

Original Research Article

Tarantula cubensis alcohol extract enhances the tumoricidal effect of capecitabine via multiple pathways in azoxymethane-induced colorectal cancer in rats

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Abstract

Purpose: To evaluate the effect of a combination of *Tarantula cubensis* alcohol extract (TCAE) and capecitabine (CAP) in the treatment of azoxymethane (AOM)-induced colorectal cancer (CRC).

Methods: Forty-two Wistar albino rats were divided into 7 groups with 6 rats in each group. The groups consisted of Control (C), Control+TCAE (C-TCAE), Control+CAP (C-CAP), Cancer control (CC), Cancer+TCAE (CC-TCAE), Cancer+CAP (CC-CAP) and Cancer+CAP+TCAE (CC-CAP+TCAE). To induce CRC, AOM (15 mg/kg) was administered to rats subcutaneously (sc) twice at a one-week interval to all the groups except control. From the 15th week, TCAE (0.2 mL/rat sc) was administered to CC-TCAE group every 3 days for 4 weeks, and CAP (40 mg/kg/day) was administered by gavage to CC-CAP group for 4 weeks. In CC-CAP+TCAE group, TCAE (0.2 mL/rat sc) was administered every 3 days for 4 weeks, and CAP (40 mg/kg/day) was administered gavage for 4 weeks. Animals were treated for 18 weeks. Aberrant crypt foci (ACF) were evaluated histopathologically among CC, CC-TCAE, CC-CAP, and CC-CAP+TCAE groups. β -catenin, CD15, Proliferating Cell Nuclear Antigen (PCNA), and Nuclear Factor kappa B (NF- κ B) expression levels were immunohistochemically compared among all groups.

Results: Histopathologically, ACF scores were significantly increased in CC group, while a significant decrease in the relevant scores ($p < 0.001$) was observed in CC-CAP and CC-CAP+TCAE treatment groups, and the lowest scores were in CC-CAP+TCAE group. Immunohistochemically, in CC group, β -catenin, Nuclear Factor kappa B (NF- κ B), Proliferating Cell Nuclear Antigen (PCNA) and CD15 expressions were highly irregular. CC-CAP and CC-CAP+TCAE groups had significantly reduced expressions ($p < 0.001$), and the lowest expressions were in CC-CAP+TCAE group.

Conclusion: The combined use of TCAE and CAP in treatment of CRC has a synergistic effect and increases the anticancer efficacy of TCAE, and CAP. More studies at the molecular level are needed in the future to demonstrate the clinical benefit of TCAE supplementation during the treatment of CRC with CAP.

Keywords: Colorectal cancer, Azoxymethane, β -catenin, NF- κ B, PCNA, CD15

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INTRODUCTION

Colorectal cancer (i.e., colon cancer, CRC) is cancer of the colon and rectum. While the majority of CRC cases occur sporadically (more than 80 %), approximately 5 – 10 % are associated with inherited mutations in genes and the remainder are associated with inflammatory bowel disease [1,2].

Multiple genetic and epigenetic changes, including hereditary and environmental factors, facilitate the pathogenesis of CRC. The genetic and epigenetic changes underlying colorectal neoplasia are the most likely events that trigger neoplastic progression [3]. Genetic changes in tumor suppressor genes and oncogenes occur in a distinct sequence in the transformation from a normal epithelium to a dysplastic epithelium in the adenoma-carcinoma process. When genetic and epigenetic changes in the genes that are involved accumulate over time in the long term, they undergo changes from normal epithelial cells to the appearance of Aberrant crypt foci (ACF) of a preneoplastic lesion characterized by abnormal colonocyte growth and destruction. Such ACF progress into adenoma and adenocarcinoma [3-5]. CRC develops in a multistep process because of the progressive accumulation of these changes and mutations in the Wnt/ β -catenin signaling pathway [2,4,5].

Tarantula cubensis alcoholic extract (TCAE, Theranekron D6 enj®, Richter pharma AG, Wels, Austria) is a homeopathic product used in veterinary medicine. Many researchers have stated that TCAE has therapeutic effects against various types of cancer [5-8]. Capecitabine (CAP) is designed to mimic continuous 5-Fluorouracil (5-FU) infusion. CAP has an activation mechanism that takes advantage of the elevated activity of thymidine phosphorylase in tumoral tissue and subsequently results in the formation of 5-FU in the tumoral tissue. It was stated that CAP prepared in an oral formulation is a prodrug that is converted to 5-FU, which is frequently used in treatment of CRC and breast cancers [9].

CAP is one of the commonly used chemotherapeutic agents in treatment of CRC. To develop more effective therapeutic strategies, it is necessary to understand the pathophysiological mechanisms associated with CRC. Combinations of CAP with other agents may enhance its therapeutic effect, which seems to be a potential approach to achieving such improvements. In this context, the combined use of CAP and TCAE may be promising, and this consideration formed the focus of this study. In

this study, the effect of the combined use of CAP and TCAE on ACF, β -catenin, CD15, Proliferating Cell Nuclear Antigen (PCNA), and Nuclear Factor kappa B (NF- κ B) in CRC induced with azoxymethane (AOM) were investigated.

EXPERIMENTAL

Animals

Forty-two male Wistar Albino rats, 12 - 16 weeks old, weighing 220 - 250 g were used. During the study, the animals were housed in polysulfone cages at 24 ± 1 °C, 60 % atmospheric humidity, and in a 12/12-hour light/dark cycle. Feed and water were given *ad libitum*. The study was approved by the Ethics Committee of Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center (approval no. 2023/074), and followed international guidelines for animal studies.

Groupings and treatments

The animals were divided into 7 groups of 6 rats in each group. No treatment was given to the healthy control (C) group. In control + TCAE (C-TCAE) group, animals were administered TCAE (Theranekron D6 enj®, Richter pharma AG, Wels, Austria) at a dose of 0.2 mL/rat subcutaneously (sc) at 3-day intervals for 4 weeks from the 15th week. In control + capecitabine (C-CAP) group, from the 15th week onwards, CAP (Kapeda tablet, Koçak Farma, Tekirdag, Turkey) was administered to the animals by gavage at a dose of 40 mg/kg/day, for 4 weeks [10]. In cancer control (CC) group, AOM (Sigma-Aldrich, Darmstadt, Germany) at a dose of 15 mg/kg was administered sc to the animals in 2 doses at a one-week interval [10].

In cancer + TCAE (CC-TCAE) group, the animals were administered sc azoxymethane at a dose of 15 mg/kg twice at a weekly interval. From the 15th week on, TCAE was administered at a sc dose of 0.2 mL/rat at 3-day intervals for 4 weeks. In cancer + CAP (CC-CAP) group, AOM was administered to the animals at a dose of 15 mg/kg twice at an interval of one week, and from the 15th week, it was administered by gavage at a dose of 40 mg/kg/day for 4 weeks. In cancer + TCAE + CAP (CC-CAP + TCAE) group, the animals were administered sc AOM at a dose of 15 mg/kg in 2 doses, one week apart. Starting from the 15th week, TCAE was administered at a dose of 0.2 mL/rat sc at 3-day intervals for 4 weeks, and CAP was administered at a dose of 40 mg/kg/day by gavage for 4 weeks. The animals in all groups were sacrificed by cervical dislocation 1 hour after the last administration

under ketamine (95 mg/kg, sc) and xylazine (5 mg/kg, sc) anesthesia [5]. The colon tissues of all animals were withdrawn, washed with normal saline, and then placed in a neutral formaldehyde solution.

Histopathological examination

Colon tissues were subjected to routine tissue processing steps after 24 - 48 hours of neutral formaldehyde fixation procedures. Then, paraffin blocks were obtained and sections of 5 µm from the paraffin blocks were taken on an adhesive slide, stained with Hematoxylin-Eosin and examined under a light microscope. In the histopathological examinations, ACF scoring was performed according to a previously reported scoring system [5]. ACF in 10 different areas from each section were scored by a blinded pathologist and averaged.

Immunohistochemical examination

Sections were taken from paraffin blocks onto adhesive slides. Immunohistochemical staining was carried out according to the UltraVision Detection System Anti-Polyvalent, Horseradish peroxidase (HRP) (Ready-To-Use, TP-060-HL, Lab Vision, USA) kit procedure. The β -catenin (Proteintech, 51067-2-AP, 1:1000 dilution), PCNA (Dako, clone PC10, M0879, 1:200 dilution), CD15 (Thermo, PA5-119027, 1:200 dilution), NF- κ B (Bioss, Bs-0465R, 1:200 dilution) antibodies were used. 3,3 diaminobenzidine (DAB) was used as the chromogen and counterstained with Mayers-hematoxylin. The Allred scoring method was used for measuring the intensity and extent of immunohistochemical staining [7]. Total scores were obtained for staining intensity (0; absent, 1; weak, 2; moderate, 3; severe) and staining degree (0; absent, 1; > 0 - 1/100, 2; > 1/100 - 1/10, 3; > 1/10 - 1/3, 4; 1/3 - 2/3 and 5; > 2/3 - 1).

Statistical analysis

The obtained data were evaluated with SPSS 25 (Inc., Chicago, USA) program. Shapiro-Wilk test was used to evaluate the normal distribution of the data, and Levene's test was used for homogeneity of variances. Histopathological and immunohistochemical data were evaluated with the Kruskal Wallis-H test. Mann-Whitney U test was used for differences between groups. Values are shown as Mean \pm standard error of the mean (SEM). $p < 0.05$ was accepted as the significance level.

RESULTS

Histopathological findings

The ACF scoring results of the groups are shown in Figure 1. The CC-CAP and CC-CAP + TCAE groups showed significant reductions in ACF scores relative to CC group ($p < 0.001$). The lowest ACF scores were found in CC-CAP + TCAE group. The intestinal sections in the healthy control groups (C, C-TCAE, C-CAP) showed normal histology. ACF formations were determined in AOM-induced groups, and these groups showed more hyperchromatic and abnormal luminal shapes than the normal crypt epithelium (Figure 2 B - E). Additionally, dysplastic crypts were widely distributed in CC group. Fewer dysplastic crypts were observed in treatment groups, especially CC-CAP and CC-CAP + TCAE, compared to CC group.

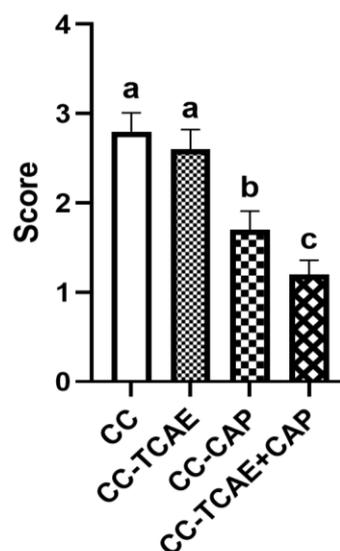


Figure 1: Effect of TCAE and CAP on Aberrant Crypt Foci (ACF). Data are presented as Mean \pm SEM. $a-c p < 0.001$ vs. control. (CC; cancer control, CC-TCAE; cancer + TCAE, CC-CAP; cancer + CAP; CC-CAP + TCAE; cancer + CAP + TCAE, TCAE; *Tarantula cubensis* alcoholic extract, CAP; capecitabine)

Immunohistochemical findings

The immunohistochemical assessment scores of control and experimental groups are shown in Table 1. Control groups showed similar immunoreactivity in the case of all relevant primers. β -catenin staining showed intense immunoreactivity in CC group. In particular, β -catenin immunoreactivity was strong in ACF structures and dysplastic crypts. CC-CAP and CC-TCAE + CAP groups had significantly lower scores ($p < 0.001$). The results of CC-CAP + TCAE and control groups did not differ to a statistically significant extent (Figure 3).

Intense immunoreactivity was determined in the PCNA staining results in CC group. CC-TCAE, CC-CAP, and CC-CAP + TCAE groups had significantly reduced expression levels (Figure 4, $p < 0.001$). Similarly, intense immunoreactivity was determined in CC group in NF- κ B staining, and CC-TCAE, CC-CAP, and CC-CAP + TCAE groups had significantly reduced expression levels (Figure 5, $p < 0.001$). Intense immunoreactivity was determined in CD15 staining in CC group, while in CC-CAP and CC-CAP + TCAE groups, a significant decrease in these scores was observed (Figure 6, $p < 0.001$).

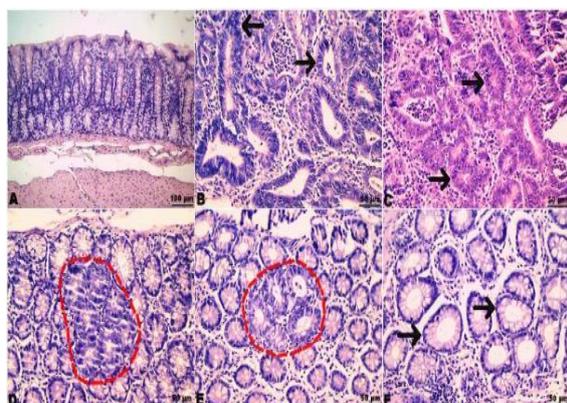


Figure 2: Microscopic examination of the effects of TCAE and CAP on Aberrant Crypt Foci (ACF), Hematoxylin-Eosin. (A) Normal histological appearance of group C, (B) Dysplastic crypts with distinctly atypical cellular features in CC group, (C) Appearance of dysplastic crypts with distinctly atypical features in CC-TCAE group, (D) Disruption of crypt epithelium and appearance of nuclear pleomorphism in CC-TCAE group, score 3, (E) The appearance of low-to-moderate dysplasia in CC-CAP group, score 2, (F) The appearance of hyperplasia without dysplasia in CC-CAP + TCAE group (C; control, CC; cancer control, CC-TCAE; cancer + TCAE, CC-CAP; cancer + CAP; CC-CAP + TCAE; cancer + CAP + TCAE, TCAE; *Tarantula cubensis* alcoholic extract, CAP; capecitabine)

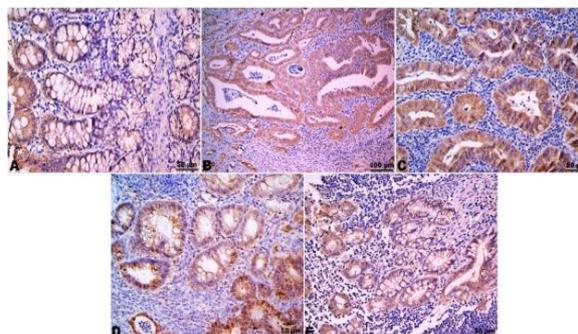


Figure 3: TCAE and CAP in AOM-induced CRC effect on β -catenin expressions (DAB) (A) C group, (B) CC group, (C) CC-TCAE group, (D) CC-CAP group, (E) CC-CAP + TCAE group (C; control, CC; cancer control, CC-TCAE; cancer + TCAE, CC-CAP; cancer + CAP; CC-CAP + TCAE; cancer + CAP + TCAE, DAB; 3,3 diaminobenzidine, TCAE; *Tarantula cubensis* alcoholic extract, CAP; capecitabine, CRC; colorectal cancer)

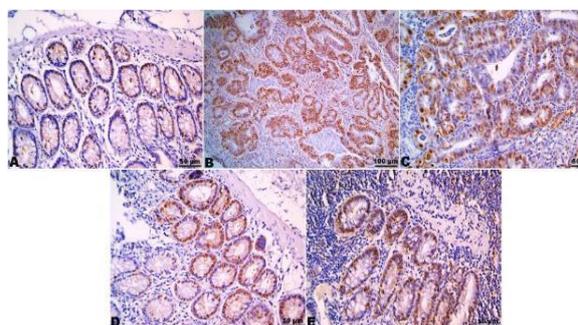


Figure 4: TCAE and CAP in AOM-induced CRC effect on Proliferative cell nuclear antigen (PCNA) expressions (DAB) (A) C group, (B) CC group, (C) CC-TCAE group, (D) CC-CAP group, (E) CC-CAP + TCAE. (C; Control, CC; cancer control, CC-TCAE; cancer + TCAE, CC-CAP; cancer + CAP; CC-CAP + TCAE; cancer + CAP + TCAE, TCAE; *Tarantula cubensis* alcoholic extract, CAP; capecitabine, DAB; 3,3 diaminobenzidine, CRC; colorectal cancer)

Table 1: Immunohistochemical scores of the effects of TCAE and CAP on β -catenin, PCNA, CD15 and NF- κ B

Primer	C	C-TCAE	C-CAP	CC	CC-TCAE	CC-CAP	CC-TCAE + CAP
β -catenin	2.66 \pm 0.33 ^d	2.50 \pm 0.22 ^d	2.50 \pm 0.22 ^d	6.50 \pm 0.34 ^a	5.83 \pm 0.17 ^a	4.33 \pm 0.21 ^b	3.33 \pm 0.21 ^{cd}
PCNA	2.33 \pm 0.21 ^e	2.16 \pm 0.17 ^e	2.16 \pm 0.17 ^e	6.33 \pm 0.33 ^a	5.16 \pm 0.30 ^b	4.00 \pm 0.26 ^c	3.17 \pm 0.16 ^d
CD15	1.67 \pm 0.42 ^d	1.83 \pm 0.40 ^d	1.83 \pm 0.40 ^d	6.17 \pm 0.30 ^a	5.50 \pm 0.30 ^a	4.33 \pm 0.21 ^b	3.50 \pm 0.22 ^c
NF- κ B	2.17 \pm 0.17 ^e	2.50 \pm 0.34 ^e	2.33 \pm 0.21 ^e	6.50 \pm 0.22 ^a	5.00 \pm 0.25 ^b	4.17 \pm 0.30 ^c	3.33 \pm 0.21 ^d

Note: Data are shown as Mean \pm SEM. ^{a-e} $p < 0.001$ vs. control. (C; Control, C-TCAE; Control + TCAE, C-CAP; control + CAP, CC; cancer control, CC-TCAE; cancer + TCAE, CC-CAP; cancer + CAP; CC-CAP + TCAE; cancer + CAP + TCAE, PCNA; proliferative cell nuclear antigen, NF- κ B; Nuclear Factor kappa B, TCAE; *Tarantula cubensis* alcoholic extract, CAP; capecitabine).

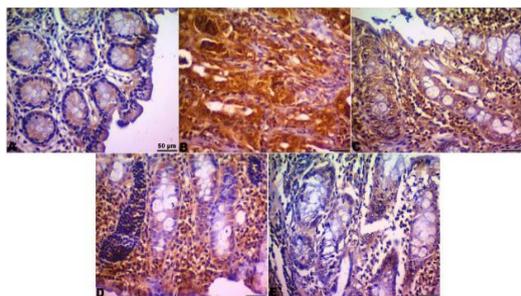


Figure 5: TCAE and CAP in Azoxymethane-induced CRC effect on NF- κ B (Nuclear Factor kappa B) expressions (DAB). (A) C group, (B) CC group, (C) CC-TCAE group, (D) CC-CAP group, (E) CC-CAP + TCAE. (C; Control, CC; Cancer control, CC-TCAE; Cancer + TCAE, CC-CAP; Cancer + CAP; CC-CAP + TCAE; Cancer + CAP + TCAE, TCAE; *Tarantula cubensis alcoholic extract*, CAP; capecitabine, DAB; 3,3 diaminobenzidine, CRC; colorectal cancer)

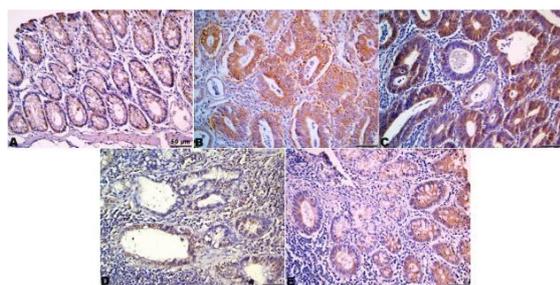


Figure 6: TCAE and CAP in AOM-induced CRC effect on CD15 expressions (DAB). (A) C group, (B) CC group, (C) CC-TCAE group, (D) CC-CAP group, (E) CC-CAP + TCAE. (C; control, CC; cancer control, CC-TCAE; cancer + TCAE, CC-CAP; cancer + CAP; CC-CAP + TCAE; cancer + CAP + TCAE, TCAE; *Tarantula cubensis alcoholic extract*, CAP; capecitabine, DAB; 3,3 diaminobenzidine, CRC; colorectal cancer)

DISCUSSION

Despite the many advancements that have been made in the diagnosis and treatment of CRCs in recent times, the disease is progressing with a high incidence and mortality on a global scale [1,2]. Surgical removal of CRCs is the most effective therapeutic approach, but early diagnosis is required. Radiotherapy and chemotherapy are other alternative treatment approaches in advanced stages of CRC [11]. Studies are currently carried out using many different agents to increase the effectiveness of chemotherapeutic drugs used in treatment of CRC [12,13]. In this study, the efficacy of the combined use of CAP and TCAE in CRC induced by using AOM was investigated.

ACF are considered precancerous within the scope of biochemical, histopathological, and immunohistochemical changes, as well as genetic and epigenetic changes. ACF also shows

pathomorphological similarity as in the development of sporadic CRCs. In general, ACF displays features ranging from hyperplasia to dysplasia in histological examinations. ACF is considered to be an important biomarker of CRC [14,15]. In this context, AOM-induced ACF is frequently preferred today in examining the anticarcinogenic effects of various agents [5,10,16]. In the present study, ACF formation was observed in the histological examinations of all AOM-treated groups, and these crypts were highly hyperchromatic and had a highly irregular luminal structure. The presence of hyperplastic and dysplastic crypts and increased numbers of ACF in AOM-treated groups revealed the development of CRC. These findings were consistent with those of previous AOM-induced CRC studies [10,16].

Although treatment with TCAE alone reduced the mean ACF scores of the groups compared to CC group, the therapeutic effect of TCAE was lower than that of CAP. A significant decrease was found in ACF scores in CC-CAP and CC-CAP + TCAE groups compared to CC group. The combined use of TCAE and CAP significantly reduced the ACF scores of the histopathologically precancerous lesions, thus showing a synergistic effect between TCAE and CAP. It has been reported in a previous study that TCAE given simultaneously with AOM significantly reduced ACF scores [5]. In comparison to the results in this study, this exhibited that chemopreventive efficacy of TCAE alone is superior to the efficacy of simultaneous treatment. However, the results revealed that CAP increased the effectiveness of the overall treatment in this study. This effect of TCAE, which is a homeopathic product, may be due to its effect on some irregular cumulative genetic and epigenetic changes that occur with the influence of AOM.

Abnormal activation of the Wnt/ β -catenin pathway plays a pivotal role in the initiation and progression of CRC. In this context, β -catenin has recently become a promising target for the chemoprevention and treatment of cancer [12,17]. In this study, elevated β -catenin expression levels were determined in CC group, confirming the view that the pathway is highly active in CRC. Several studies on cancers induced by AOM have reported increase in β -catenin gene and/or protein expressions [5,12,17]. The strong β -catenin expression levels in AOM-treated groups in this study, especially in terms of ACF, showed that Wnt/ β -catenin signaling caused tissue-wide abnormality, and this result was consistent with the findings of previous studies [12,17]. In this study, CC group

showed the highest expression levels, followed by CC-TCAE group, while the lowest scores were found in CC-CAP + TCAE group. The use of TCAE in combination with CAP, rather than alone, clearly demonstrated positive effects on β -catenin expressions and inhibited cancer progression. The NF- κ B is a very important transcription factor that plays a role in different processes such as apoptosis, inflammatory responses, cell proliferation, metastasis, and drug resistance. Since the aberrant regulation of NF- κ B in tumor cells has been frequently reported, the inhibition of this cascade limits cell proliferation. This factor has been stated to play a role in tumor development by activating proliferative and antiapoptotic processes, especially in cancer cells. Additionally, the dysregulated activation and overactivation of the NF- κ B pathway is an important characteristic of CRC [18,19]. Recently, the suppression of the NF- κ B signaling pathway using several inhibitory agents has been seen as a potential therapeutic approach in CRC. Additionally, high NF- κ B expression levels were reported in cancer control groups in CRC studies, and it was stated that this pathway is very important in the development of CRC [18,19]. In the presented study, in parallel with the findings of previous studies, high NF- κ B expression levels were determined in CC group, further confirming the importance of this factor in cancer development. Although TCAE suppressed NF- κ B expression, its effect was not as significant as that of CAP. Nevertheless, the combined use of TCAE and CAP was found to significantly reduce NF- κ B expression.

The PCNA is known as a marker of cellular proliferation in healthy and tumor tissues [14,20]. It has been frequently studied to evaluate tumor proliferation in many different studies [7,21]. Strong PCNA expressions were determined in CC group, in line with the findings of previous studies. The TCAE and CAP treatments significantly reduced these expression levels. The combined synergistic effect of TCAE and CAP, rather than TCAE or CAP monotherapy, revealed that this combination inhibited tumor proliferation more effectively. CD15 is found in various types of cancers, including acute leukemias, kidney cancer, hepatocellular carcinoma, breast cancer, and prostate and bladder cancers [22]. The CD15 expressed in tumor cells was reported to mediate adhesion to endothelial cells and its high expression parallels its invasive potential [22,23]. Jang *et al* [23] stated that CD15 expressions gradually increase in the development and progression of cancer. In recent years, CD15 has been stated to be a powerful target for cancer therapy [22]. In the presented study, high CD15 expression levels

were determined in CC group, in parallel with the findings of previous studies [22, 23]. The lowest expression scores in the study were found in the C-CAP and CC-CAP + TCAE groups. This further points to a synergistic effect of TCAE and CAP in lowering CD15 expression levels and increasing treatment efficacy.

CONCLUSION

In AOM-induced CRC, TCAE and CAP work synergistically to enhance the anticancer activity of CAP by modulating aberrant crypt foci (ACF), β -catenin, nuclear factor kappa B (NF- κ B), proliferating cell nuclear antigen (PCNA) and CD15. More studies at the molecular level are needed in the future to demonstrate the clinical value of TCAE supplementation during treatment of CRC with CAP.

DECLARATIONS

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None provided.

Ethical approval

This study was approved by Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center (approval no. 2023/074).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Gokhan Akçakavak, Mehmet Tuzcu and Ozgur Ozdemir designed the study. Osman Dogan, Zeynep Celik, Ozhan Karatas completed the laboratory procedures. All authors read and approved the final version for publication.

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