon cancer, so it can be used as a relevant model for colon cancer research.

3.2 Effect of VAEE on Increasing Endogenous Antioxidant and Apoptosis Levels

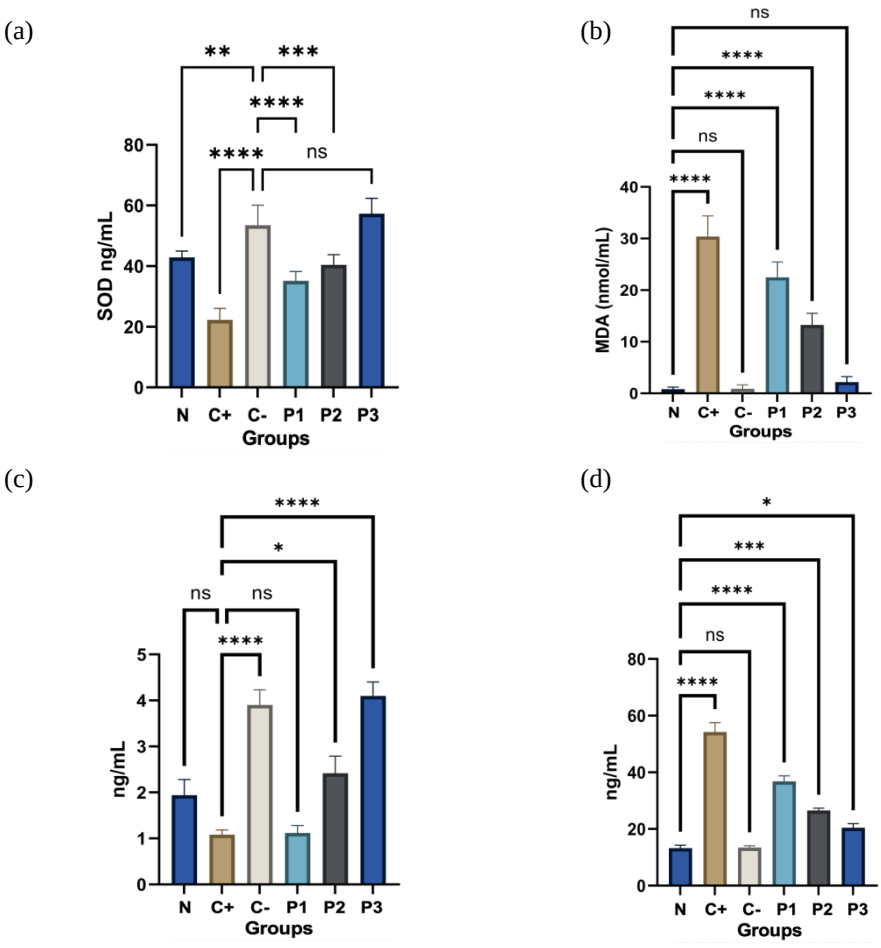

Figure 3. (a) SOD levels; (b) MDA levels; (c) Caspase-3 levels; (d) Bcl-2 levels

(a) SOD Levels

Fig.3 above shows that the normal SOD level in the normal group is 42.912 ± 2.02 ng/mL. In the negative control group, SOD levels decreased to 22.271 ± 3.85 ng/mL, indicating an increase in oxidative stress in the colon cancer mice model on the positive control group, the levels increased very significantly to 53.484 ± 6.63 ng/mL, indicating the strong antioxidant ability of quercetin in reducing oxidative stress. In this study, the 100 mg/kg BW VAEE group had given a statistically significant different value when compared to the negative control group, which was 35.140 ± 3.12 ng/mL. The VAEE dose of 30 mg/kg BW produced better SOD levels than the VAEE dose of 100 mg/kg BW but was still below the normal group value, which was 40.433 ± 3.34 ng/mL. VAEE dose of 500 mg/kg BW produced the highest SOD levels among the other groups and was not statistically significantly different from the positive control group, which was 57.289 ng/mL, indicating that a higher dose of VAEE can provide the best protective effect against oxidative stress. This can happen because VAEE is rich in secondary metabolites that support increased SOD levels in colon cancer mice models. *Vernonia amygdalina* leaf extract has secondary metabolites including; flavonoids, terpenoids, alkaloids, tannins, saponins, glycosides, steroids, and phenols [24]. A phytochemical study of VAEE showed the presence of several bioactive components, including saponins, flavonoids, and polysaccharides, which showed various beneficial effects, such as antioxidant, anti-inflammatory, antidiabetic, and antineoplastic activities [25]. The results of this study are also in line with previous studies showing an increase in SOD levels along with increasing doses of VAEE in doxorubicin-induced mice [11]. Other studies have also shown that African leaves increase SOD levels such as in the occurrence of liver damage in carbon tetrachloride-induced mice, in STZ-induced diabetic mice, and also in mice that experience kidney damage due to gentamicin induction.

SOD (*superoxide dismutase)* is an antioxidant enzyme that plays an important role in protecting cells from oxidative stress by breaking down superoxide radicals into oxygen and hydrogen peroxide. VAEE contains flavonoid compounds which are known to have strong antioxidant properties so that they can increase SOD enzyme activity and play a role in reducing oxidative stress and have direct activity as direct free radical scavengers because flavonoids have OH groups on rings A and B in the general structure of flavonoids. One of the flavonoid compounds contained in VAEE is Luteolin which is known to have anti-inflammatory, antioxidant, and anticancer properties [11]. Luteolin causes the release of Nrf2 from the inhibitory protein Keap1 in the cytoplasm, this release allows Nrf2 to move to the nucleus. Activated Nrf2 then moves to the nucleus and binds to the antioxidant response element (ARE) in the SOD gene promoter which can increase SOD gene transfer [26]. Luteolin can also increase the stability and activity of Nrf2 by activating the *phosphoinositide 3-kinase* (PI3K)/Akt pathway [27]. In addition, increasing SOD levels by luteolin can also be through certain MAPK inhibitor pathways that can downregulate Nrf2, thereby increasing Nrf2 stability [28]. Increasing SOD levels can help reduce the risk of cancer by reducing oxidative stress and cell damage [29].

(b) MDA Levels

MDA (Malondialdehyde) is a lipid peroxidation product that is often used as a marker of oxidative stress in cells. Increased MDA levels indicate cell damage due to free radicals, which can contribute to the carcinogenesis process, including colon cancer. Fig.3 above shows that MDA levels in the normal group were 0.8142 ± 0.41 nM/mL, indicating low levels of oxidative stress under normal conditions. MDA levels in the negative control group were statistically very significantly different from the normal group, namely 30.3870 ± 4.0 nM/mL, indicating a very high increase in oxidative stress. AOM/DSS is an agent that is often used to induce colon cancer in experimental animals, indicating that oxidative stress plays a role in the development of colon cancer. The combination of AOM and DSS activates various inflammatory pathways, including the NF-κB pathway. Activation of these pathways increases the expression of pro-inflammatory cytokines and pro-oxidant enzymes that worsen ROS production. NF-κB, for example, induces the expression of enzymes such as NADPH oxidase that produce ROS. Excessive ROS causes lipid peroxidation, which damages cell membranes and produces reactive lipid peroxidation products. These products, such as MDA and *4-hydroxynonenal*, can cause further damage to DNA and proteins, worsen oxidative stress and accelerate carcinogenesis [30]. MDA levels in the positive control group and VAEE at a dose of 500 mg/kg BW were not statistically significantly different from the normal group, namely 0.8767 ± 0.77 nM/mL and 2.1712 ± 1.07 nM/mL, respectively. Quercetin is a flavonoid known to have antioxidant properties, which helps reduce oxidative stress and has a protective effect against the development of colon cancer. The chemical structure of quercetin contains many hydroxyl groups (-OH) and is strategically located allowing quercetin to neutralize free radicals through the donation of hydrogen atoms or electrons, which convert free radicals into less reactive molecules, its resonance-stable flavonoid structure also contributes to reducing oxidative stress effectively. While in the VAEE group at a dose of 100 mg/kg BW and 300 mg/kg BW, it was able to reduce MDA but was not

better and was very significantly different when compared to the normal group, namely 22.4670 ± 2.99 nM/mL and 13.2339 ± 2.29 nM/mL, respectively, this shows that VAEE with a higher dose has a more optimal effect in reducing oxidative stress. The flavonoid compound content of VAEE can inhibit the activity of ROS-producing enzymes, such as NADPH oxidase and xanthine oxidase. Inhibition of these enzymes reduces ROS production in cells. For example, apigenin and luteolin are known to inhibit NADPH oxidase [31]. The results of this study are also in line with previous studies, namely that the antioxidant activity of flavonoids contained in the water extract of Gyrinops versteegii leaves showed a significant decrease in MDA levels in mice [32].

(c) Caspase-3 Levels

Decrease or increase in caspase-3 levels in cells can indicate various conditions and biological processes that occur in cells. Caspase-3 is the main protease that plays a role in the execution of apoptosis, so changes in its levels are often associated with the apoptosis process. Fig.3 above shows that caspase-3 levels in the normal group and VAEE dose of 100 mg/kg BW were not statistically significantly different from the negative control group with values of 1.94 ± 7.60 ng/mL; 1.12 ± 3.63 ng/mL and 1.08 ± 2.39 ng/mL, respectively. The combination of AOM/DSS causes chronic inflammation in the colon. This chronic inflammation can activate inflammatory signaling pathways such as NF-κB, which can increase the expression of anti-apoptotic proteins such as Bcl-2. Increased Bcl-2 can inhibit the intrinsic apoptosis pathway, which ultimately suppresses caspase-3 activation. Low levels of caspase-3 in the normal group can be influenced by several factors related to physiological functions and regulation of apoptosis in healthy cells. Under normal conditions, body cells are in a state of homeostasis where the process of cell division and cell death is well regulated [33]. Caspase-3 is activated in response to severe DNA damage or significant oxidative stress. In healthy cells and in a non-stressful environment, the level of oxidative stress and DNA damage is minimal, so there is no trigger for caspase-3 activation [31]. Luteolin, one of the flavonoid compounds contained in VAEE can increase caspase-3 levels by modulating various signaling pathways involved in apoptosis, including the p53 pathway, the MAPK (*Mitogen-Activated Protein Kinase*) pathway, and the PI3K/Akt pathway. Activation of p53 by flavonoids can increase the expression of pro-apoptotic genes leading to caspase-3 activation. In addition, flavonoids can inhibit the PI3K/Akt pathway which usually inhibits apoptosis, thereby increasing caspase-3 activity [33]. In this test, VAEE at a dose of 300 mg/kg BW was able to provide a significant increase in caspase-3 levels compared to the negative control group, which was 2.42 ± 8.26 ng/mL but was not more optimal when compared to VAEE at a dose of 500 mg/kg BW and the positive control group, which were 4.10 ± 6.71 ng/mL and 3.90 ± 7.42 ng/mL, respectively, this shows that caspase-3 levels increase with the addition of VAEE doses, indicating that with higher doses VAEE has the potential to increase apoptosis. On biosynthetic silver nanoparticles against apoptosis of breast cancer cells, showed that a 14.5-fold increase in caspase-3 expression after treatment with silver nanoparticles (AgNPs) was directly related to an increase in the amount of programmed cell death or apoptosis [34].

Bcl-2 is an anti-apoptotic protein that prevents cell death by inhibiting the release of pro-apoptotic factors from mitochondria. This protein works by binding and neutralizing pro-apoptotic proteins such as Bax and Bak, which when activated will cause the release of cytochrome c and caspase activation, leading to apoptosis. Fig.3 above shows that the Bcl-2 levels in the normal group were 13.20 ± 2.39 ng/mL. The positive group statistically gave a value that was not significantly different from the normal group, namely 13.40 ± 1.52 ng/mL, indicating that quercetin can play a role in normalizing or stabilizing Bcl-2 expression. Quercetin can inhibit signaling pathways that promote cell survival and Bcl-2 expression by inhibiting the PI3K/Akt pathway which is often active in cancer cells and plays a role in increasing Bcl-2 expression. Inhibition of this pathway by quercetin can reduce Bcl-2 expression and make cells more susceptible to apoptosis [31]. In the negative control group, the increase in Bcl-2 levels was very significantly different from the normal group, which was 54.20 ± 7.53 ng/mL, indicating that the induction of colon cancer with AOM/DSS caused upregulation of Bcl-2 protein. The PI3K/Akt pathway is a cellular signaling pathway that plays an important role in cell survival and resistance to apoptosis. AOM/DSS can activate this pathway, which in turn increases Bcl-2 expression. Akt activation stimulates transcription factors that increase Bcl-2 gene transcription, helping cells survive under stressful conditions. The role of DSS in the induction of inflammation causes damage to the intestinal epithelium, increases intestinal permeability and triggers chronic inflammation. This inflammation leads to the activation of various pro-inflammatory signaling pathways such as NF-κB, which is known to increase Bcl-2 expression. NF-κB induces Bcl-2 gene transcription in response to cellular stress and inflammation to protect cells from apoptosis [30]. In the test group, VAEE at a dose of 100 mg/kg BW was able to provide a significant decrease in Bcl-2 levels compared to the normal group, but was not better than VAEE at a dose of 300 mg/kg BW and a dose of 500 mg/kg BW which obtained Bcl-2 levels of 26.57 ±

1.79 ng/mL and 20.40 ± 3.36 ng/mL, respectively, indicating that at higher doses, VAEE is more effective in reducing the upregulation of Bcl-2 levels. The luteolin compound contained in VAEE, in addition to inhibiting the PI3K/Akt pathway, can also inhibit NF-κB activity which plays a role in regulating Bcl-2 expression, thereby suppressing Bcl-2 gene expression and inducing apoptosis [33]. On adipose tissue-derived stromal cells (ADSCs) into neurons, showed that decreased Bcl-2 expression and increased Bax expression play an important role in the regulation of mitochondria-mediated apoptosis during ADSCs differentiation into neurons. Activation of these apoptotic pathways, both caspase-dependent and independent, causes significant mitochondrial structural changes and cell death (apoptosis) after 5 hours of differentiation induction [35].

4. Conclusion

In conclusion, our study highlights the potential of *V. amygdalina* extract (VAEE) as a promising natural treatment for colon cancer induced by *azoxymethane*/*dextran sulfate sodium (*AOM/DSS) in mice. In vivo examination showed that VAEE has the potential as an anticancer agent. Several doses of treatment resulted in significant values on endogenous antioxidant levels and apoptosis compared to quercetin control ($p<0.05$). A dose of 500 mg/kg BW has the potential to be a candidate anticancer agent.

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