

Effect of Blueberry and Raspberry on Activin-AB and Oxidative Stress Levels in Mice Given Azoxymethane and Dextran Sulfate Sodium

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ABSTRACT

There are insufficient clear studies on activin-AB (act-AB) as one of the isomers of activins that plays role in development of cancer. In this study aimed to investigate the effect of azoxymethane (AOM) and dextran sulfate sodium (DSS) as chemical agents on the act-AB level of colon tissue with oxidative stress and tumor necrosis factor- α (TNF- α) levels. A comparison of the effects of blueberries and blackberries was also planned. It was formed six equal groups of mice. Only intraperitoneal (ip) physiological serum was administered to control group (CNT). The second group (AOMD) received ip AOM/DSS combination. Third and fourth groups received oral aqueous raspberry (*Rubus idaeus* L.) and blueberry (*Vaccinium myrtillus* L.) extracts (RAE and VAE) in drinking water, respectively. Others received VAE and RAE plus AOM/DSS. While enzyme-linked immunosorbent assay was used for act-AB and TNF- α measurements, spectrophotometric methods were used for analyses of malondialdehyde (MDA) and reduced glutathion (GSH). AOMD act-AB levels were lower than other groups but significantly higher in VAE ($p<0.05$). MDA levels of AOMD were significantly higher than CNT, RAE and VAE while MDA levels of AOMD+VAE were lower than AOMD. Plasma GSH levels were higher in VAE compared to AOMD ($p<0.01$). TNF- α levels were lower in VAE ($p<0.01$) and AOMD+VAE groups. As a result, it was determined that VAE may normalize act-AB level in AOMD group. VAE was also found to be more effective than RAE for anti-inflammatory response associated with oxidative stress and TNF- α level during AOM and DSS application.

Keywords: Activin AB, Azoxymethane, Oxidative stress, *Rubus idaeus*, *Vaccinium myrtillus*



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1. Introduction

Nowadays, many serious human diseases are experimentally induced in laboratories using specific chemical agents and treatment strategies are developed accordingly. Azoxymethane (AOM) and dextran sulfate sodium (DSS) applications are frequently preferred in experimental studies as chemical agents for colorectal cancer which is associated with inflammation and is more common in developed countries, aiming to make treatments more effective [1,2].

Malignant cells or tumors are shaped by extracellular matrix (ECM) elements as they affect their environment. If conditions such as an abundance of pathogenic chemical agents are present, the ECM can trigger all stages of carcinogenesis, with an increase in signaling molecules, including activins [3-5]. Activins (Act-A, -B, -AB, -C, and -E) are a family of cytokine proteins that are members of the transforming growth factor- β (TGF- β) family. Act-AB is a heterodimer form consisting of a β_A subunit and a β_B subunit via disulfide bonds. [6]. Activins exert multifaceted effects on important tissue growth processes such as embryogenesis and wound healing by binding to type I and type II serine/threonine kinase receptors in membranes [4, 6-8]. The biological limits of activins are regulated by follistatin inhibition [9]. Gene transcription is regulated by the binding of

phosphorylated receptors to mediating proteins and transmission of activin signals to the nucleus [10,11]. When investigating the role of activins in vitality and health, it is generally observed that only isoforms other than act-AB are considered [5,9-12]. Therefore, there is a need to elucidate the molecular pathways of act-AB through different experimental applications.

TNF- α is a proinflammatory cytokine that plays an important role in the pathogenesis of inflammatory bowel disease [13]. Considering the studies conducted, there is insufficient research on the direct regulation of the TNF- α by act-AB, especially in the colorectal cancer induction process. The accumulation of reactive oxygen species (ROS) which is important in the pathogenesis of many diseases in the tissues of living beings can induce protein denervation, lipid peroxidation, DNA damage, apoptosis, ferroptosis and pyroptosis in intestinal tissues. Increasing evidence shows that antioxidative treatments are beneficial against colon tissue damage [14]. Reduced glutathione (GSH) which is very effective in the antioxidant defense system of living beings and malondialdehyde (MDA) which is the end product of lipid peroxidation formed by ROS are important as markers of oxidative stress [15, 16]. Assessment of TNF- α and oxidative stress levels together with activin-AB profiling

can help investigate their effects on the ECM of colon tissue, which is prone to inflammation and may subsequently become cancerous.

Epidemiological studies support that the health benefits of fruit and vegetable consumption are largely due to the flavonoids found in these products. Blueberries and raspberries which are frequently encountered in studies against ROS are rich in flavonoids [17]. Studies have confirmed that these substances found in berries such as raspberries have significant contributions to high-density lipoprotein (HDL), blood pressure and the prevention of cardiovascular diseases [18, 19]. Blueberries contain abundant phenolic compounds (anthocyanins, catechins, quercetin, tannins, phenolic acids and ellagitannins), pectins, sugars and vitamins [20]. Due to these rich bioactive contents, it is noteworthy that there are many studies on the antimicrobial and antioxidant potential of blueberries [21,22].

The main objectives of this study are (i) to evaluate the effects of AZO and DSS on activin-AB levels which are rarely studied and (ii) to measure the levels of MDA, GSH, and TNF- α which are closely related to inflammation and oxidative stress in the disease process and (iii) to comparatively analyze the effects of blueberries and raspberries which are important functional foods on the levels of these parameters. Thus, it was aimed to make an experimental contribution to the literature for the fields of molecular therapy and metabolism.

2. Materials and Methods

2.1. Experimental Animals and Liquid Extracts

In the animal house of the Kafkas University (KAU) Experimental Animal Research Center a total average ten-week-old weighing 21 ± 2 g female *Mus musculus* mice were housed at $22 \pm 2^\circ\text{C}$ and 50-70 % relative humidity with a 12-hour light/dark cycle. A standard complete pellet mouse chow with 26 % crude protein (Bayramoğlu Feed and Flour Industry Trade Inc., Erzurum Turkey) and water were also available to the animals during the trial. At every stage of the investigation, the intended ethical regulations were upheld (KAU-HADYEK 2019/024), and all animals were treated in accordance with the standards of the National Experimental Animal Research Council of Kafkas University.

Blueberry (*Vaccinium myrtillus* L.) and raspberry (*Rubus idaeus* L.) fruits were collected from Serinkuyu Village of Hanak District of Ardahan Province in the North-Eastern Anatolia Region of Turkey in August 2020. Species confirmation of the plants was made by a botanist at the Biology Department in Kafkas University. The blueberry and raspberry fruits were dried at 40°C in a shaded and airy place for 4 days and then ground in a shredder. 30 g of both blueberry and raspberry were weighed separately and 250 ml of distilled water was added and left for

maceration for 1 hour. The mixture was then boiled for 190 minutes and the resulting liquid after condensation of the vapor collected in the cooler was transferred to a sterile dark-colored glass container. Liquid extracts were stored in the refrigerator at 4°C and prepared fresh every 2 days for experimental applications.

In study, 42 experimental mice were equally divided into six groups in the experimental animal housing and application center. The first group was determined as the control group (CNT) and was fed with standard mouse chow and water and given physiologic saline solution intraperitoneal injection (IP). The second group (AOMD) was given a single dose of 10 mg/kg azoxymethane (CAS: 25843-45-2, CAT: A5486, Sigma Aldrich) dissolved in physiologic saline solution by IP route and 7 days later, 2.5% dextran sulfate sodium (CAS: 9011-18-1, CAT: J603606, Alfa Aesar by Thermo Fisher Scientific) was given with drinking water for 1 week. Then, following normal drinking water for 14 days, this cycle was continued for a total of three times [23]. The third and fourth groups were given *Rubus idaeus* L. and *Vaccinium myrtillus* L. fruit liquid extracts (RAE and VAE, respectively) orally at a dosage of 100 mg/kg/day for 10 weeks. The dosage was based on the amounts that had previously yielded positive results in studies on mice [24, 25]. Animals in the fifth and sixth groups that received AOM/DSS were given 100 mg/kg/day RAE and VAE orally for 10 weeks, respectively.

At the end of the experimental applications, mice were injected with a mixture of 10 mg/kg ketamine (10%) and 80 mg/kg xylazine (2%) and euthanized by decapitation to obtain tissue samples. After that, half of the colon tissue samples from the distal part above the anus were removed. The obtained tissue samples were diluted 5-fold with a homogenizer (Wiggen Hauser) on ice using 0.1 M phosphate buffer solution with pH: 7.3 (Cat no: X6571D, England). After the homogenates were centrifuged at 1500 rpm for 10 min at 4°C , the supernatants obtained were placed in eppendorf tubes and stored in the deep freezer (-20°C) until analysis. The analysis process was completed within the following week.

2.2. ELISA and Spectrophotometric Analyses

Activin-AB (Cat No: E-EL-M2660, mouse ACV-AB, Elabscience, USA) and TNF- α (Cat No: SEA133Ra, Cloud Clone Corp., Wuhan, PRC) analysis was performed with a commercially produced assay based on an enzyme-linked immunosorbent assay for antigen detection using a microplate reader (BioTek Epoch, USA). Calculations were made using a standard curve and the means are expressed relatively. The levels of MDA were measured as method described by Yoshioka et al. [26] and the levels of GSH were measured as method described by Beutler et al. [27] spectrophotometrically (T60 UV/VIS Spectrophotometer, PG Instruments Ltd, UK). The color absorbance values of standard solutions prepared with different concentrations of 1,1,3,3-tetramethoxypropane (CAS: 122-31-6, EC

Number: 204-533-1, Sigma-Aldrich, China) and reduced L-glutathione (CAS: 70-18-8; EC: 200-725-4, Sigma-Aldrich, Japan) were used for MDA and GSH level calculations.

2.3 Statistical Analyses

Data were expressed as mean (X) and standard deviation (SD) values. Means were compared using computer software (SPSS 20.0 for Windows, IBM). One-way analysis of variance (ANOVA) test was used to determine whether there were statistical differences in the groups. Tukey's multiple comparison test was used to determine whether the mean value of which group was different from the mean value of the other group. The negative or positive relationships of the parameters of groups were examined using Spearman's two tailed correlation test. p values <0.05 were considered significant

3. Results and Discussion

The concentration of act-AB in the AOMD colon tissue was found to be lower than the average of the VAE group ($p=0.005$). Although there were changes in the means in the RAE treatment, these changes were not significant ($p>0.05$). It was determined that VAE application had an

increasing effect on the colon act-AB levels compared to the other group mean values and had a normalizing effect by bringing the AOMD values closer to the control (Figure 1).

There were significant differences in the mean plasma MDA, GSH and TNF- α of the groups according to one-way analysis of variance ($p=0.003$, $p=0.014$ and $p=0.023$, respectively). According to Tukey multiple comparison test, it was found that MDA levels of AOMD were significantly higher than CNT, VAE and RAE, ($p=0.010$, $p=0.005$ and $p=0.008$, respectively), while MDA levels of AOMD+VAE were lower than AOMD at $p=0.088$ significance level. There was a decrease in MDA levels of AOMD+RAE compared to AOMD ($p=0.455$), but this decrease was not as remarkable as in AOMD+VAE. It was found that mean plasma GSH levels were significantly higher in VAE compared to AOMD ($p=0.012$), and in the other groups, mean plasma GSH levels were higher than AOMD, although not statistically significant ($p>0.05$). It was determined that mean plasma TNF- α levels, unlike GSH levels, were significantly lower in VAE compared to AOMD ($p=0.043$), and in the other groups, mean plasma TNF- α levels were lower than AOMD, although not statistically significant ($p>0.05$) (Figure 2).

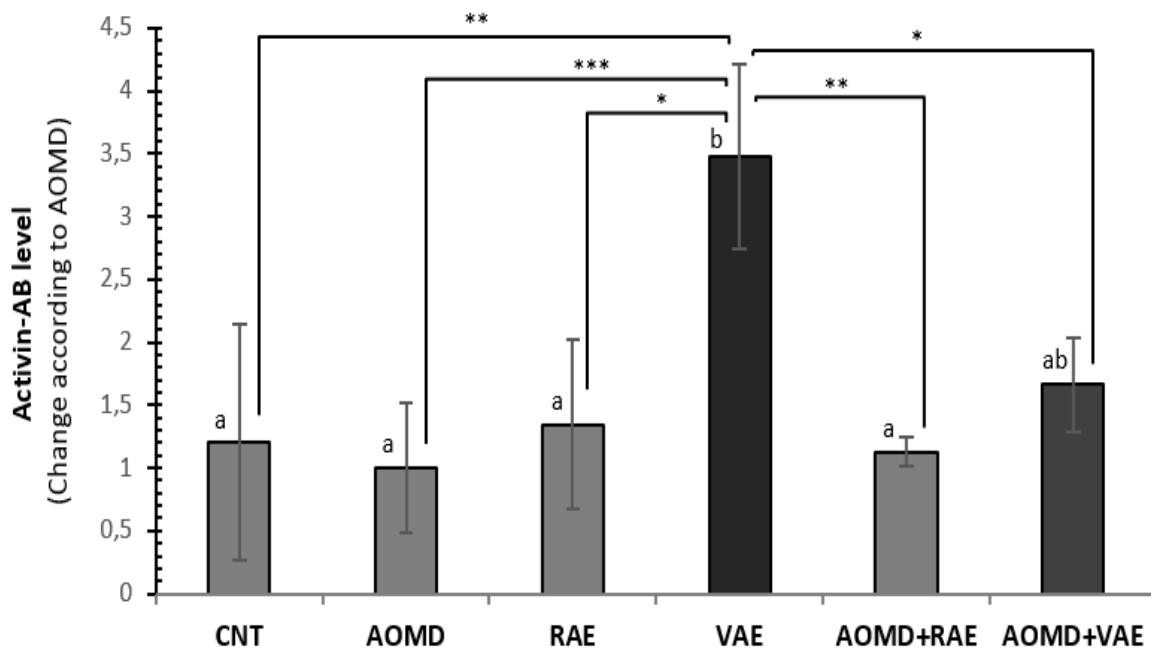


Figure 1. Act-AB levels of mouse colon tissue samples were higher in VAE (*Vaccinium myrtillus* L. aqueous extract) group according to other groups. (CNT: Control, AOMD: Azoxymethane/dextran sulfate sodium, RAE: *Rubus idaeus* L. aqueous extract, AOMD+VAE and AOMD+RAE ($^{a,b}p<0.05$ according to one-way analysis of variance and $*p<0.05$, $**p\leq 0.01$ and $***p\leq 0.005$ according to Tukey test).

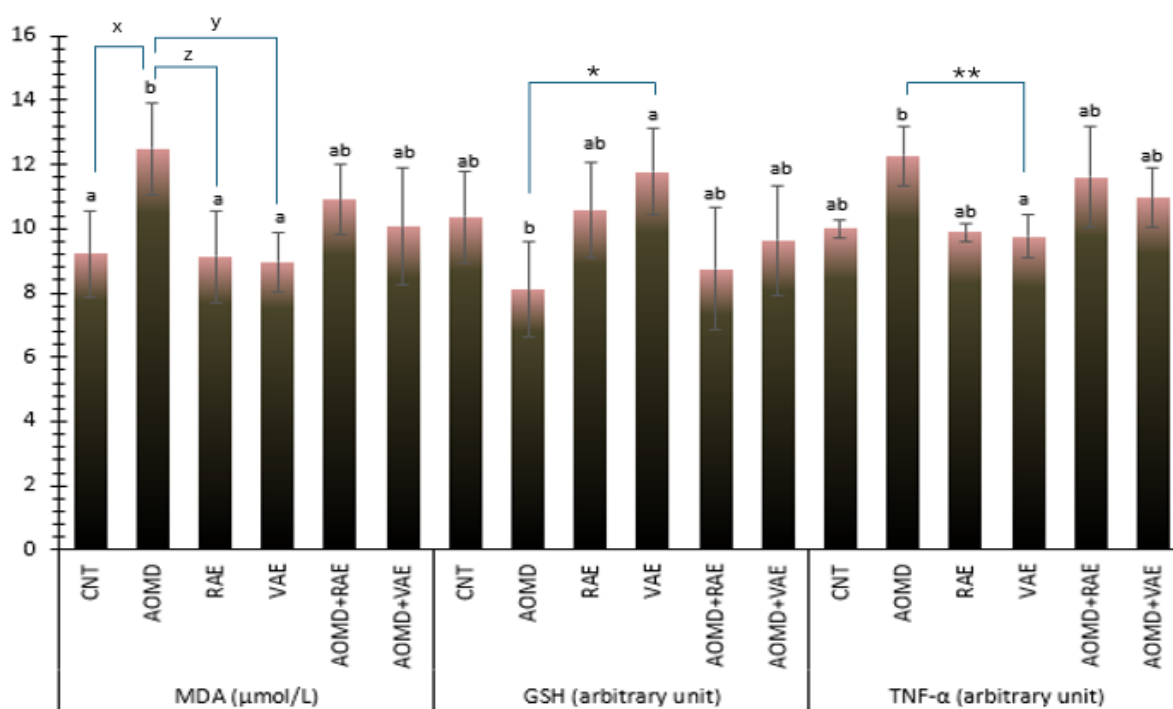


Figure 2. MDA, GSH and TNF-α levels in plasma samples of CNT, AOMD, RAE, VAE, AOMD+RAE and AOMD+VAE mouse groups. (^{a,b}*p*<0.05 according to one-way analysis of variance and ^x*p*=0.010, ^y*p*=0.005, ^z*p*=0.008, ^{*}*p*=0.012 and ^{**}*p*=0.043 according to multiple comparison Tukey test).

Table 1. Correlations between biochemical parameters

Parameters	MDA	GSH	TNF-α
Act-AB	-0.443	0.336	-0.456*
TNF-α	0.540*	-0.496*	
GSH	-0.407		

Abbreviations: Act-AB, activin-AB; GSH, reduced glutathione; MDA, malondialdehyde; TNF-α, tumor nekroz factor-α (**p*<0.05)

In this study conducted to determine the act-AB relationship in the colon cancer induction case, the act-AB level in the AOMD group decreased slightly compared to the CNT group. While the act-AB protein expression levels in the VAE group increased significantly compared to the CNT and AOMD groups, a significant increase was found in the act-AB level in the AOMD+VAE group compared to the AOMD group, approaching the CNT average level. Although there was an increase in act-AB protein expression levels in the RAE and AOMD + RAE groups compared to the AOMD group, it did not reach statistical significance (Figure 1). Refaat et al. [9] analyzed act-A, -B, -AB and follistatin proteins in human colon cancer tissue with enzyme-linked immunosorbent assay and claimed that act-A averages increased during colon cancer, but this level was opposite to act-AB averages. Although no predictions have been made regarding Act-AB, a significant increase in late-stage Act-A expression has been associated with triggering malignant cell proliferation and malignant cell metastasis. An experimental study reported that metastatic lesions were observed in rat colon tissue with long-term (35 weeks) AOM administration, act-AB tissue concentration was significantly inversely correlated with lesion number and

size. It was also noted that further studies are needed to determine the clinical value of activins and related molecules [28]. Jueckstock et al. [29] in their study on HER2 positive breast cancer patients, they found that the serum act-AB levels of the patients were low, but the sudden acute increase in act-A caused cancer recurrence. Tsuchida et al. [30] found that act-AB and act-B use the combination of ActRIIA and ALK7 in the metabolic pathway, but act-A does not use this pathway in signaling. While activin A usually signals through ALK4, activin B and activin AB use both the ALK4 and ALK7-mediated signaling pathway which results in the activation of the SMAD2/3 pathway. These findings suggest that act-AB may utilize different metabolic signaling pathways compared to other isoforms of activin. In addition, previous studies have reported that oxidative stress can increase the activin expressions [31, 32]. Hesperidin, a natural bioflavonoid used as a dietary supplement to support vascular health due to its strong antioxidant and anti-inflammatory properties, has been reported to reduce oxidative stress in mice given dimethylhydrazine (DMH) as a cancer inducer while altering levels of the act-A as an isoform of the act-AB differently depending on whether DMH is administered concurrently or after the treatment [32]. Furthermore, it has been noted that bioflavonoids, such as chlorogenic acids which are abundant in blueberries have oxidative stress-reducing effects against arsenic exposure [33]. This study suggests that VAE may normalize act-AB levels by reducing inflammatory factors such as TNF-α and improving levels of oxidative stress parameters such as GSH and MDA. The fact that act-AB levels can show a negative correlation with MDA and TNF-α (Table 1) suggests that Act-AB may act independently of binding

proteins or factors for activin-A and -B which are involved in the controllable metabolic pathways of native cells in AZO and DSS exposure. More detailed studies on molecules involved in the activin pathway are needed to clarify this alternative pathway possibility.

A preclinical study using mice by Popivanova et al. [34] found a positive association between treatment with AOM and DSS and increased TNF- α expression in colon tissue, leading to colon tumor formation. Furthermore, it was demonstrated that bone marrow transplantation deficient in TNF-receptor p55 and treatment with a TNF- α antagonist resulted in a reduction in inflammation in the mice [34]. It has also been reported that activins reduces TNF- α -induced phosphorylation of factors such as extracellular signaling regulatory kinase (ERK) and mitogen-activated protein kinase (MAPK) in molecular signaling pathways [14]. TNF- α levels measured by enzyme-linked immunosorbent assay were significantly reduced in the AOMD+VAE group compared to the AOMD group and approached those of the CNT and VAE groups. Although the results in the RAE-derived *Rubus idaeus* L. group showed a very similar effect to VAE, these similar values were not statistically significant at the dose applied to mice (Figure 2). In this study, the combined evaluation of activins act-AB and TNF- α may shed light on research into signaling pathways in molecular pathogenesis. The antagonistic effect of VAE in response to increased TNF- α levels is also noteworthy.

Vaccinium myrtillus L. has been reported to have a potential inhibitory effect against cell adhesion by reducing the release of proangiogenic factors while also regulating the expression of cell adhesion molecules [35]. Activins are potent regulators of cell adhesion, in addition to cell migration and invasion [36]. While VAE increases act-AB levels due to its inhibitory effect on cell adhesion, this level is downregulated in the VAE+AOMD treatment group where they are administered together (Figure 1). The combined application of act-AB and VAE may be normalizing in terms of cell adhesion, but this needs to be clarified with further studies.

In the current study, MDA and GSH levels which are relevant parameters were also examined because ROS are directly linked to colitis or cancer. Excessive ROS accumulation is characterized by effects that increase during oxidative stress such as high MDA levels related to lipid peroxidation and low GSH levels [37, 38]. A decrease in the GSH/oxidized glutathione (GSSG) ratio in the liver indicates that severe oxidative stress is induced [36]. MDA levels were statistically significantly increased in the AOMD group compared to the CNT group, while GSH levels decreased. In the AOMD+VAE group, MDA levels were significantly decreased compared to the AOMD group while GSH levels increased significantly, approaching the CNT and VAE groups. The results in the RAE group were quite similar to the VAE group but were not statistically significant at the dosage administered to the mice (Figure 2). In a study related with mice with DSS-induced colitis, the effects of phenolic acids which are also found abundantly in VAE and RAE on oxidative stress

parameters in colon tissues were noted that caffeic acid reduced MDA levels but increased glutathione peroxidase (GSH-Px) levels and sinapic acid reduced MDA and TNF- α levels [40]. Similarly, in a similar study on DSS-induced colitis in mice, vanillic acid treatment reduced MDA levels by attenuating DSS-induced lipid peroxidation, and the decrease in GSH levels was normalized by vanillic acid treatment [41]. In a study in mice with AOM-induced colitis, it was determined that mango, with its high antioxidant content, led to significantly higher GSH-Px and lower MDA concentrations [42]. In indomethacin administration as a chemical agent for gastrointestinal damage, abundant ellagic acid in blackberries and blueberries has been found to be significantly effective in suppressing oxidative stress [43]. Blueberries and raspberries also contain high levels of many different phenolic acids. The coumaric acid and syringic acid with anti-inflammatory and antioxidant effects is particularly abundant among them [44]. In a study related with rats given the acetic acid as a chemical agent of colitis, it was reported that syringic acid and coumaric acid increased the mRNA expression of heme oxygenase-1 (Ho-1), NAD(P)H:quinone reductase 1 (NQO1) and the nuclear factor erythroid 2-related factor 2 (NRF2) for the electron reduction when TNF- α and IL-1 β protein levels in immun system response decreased in colon tissue [45]. Considering the data obtained, it is understood that providing cellular resistance against oxidants by catalyzing the reduction of electrons may have a protective effect in inflammation and carcinogenesis. The current study demonstrates that oxidative stress related to MDA and GSH together TNF- α levels was intense in the AOMD group and that this intensity could be reduced by administering both VAE and RAE, suggesting that VAE is more protective against oxidative stress.

According to many previous studies, if there are no pathological conditions except for follicular fluid, the concentrations of act-AB in body fluids and tissues are much lower than other activin isoforms (in pg/mL samples), making it difficult to analyze [28, 46, 47]. Therefore, the analyses related with activin levels in many studies usually involve activin A rather than activin AB [10, 28, 31, 32, 47]. However, findings have been obtained in a few studies and it has been reported that although it can be measured more easily in the follicular fluid of cattle, sheep and pigs, it is more difficult to measure in humans [46]. The idea that the reason for the low concentrations of act-AB which are difficult to measure may also be related to its very rapid metabolism is also important [47]. According to the results of the present study, although act-AB concentrations were highly variable in each group, statistical analyses were used to correlate the rates of these changes with experimental applications, contributing to the resolution of this important gap. It is thought that further elaborating on this topic with metabolic data obtained from different physiological and pathological conditions would be much more beneficial.

In conclusion, it was determined that *Vaccinium myrtillus* L. administered concomitantly with AOM and DSS as chemical carcinogens in mice was effective in normalizing act-AB levels which are important for cell proliferation in colon tissue. This plant has a protective effect because it leads to a parallel reduction in oxidative stress and inflammation with treatment. This protective effect needs to be elucidated by further studies involving gene expression analyses of different phenolic compounds and metabolic signaling pathways of *Vaccinium myrtillus* L. according to the literature review, this study is the first to evaluate the effectiveness of blueberries and raspberries against chemical agents used in inflammation-related colon cancer formation, both *in vivo* and comparatively. This study directly provides a basis for the effectiveness of berry fruits in future studies, especially in protection against chemical carcinogens. Furthermore, it is anticipated that the combined evaluation of act-AB, TNF- α , MDA, and GSH levels in the fruits of *Vaccinium myrtillus* L. and *Rubus ideaus* L. could significantly contribute to focusing treatments on understanding the causes of the pathogenesis of stimulation with chemical agents.

Conflict of Interest

There are no conflicts of interest in this work.

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Ethical Approval Statement

This study was approved by the Ethics Committee of Kafkas University Animal Experiments Local Ethics Committee (Ethical approval code: KAU-HADYEK 2019/024).

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