

## Effect of *Tarantula cubensis* alcoholic extract on tumour pathways in azoxymethane-induced colorectal cancer in rats

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Received April 3, 2022

Accepted December 8, 2022

### Abstract

The aim of this study was to determine the effects of *Tarantula cubensis* alcoholic extract (TCAE) on tumour development pathways in azoxymethane (AOM)-induced colorectal cancer in rats by molecular methods. Eighteen paraffin-embedded intestinal tissues, six from each group, were studied in the healthy control (C), cancer control (CC), cancer + TCAE (C-TCAE) groups. Sections of 5 µm thickness were taken from the paraffin blocks and submitted to staining with haematoxylin-eosin. In the histopathological examination, the number of crypts forming aberrant crypt foci (ACF) and the degree of dysplasia in the crypts were scored. Real-time PCR analysis was completed to determine β-catenin, KRAS (Kirsten rat sarcoma virus), APC (adenomatous polyposis coli) and P53 expressions on samples from each paraffin block. The grading scores of the number of crypts forming ACF and dysplasia in the crypts showed an evident decrease in the C-TCAE group in comparison to the CC group ( $P < 0.05$ ). In real-time PCR analysis, mRNA expression levels of P53 ( $P > 0.05$ ) and APC ( $P < 0.001$ ) genes were found to be increased in the C-TCAE group according to the CC group. The expression levels of KRAS ( $P < 0.01$ ) and β-catenin ( $P < 0.005$ ) mRNA were found significantly decreased in the C-TCAE group. In conclusion, the effects of TCAE on AOM-induced colorectal cancer (CRC) in rats were evaluated molecularly; TCAE was found to modulate some changes in CRC developmental pathways, inhibiting tumour development and proliferation, and stimulating non-mutagenic tumour suppressor genes. Thus, it can be stated that TCAE is an effective chemopreventive agent.

*APC, P53, KRAS, β-catenin, chemoprevention*

Colorectal cancer (CRC) is defined as the cancer of portions of the large intestine, colon, and rectum. In CRC, symptoms such as pain during defecation, fatigue, weight loss, changes in bowel movements, added to mucus and blood in the stool are observed (Tantoy et al. 2016; Qureshi et al. 2018). Among the deaths related to cancer in Europe, colorectal cancer ranks second (Jemal et al. 2008). Worldwide, it occupies the place of the fourth most common malignant neoplasm (Mattiuzzi and Lippi 2019).

The pathogenesis of CRC is a complex web of multiple genetic and epigenetic changes. It includes hereditary and environmental factors (Migliore et al. 2011). Colorectal cancers occur related to a series of well-characterised histopathological changes resulting from some tumour suppressor genes and oncogenes suffering specific mutations (Fearon and Vogelstein 1990). The main step in the development of CRC, primarily in adenoma formation, is the constitutive activation of the signalling pathway of Wnt, known as the gatekeeper. It is caused by the loss of the function of adenomatous polyposis coli (APC), known as the tumour suppressor gene, and, less commonly, by mutations in the gene in charge of activating β-catenin, the β-catenin-encoding gene (CTNNB1) (Schulz 2005). Nearly 90% of the sporadic CRCs involve mutations in sections of the signalling pathway of Wnt/β-catenin (Giles et al. 2003). Other pathways in cancer progression include mutations that increase Kirsten rat sarcoma virus (KRAS) expression, the decline of P53

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function, inactivation of transforming growth factor-beta (TGF- $\beta$ ) cellular response and activation in the phosphoinositide 3-kinase (PI3K) pathway (Schulz 2005).

*Tarantula cubensis* alcoholic extract (TCAE) is obtained from a spider named *Tarantula cubensis*. *Tarantula cubensis* alcoholic extract has been widely used as a homeopathic product in veterinary medicine (Theranekron® D6, Richter Pharma, Wels, Austria) in recent years (Richter Pharma 2018). Among the possible utilisations of TCAE as a homeopathic product in the veterinary field are the treatment of gangrene, septicemia and toxemia (Richardson-Boedler 2002). The mechanism of action of TCAE is not fully known (Day and Saxton 1998). It has been reported that the use of TCAE results in the regression of both benign and malignant mammary tumours in dogs (Gültiken and Vural 2007; Gultiken et al. 2015).

Lately, azoxymethane (AOM) has been frequently used in order to induce the experimental CRC model (Takahashi and Wakabayashi 2004; Ferraz da Costa et al. 2020). Chemoprevention is the term used to define the use of substances, both natural or synthetic, to minimise or eliminate the risk of developing the disease in many conditions, including cancer. In this context, it is very important to inhibit some genetic and epigenetic changes that induce clonal proliferation in cancer (Singh et al. 2019). Since CRC is among the leading causes of death globally, studies to find different methods from existing chemotherapy options have been focused on for many years (Norazalina et al. 2010; Ashokkumar and Sudhandiran 2011). During the literature review, it was found that TCAE, a homeopathic product whose mechanism of action is unknown and which is frequently used in the veterinary field, reduces free oxygen radicals (Dik et al. 2014), can activate apoptosis by inducing the caspase-3 pathway *in vitro* (Ghasemi-Dizgah et al. 2017) and, in this context, it has been noted that it has a positive effect on mammary tumours in dogs (Gultiken et al. 2015). It has also been stated that it reduces the Bcl-2 and Ki-67 expression in mammary adenocarcinomas in dogs. No study has been found on the experimental efficacy of TCAE on the development pathways of CRC. The aim of this study was to determine the effects at the molecular level on  $\beta$ -catenin, KRAS, APC, P53 of simultaneous TCAE administration as chemopreventive in AOM-induced colorectal cancer in rats.

## Materials and Methods

### Animals

The study material consisted of 18 rat intestinal (colon) paraffin blocks, which were taken from a study on experimental colon cancer using *Tarantula cubensis* alcoholic extract and *Nerium oleander* distillate (Er et al. 2019), the trial design of which is explained below. The study was approved by the Ethics Committee of the Experimental Animal Production and Research Center of the Veterinary Faculty of Selçuk University (SÜVDAMEK) (2021/20).

### Study design

The study of Er et al. (2019) included a total of 28 rats; 10 rats in each experimental group (cancer control, cancer + TCAE), and 8 rats in the healthy control group (C). In order to induce cancer in the experimental groups, azoxymethane was administered to the cancer control group (CC group, n = 10) at the beginning of the experiment at a dose of 15 mg/kg s.c. twice a week (Refaat et al. 2015). Azoxymethane was administered to the treatment group cancer + TCAE (C-TCAE, n = 10) at a dose of 15 mg/kg s.c. twice a week at the beginning of the experiment (Refaat et al. 2015), while TCAE was administered once a week from the beginning of the experiment and for 18 weeks at a dose of 0.2 ml/kg s.c. (Karabacak et al. 2015). Towards the finalisation of the experiment, ketamine (95 mg/kg s.c.) + xylazine (5 mg/kg s.c.) were used to anaesthetise the rats and then they were euthanised by the cervical dislocation method. Colon tissues removed after the necropsy were fixed in 10% formaldehyde solution for 24 h. The embedding of the tissue in paraffin was performed by the routine tissue follow-up procedure. In this study, the paraffin blocks of the intestinal samples obtained from the study whose design was described above were used (colon tissue of 18 rats in total, 6 in each group).

### Histopathological analysis

Sections of 5  $\mu$ m thickness were taken from the existing paraffin blocks and stained with haematoxylin-eosin. Preparations were examined under the light microscope (Olympus BX51, Tokyo, Japan). In the histopathological examination, both the number of crypts forming aberrant crypt foci (ACF) in the groups and the classification

of ACF were made from 10 different areas randomly selected from each preparation according to the criteria given in Table 1 (Papanikolaou et al. 2000), and their averages were calculated.

Table 1. Scoring of the presence and grading of dysplasia in the crypts.

Score	Criterion
1. Hyperplasia without dysplasia	Increased cellular number in the crypt, presenting the crypts typical nuclear morphology and goblet cell composition
2. Low-moderate dysplasia	In addition to the nuclear stratification in crypts, presence of dark elongated nuclei (elongated spindle-like appearance) and moderate goblet cell differentiation
3. High-grade dysplasia	Nuclear enlargement in the crypt epithelium and disruption of crypt morphology by nuclear pleomorphism

#### RNA isolation and cDNA synthesis

The paraffin blocks which contained the best representative preparations of the tumour were determined by histopathological examination and used in the molecular examination. The wiped and dried Leica RM2255 model microtome device was used for, each time with a clean microtome knife, taking 4–5 sections of 5 µm thickness from the blocks, that were then placed in sterile Eppendorf tubes and submitted to molecular examination. Paraffin removal from the samples was performed by going through xylene and ethanol series, respectively, according to the procedure of the relevant kit (Roche Life Science, High Pure FFPE RNA Isolation Kit Cat. No: 06650775001). Total RNA was obtained by applying the isolation kit procedure according to the manufacturer's instructions (Roche Life Science, High Pure FFPE RNA Isolation Kit Cat.No: 06650775001). The cDNA synthesis from the RNA samples was performed with the High Fidelity Transcriptor cDNA synthesis kit (Roche, Cat. No: 0508995001, Mannheim, Germany) in concordance with the recommendations of the manufacturer. The master mix was calculated based on the number of samples studied. For the cDNA master mix, protector RNase inhibitor 0.5 µl, transcriptase reaction buffer 4 µl, reverse transcriptase 1.1 µl, dNTP 2 µl, DTT 1 µl in a total of 8.6 µl were prepared. The obtained samples were stored at -20 °C until the PCR step.

#### Quantitative Real-time PCR

Real-time PCR was performed with the TaqMan probe method on a Roche Light Cycler 2.0 device. For this purpose, the Roche Light Cycler TaqMan Master (Roche, Cat No: 04735536001) was used. For the reaction mixture solution, forward primer 2 µl, master mix 4 µl, reverse primer 2 µl, probe 2 µl and dH<sub>2</sub>O 5 µl resulted in 15 µl of total volume. A separate reaction mixture was prepared for each gene investigated. In the capillary tube for each sample, 15 µl of the reaction mix and 5 µl of the cDNA master mix were placed. The β-catenin, KRAS, APC and P53 primers used are shown in Table 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. First, a single cycle pre-incubation step was performed at 95 °C for 10 min. Afterwards, in the amplification step, a quantification protocol consisting of 50 denaturation cycles of at 95 °C for 10 s, annealing at 54 °C for 30 s, and elongation for 3 s at 72 °C was applied. Finally, cooling was executed at 40 °C for 30 s in a single cycle. The expression levels of the genes investigated in the study were analysed with the Delta delta Ct ( $\Delta\Delta C_t$ ) method. The calculation was performed by the division of the threshold cycle (C<sub>t</sub>) value obtained for each primer investigated in the study by the C<sub>t</sub> value obtained for GAPDH in the same tissue.  $2^{-\Delta\Delta C_t}$  represents the fold expression change in relation to the control (Pfaffl 2001).

Table 2. Sequences of the primers used in the amplification process.

P53	Forward	5'-CCCAACGAAATTACTGCCTTTC-3'	(103 bp)
	Reverse	5'-TAGTGGCCTTGGTGCATTAG-3'	
KRAS	Forward	5'-GGACTCTGAAGATGTGCCTATG-3'	(103 bp)
	Reverse	5'-CCCATAACTCCTTGCTAACTCC-3'	
APC	Forward	5'-CACCATGTGGATCAGCCTATT-3'	(104 bp)
	Reverse	5'-CCCTGTACTGATGGAGTCTTTG-3'	
β-CATENIN	Forward	5'-CAAGCCACAGGACTACAAGAA-3'	(107 bp)
	Reverse	5'-CAATGTCCAGTCCGAGATCAG-3'	
GAPDH	Forward	5'-GGGCCAAAAGGGTCATCATC-3'	(500 bp)
	Reverse	5'-AACCTGGTCTCAGTGTAGC-3'	

APC - adenomatous polyposis coli; KRAS - Kirsten rat sarcoma virus; GAPDH - glyceraldehyde 3-phosphate dehydrogenase)

### Statistical analysis

The statistical program SPSS (Inc., Chicago, USA 25.0) was used to analyse the obtained data. The ACF scoring and crypt counts were evaluated using *t*-test, and the data obtained from the real-time PCR analysis were evaluated with ANOVA and *post hoc* Duncan test. The accepted significance limit was  $P < 0.05$ .

## Results

### Histopathological findings

Six animals from each group were evaluated in the histopathological examination. While normal intestinal tissue was seen in the C group (Plate VII, Fig. 1-A), the presence of ACF was seen in the AOM-treated groups (CC and C-TCAE) (Plate VII, Figs 1-C, 2 B-C). The number of crypts forming ACF in the C-TCAE group was found to be significantly lower (21.6%) compared to the CC group (Table 3,  $P < 0.05$ ). When the presence and grading of dysplasia in the crypts forming ACF was evaluated, it was determined that the scores in the C-TCAE group (Plate VII, Fig. 1-C) were significantly lower (23%) compared to the CC group (Plate VII, Fig. 2 B-C, Table 3,  $P < 0.01$ ).

Table 3. ACF classification (dysplasia scores of ACF-forming crypts) and crypt numbers in azoxymethane administered groups.

	CC (Mean $\pm$ SE)	C-TCAE (Mean $\pm$ SE)	<i>P</i> value
Number of crypts forming ACF	81.83 $\pm$ 4.57 <sup>a</sup>	64.16 $\pm$ 3.52 <sup>b</sup>	$P < 0.05$
Dysplasia scores of crypts forming ACF	2.15 $\pm$ 0.08 <sup>a</sup>	1.65 $\pm$ 0.12 <sup>b</sup>	$P < 0.01$

<sup>a,b</sup> Significant difference between values within the row  
ACF - aberrant crypt foci; CC - cancer control; C-TCAE - cancer + TCAE

### Real-time PCR findings

Intergroup Ct and  $2^{-\Delta\Delta Ct}$  values are shown in Table 4. When the expression of p53 mRNA was evaluated, it was found that it was lower in the CC group than in the C group, while the expression value in the C-TCAE group was, although close to the C group, non-significant (Table 4,  $P > 0.05$ ). In the evaluation of KRAS mRNA expression, it was determined that the expression level in the AOM-treated CC group according to the C group was increased, while it was determined lower in the C-TCAE group compared to the C and CC groups (Table 4,  $P < 0.01$ ). In the case of the expression of APC mRNA, it was found that the values of the CC group were quite low compared to the C group (Table 4,  $P < 0.001$ ). Compared to the CC group, a significant increase in the expression level was detected in the C-TCAE group, whose values approached those of the C group. In the evaluation of the  $\beta$ -catenin expression, the highest expression level was detected in the CC group (Table 4,  $P < 0.005$ ). Although the expression level was not significantly different in the C-TCAE group, it was found to be lower than in the C group.

## Discussion

Due to its mortality and morbidity, colorectal cancer is considered a major problem in many developed countries (Jemal et al. 2008; Mattiuzzi and Lippi 2019). On a global scale, it is the third most common cause of death among cancers, with more than 1 million new diagnoses every year and its prevalence increasing in the last 10 years; therefore, colorectal cancer should not be ignored (Cunningham 2010; Chen et al. 2016). In this study, the effects at the molecular level of simultaneous TCAE administration as chemopreventive over KRAS, P53,  $\beta$ -catenin and APC in AOM-induced colorectal cancer in rats were determined.

Table 4. Effect of TCAE on tumour growth pathways in experimental colorectal cancer (mean  $\pm$  SE).

		C	CC	C-TCAE
	Ct*	30.76 $\pm$ 1.18 <sup>b</sup>	34.30 $\pm$ 3.33 <sup>a</sup>	32.07 $\pm$ 0.94 <sup>ab</sup>
P53	2 <sup>-<math>\Delta\Delta</math>Ct</sup> ( $\times 10^{-8}$ )	3835.57 $\pm$ 2005.67 <sup>a</sup>	2139.00 $\pm$ 2547.92 <sup>a</sup>	3499.33 $\pm$ 1193.83 <sup>a</sup>
	Ct*	26.21 $\pm$ 1.79 <sup>ab</sup>	25.41 $\pm$ 1.41 <sup>b</sup>	27.40 $\pm$ 1.68 <sup>a</sup>
KRAS	2 <sup>-<math>\Delta\Delta</math>Ct</sup> ( $\times 10^{-5}$ )**	766.29 $\pm$ 826.10 <sup>ab</sup>	1213.00 $\pm$ 290.63 <sup>a</sup>	255.71 $\pm$ 230.16 <sup>b</sup>
	Ct****	27.79 $\pm$ 0.48 <sup>b</sup>	35.49 $\pm$ 2.15 <sup>a</sup>	29.78 $\pm$ 2.60 <sup>b</sup>
APC	2 <sup>-<math>\Delta\Delta</math>Ct</sup> ( $\times 10^{-5}$ )****	227.29 $\pm$ 69.77 <sup>a</sup>	2.71 $\pm$ 3.72 <sup>c</sup>	98.71 $\pm$ 82.91 <sup>b</sup>
	Ct*	28.24 $\pm$ 0.23 <sup>ab</sup>	27.08 $\pm$ 0.78 <sup>b</sup>	28.82 $\pm$ 1.75 <sup>a</sup>
$\beta$ -catenin	2 <sup>-<math>\Delta\Delta</math>Ct</sup> ( $\times 10^{-8}$ )****	111535.71 $\pm$ 60488.06 <sup>b</sup>	359514.14 $\pm$ 244362.05 <sup>a</sup>	65733.29 $\pm$ 55736.64 <sup>b</sup>

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ , \*\*\*\*  $P < 0.001$ ; <sup>a, b, c</sup> significant difference between values within the row  
 C - control; CC - cancer control; C-TCAE - cancer + TCAE; Ct - threshold cycle; APC - adenomatous polyposis coli; KRAS - Kirsten rat sarcoma virus

Aberrant crypt foci are defined as precancerous lesions that comprise the early stage of colon cancer and are considered an important biomarker of CRC (Orlando et al. 2008). Azoxymethane also induces ACF, described as preneoplastic colonic lesions in humans and animals (Takahashi and Wakabayashi 2004). In our study, in which CRC was induced by AOM, ACF formation was observed in the histopathological examination of all the individuals of the CC and C-TCAE groups (Plate VII, Figs 1-B, 2 B-C). No ACF formation was observed in the C group (Plate VII, Fig. 1-A). A significant difference was noted in the number of crypts forming ACF ( $P < 0.05$ ) and the presence and grade of dysplasia in the crypts ( $P < 0.01$ ) between the CC and C-TCAE groups (Table 3). In terms of ACF numbers and grade of dysplasia, it was determined that scores decreased by 21.6% and 23.0%, respectively, in the C-TCAE group compared to the CC group. These findings are consistent with the findings in the cancer control group in previous similar studies (Norazalina et al. 2010; Sepporta et al. 2016). In the histopathological examination, it was observed that ACFs in AOM-induced groups were more hyperchromatic and had different luminal shapes compared to normal crypt epithelium. Previous studies reported that AOM application increased ROS levels due to decreased antioxidant enzyme activity and depleting glutathione, thus inducing colon cancer with a mechanism in which the total antioxidant capacity is impaired (Al-Numair et al. 2011; Lai et al. 2013). As stated by Dik et al. (2014), it is possible to interpret that the antioxidant activity of TCAE in our study also played a role in the decrease of both the number of ACFs and the scores of dysplasia in the C-TCAE group.

The KRAS proto-oncogene, encoded by the KRAS gene, performs an essential function as a signalling molecule in normal homeostasis. In most cell types, the KRAS proto-oncogene arranges cellular proliferation; therefore, mutations in the KRAS gene cause, by disrupting its normal function, continuous signalling and proliferation (Schulz 2005). It has been stated that KRAS oncogenic activation causes activation of the signalling pathways RAF/MEK/ERK and PI3K/AKT (Kure et al. 2009). On the other hand, it has been demonstrated that oncogenic KRAS increases nuclear  $\beta$ -catenin levels and  $\beta$ -catenin-TCF4 formation by causing GSK-3 $\beta$  inhibition via the PI3K/AKT pathway (Li et al. 2005). In our study, the increase in KRAS expression levels in the AOM-induced CC group shows that the KRAS proto-oncogene is important in tumour formation in this model. Mutations in the KRAS gene have been shown at different rates in the model of AOM-induced CRC. In the study conducted by Hu et al. (2009) in rats, 33% of mutations were recorded in ACFs. In the studies in rats of Khare et al. (2003), the mutation rate was determined as 27%, and it was shown that wild-type KRAS was activated at a rate of 23%. The modulation of KRAS mutations and/or wild-type KRAS constitutive activations by chemopreventive agents may

constitute a good cancer prevention strategy. In our study, we think that the induction of CRC by AOM in rats increased the mutation in the KRAS proto-oncogene and/or wild-type KRAS gene expression. A lower Ct value (25.41) in the CC group according to the healthy control group indicates that the expression level increased, while a higher (27.40) value in the C-TCAE group compared to CC indicates a reduction of the expression level (Table 4,  $P < 0.05$ ). In this context, considering the low expression in the C-TCAE group, it can be assumed that TCAE reduces the mRNA expression levels of the KRAS proto-oncogene, which is correlated with the decrease in the number of ACFs in order to eliminate some of the dysfunctions in normal homeostasis induced by AOM.

Since the expression levels of KRAS mRNA in TCAE were similar to those of the C group in our study, it can be said that TCAE positively affects the expression level of this gene. According to the literature, AOM causes KRAS gene mutations (Khare et al. 2003; Hu et al. 2009). However, since the data obtained in the study are based on the measurement of wild-type KRAS expressions, no interpretation can be made about the mutations. In different studies, it has been reported that some chemopreventive agents may display certain effect by reducing the mutation frequency in experimental colorectal cancer models induced by carcinogens (Hu et al. 2009; Pallem et al. 2020). More molecular studies are required in the future to determine whether TCAE has any effect on the mutation frequency. The KRAS proto-oncogene is known as the epidermal growth factor (EGFR) down-effectors, and overexpression of EGFR is considered an important step in the progression of many carcinomas (Schulz 2005). In an AOM-induced experimental study by Fichera et al. (2007), it was reported that EGFR signals were increased, thereby inducing wild-type KRAS. In our study, we think that the decrease of KRAS mRNA expression levels in the C-TCAE group was due to the positive effect of TCAE on EGFR signalling.

p53 is an antioncogene and acts as a gene transcription factor, causing arrests of the cell cycle and/or induction of many genes that promote apoptosis. The p53 tumour suppressor gene function relies on a cellular pathway network that detects DNA damage, cellular stress, and inappropriate mitogenic stimulation (Erster et al. 2004; Halaby et al. 2015). In our study, although it was remarkable that the CC group had the lowest p53 mRNA expression level and the C group presented the highest p53 mRNA expression level, no significant difference was observed (Table 4,  $P > 0.05$ ). Although there was a significant difference in Ct values, we think that the lack of significant difference in  $2^{\Delta\Delta Ct}$  values is due to the number of samples. The CC findings in our study overlap with the findings of studies in which the AOM-induced CRC model was created (Hernández-Salazar et al. 2013; Song et al. 2019). The p53 also supports apoptosis through the transactivation of target genes along with transcription-independent mechanisms. The p53 presents the capacity to induce the transcription of several proapoptotic genes, including those encoding members of the Bcl-2 family such as Puma, Bax, and Noxa (Amaral et al. 2010). Previous studies have stated that apoptotic genes (especially Fas, Bid, and Bax) are up-regulated by wild-type p53 (Müller et al. 1998; Sax et al. 2002). In addition, in a different study induced by AOM/DSS, it was stated that the expression levels of p53 and Bax mRNA decreased, and Bcl-2 expression increased (Song et al. 2019). It has been reported that there is no mutation in the p53 gene in CRCs induced by AOM (Erdman et al. 1997). As mentioned above, in our CRC model created with AOM, when no mutation is present in the p53 gene; it can be thought that TCAE administration increases the level of p53 mRNA expression, resulting in the incremented expression of proapoptotic genes, decreased anti-apoptotic genes, and thus leading to the apoptosis of tumour cells.

The cytoplasmic  $\beta$ -catenin is known as the key component of the Wnt pathway and represents a major role in the proliferation control and CRC. The destructive complex consisting of APC, Axin, GSK-3 $\beta$ , CKI in the cytoplasm are responsible for the phosphorylation and proteasomal degradation of  $\beta$ -catenin during its normal homeostasis.

In the case of the inactivation of the Wnt pathway,  $\beta$ -catenin translocates to the nucleus. In the nucleus,  $\beta$ -catenin binds to the members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, acting as a co-activator of the transcription of the target gene. The hyperactivation of the regulated gene transcription of the TCF/LEF,  $\beta$ -catenin, is a hallmark of colorectal cancer development (Schulz 2005; Bian et al. 2020). In the nucleus, and after the interaction of TCF/LEF family members,  $\beta$ -catenin performs the role of a transcription trigger, increasing the expression levels of genes such as Cyclin D1 and c-Myc, thus ensuring that the pathway acquires an oncogenic feature. The pathway of Wnt is a very important signalling pathway for the initiation and progression of many tissues and/or organ cancers. In this context, it has recently become an encouraging target for cancer chemoprevention (Herbst and Kolligs 2007). In terms of the APC gene expression in our study, although the APC mRNA gene expression level was low in the CC group, the expression level was increased in the C-TCAE group (Table 4,  $P < 0.001$ ). The decrease in APC expression in the CC group is consistent with the findings of previous experimental studies (Kishimoto et al. 2000; Kishimoto et al. 2002). In the case of the  $\beta$ -catenin gene expression, it was found to be quite high in the CC group compared to the other groups, while the expression level was considerably reduced in the C-TCAE group (Table 4,  $P < 0.005$ ). It was noted that this situation was inversely proportional to APC expressions and revealed that the pathway of Wnt/ $\beta$ -catenin works for this model. In the experimental model of CRC induced by AOM, APC mutations are rare compared to  $\beta$ -catenin (Caderni 1997; Takahashi et al. 2000). Although APC mutations were rare in previous studies, at the evaluation of the findings of our study, it is seen that the changes in the APC gene are one step ahead and this is achieved by the increase in the expression of non-mutant wild-type APC. He et al. (1998) reported that wild-type APC restricts the formation of complexes between  $\beta$ -catenin and TCF, thus preventing c-myc from becoming oncogenic. In a study by Kishimoto et al. (2002), it was stated that there is a relationship between a higher APC and decreased c-myc expression levels. In the light of the literature, it is possible to interpret that the low levels of APC expressions in the CC group in our study had an effect on tumour development and that the TCAE in the C-TCAE group prevented Cyclin D1 and c-Myc from becoming oncogenic by modulating  $\beta$ -catenin as a result of increasing the APC mRNA gene expression level. In addition, decreased APC mRNA expression in the colon in the CC group may be related to the increased ACF rather than to a mutation. In this context, the decrease in histopathological scores in the C-TCAE group related to ACF and the level of mRNA expression in our study show parallelism. In addition, the reduced  $\beta$ -catenin expression levels and thus prevention of tumour progress and proliferation may be attributed to the combined effect of decreasing mRNA expression of KRAS proto-oncogene and increasing APC mRNA expression levels.

As a result, the effects of TCAE in CRC encouraged by AOM in rats in this study were molecularly evaluated, and it is possible to say that it prevented tumour progress and proliferation by modulating some changes in CRC developmental pathways; thus, it can be considered an effective chemopreventive agent. In order to assess the efficacy of a chemotherapeutic agent in experimental cancer studies and chemotherapy trials, it may be more useful to evaluate the mutagenic and non-mutagenic types of genes in the pathways together instead of the natural genes in normal homeostasis in the pathways as was done in this study. In addition, it has been revealed that there are significant changes in KRAS, APC and  $\beta$ -catenin pathways in experimental CRC models, and changes in these pathways can be used as evaluation criteria in antitumoral treatments. Adding to the findings reported by Er et al. (2019) that TCAE increases the antioxidant capacity of the body and activates the tumour necrosis factor-mediated apoptosis pathway, with regard to the increased tumour suppressor gene expressions in our study, we can conclude that TCAE causes apoptosis in tumour cells by activating non-mutagenic tumour suppressor genes and pathways, thus showing its antitumoral activity.

### Conflict of Interest

The authors declare that they have no conflict of interest.

### Acknowledgements

This research was supported by Selçuk University Scientific Research Project Coordinator with project number 19202084 within the scope of the thesis project. This article was produced from the thesis subject of the first author. The author completed his PhD as a fellow of YÖK 100/2000 (Molecular Pathology) and TUBITAK 2211-A, and would like to thank both institutions for their support during his PhD education.

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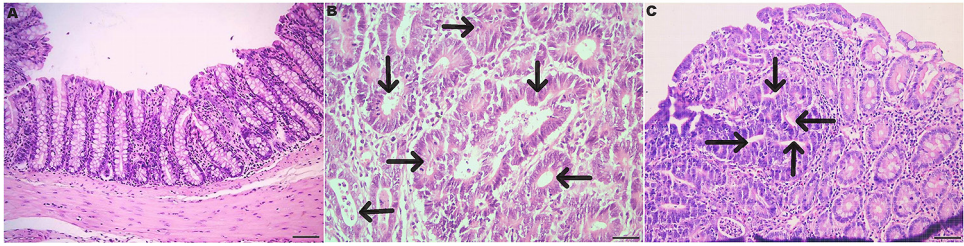


Fig. 1. A. Normal bowel section, C group, HE, bar 100  $\mu$ m. B. Dysplastic crypts with prominent atypical cell features (arrows), CC group, HE, bar 50  $\mu$ m. C. ACF formed by crypts with high-grade dysplasia (score 3), C-TCAE group, HE, bar 100  $\mu$ m

C - control; CC - cancer control; C-TCAE - cancer + TCAE; HE - haematoxylin-eosin

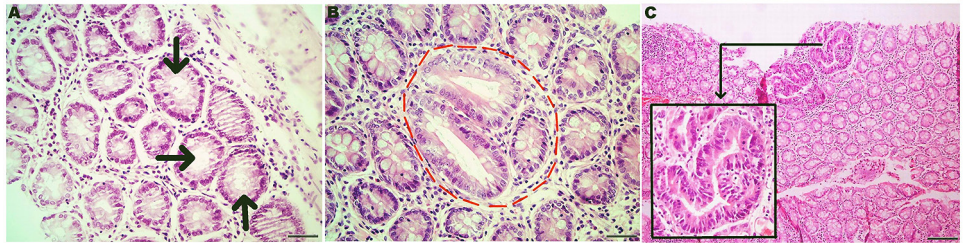


Fig. 2. A. Hyperplasia without dysplasia in crypts (arrows), CC group, HE, bar 50  $\mu$ m. B. ACF with low-to-moderate dysplasia in the crypts (red lines), CC group, HE, bar 50  $\mu$ m. C. ACF with crypts presenting high-grade dysplasia, CC group, HE, bar 200  $\mu$ m. Thumbnail as a close-up view of the dysplastic crypt (score 3)

C - control; CC - cancer control; C-TCAE - cancer + TCAE; HE - haematoxylin-eosin