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Effect of *Tarantula cubensis* alcohol extract and Capecitabin combine in Colorectal Cancer rats

Efecto de la combinación de extracto de alcohol de *Tarántula cubensis* y Capecitabina en ratas con Cáncer Colorrectal

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ABSTRACT

Colon cancer (CRC) is one of the most common types of cancer in the world. In this study, the effects of *Tarantula cubensis* alcoholic extract (TCAE) and the Capecitabine in CRC were investigated. Wistar albino rats were divided into eight groups with 12 animals in each group: untreated healthy and CRC groups, healthy and CRC groups treated with TCAE or Capecitabine, and healthy and CRC groups treated with both TCAE and Capecitabine. Azoxymethane was used in all CRC groups. TCAE and Capecitabine were administered to the relevant groups starting in the 15th week. All rats were euthanized after 18 weeks, and tissue samples were collected. The mRNA levels of Bcl-2, Bax, and Cas-3 in the harvested tissues were determined using real-time PCR and histopathologically abnormal crypt foci (ACF) scores were determined. It was found that TCAE modulated the decreased Bax/Bcl-2 expression rate in the CC group, but had the opposite effect in healthy animals, which was significantly reduced compared to the healthy groups (P<0.05). In addition, this rate was significantly lower in Capecitabine administered groups compared to other groups, and a paradoxical effect was observed (P<0.05). No significant change was observed in Cas-3 expression levels in all groups (P>0.05). Importantly, single and combined use of TCAE and Capecitabine in rats with CRC significantly reduced ACF scores (P<0.05). It can be stated that TCAE can specifically modulate the decreased Bax/Bcl-2 ratio in animals with cancer, and the therapeutic efficacy of Capecitabine is achieved at a dose of 40 mg·kg^{-1}.

Key words: Azoxymethane; Capecitabine; colorectal cancer; TCAE

RESUMEN

El cáncer de colon (CRC) es uno de los tipos de cáncer más comunes en el mundo. En este estudio, se investigaron los efectos del extracto alcohólico de *Tarantula cubensis* (TCAE) y la capecitabina en CRC. Las ratas albinas Wistar se dividieron en ocho grupos con 12 animales en cada grupo: grupos sanos y CRC sin tratar, grupos sanos y CRC tratados con TCAE o capecitabina, y grupos sanos y CRC tratados con TCAE y capecitabina. Se usó azoximetano en todos los grupos de CRC. Se administraron TCAE y capecitabina a los grupos pertinentes a partir de la semana 15. Todas las ratas fueron sacrificadas después de 18 semanas y se recogieron muestras de tejido. Los niveles de ARNm de Bcl-2, Bax y Cas-3 en los tejidos recolectados se determinaron mediante PCR en tiempo real y se determinaron las puntuaciones de focos de cripta histopatológicamente anormales (ACF). Se encontró que TCAE moduló la disminución de la tasa de expresión de Bax/Bcl-2 en el grupo CC, pero tuvo el efecto contrario en animales sanos, que se redujo significativamente en comparación con los grupos sanos (P<0,05). Además, esta tasa fue significativamente menor en los grupos a los que se administró capecitabina en comparación con otros grupos, y se observó un efecto paradójico (P<0,05). No se observaron cambios significativos en los niveles de expresión de Cas-3 en todos los grupos (P>0,05). Es importante destacar que el uso único y combinado de TCAE y capecitabina en ratas con CCR redujo significativamente las puntuaciones de ACF (P<0,05). Se puede afirmar que TCAE puede modular específicamente la disminución de la relación Bax/Bcl-2 en animales con cáncer, y la eficacia terapéutica de capecitabina se logra a una dosis de 40 mg·kg^{-1}.

Palabras clave: Azoximetano; Capecitabina; cáncer colorectal; TCAE

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INTRODUCTION

The incidence of colorectal cancer (CRC) is the third-highest of all cancers [1]. CRC is also found in Veterinary Medicine [2]. Unlike other cancer types, mutations in tumor suppressor genes (TSG) have been reported in CRC [3]. Moreover, CRC arises due to defects in oncogenes, TSG, and genes related to deoxyribose nucleic acid (DNA) repair mechanisms [4]. Capecitabine is a new oral adjuvant and palliative Fluoropyrimidine prodrug approved by The United States Food and Drug Agency (FDA) in 1998 that inhibits Thymidylate synthase [5] and is used to treat various cancer types. It has been reported that Capecitabine is converted to 5-Fluorouracil (5-FU) when prepared in oral formulation [6] with a recommended dose of 2,500 $mg{\cdot}m^{-2}$ administered for 14 out of every 21 days [7]. Capecitabine is converted to the active form after a three-step enzymatic activation process. The enzyme Cytidine deaminase, which plays a role in the catabolism of the drug in rats (*Rattus norvegicus*), is at a lower level than in monkeys (*Cnomolgus monkeys*) and humans [8]. In addition, the enzyme activity and plasma concentrations of its metabolites have been reported to decrease with repeated applications of Capecitabine [9]. Fluorodeoxyuridine monophosphate, which is formed by the enzymatic conversion of 5-FU by Thymidine kinase, is reported to inhibit Thymidylate synthetase, which is the rate-limiting step in Thymidine synthesis. In addition, without Thymidine, desoxi rribonuclec acid (DNA) synthesis is impaired, and cellular death occurs. 5-FU is also converted to Fluorouridine Triphosphate (FUTP), the antimetabolite of 5-FU, by Thymidine Phosphorylase (dThdPase), and FUTP binds to RNA and instructs the cell to undergo apoptosis [10]. Interestingly the dThdPase enzyme is found at higher levels in cancerous tissues than in normal tissues, so Capecitabine has a more effective and safer profile than 5-FU [11].

Tarantula cubensis alcoholic extract (TCAE) is a homeopathic product used in Veterinary Medicine, where it is generally used to treat conditions such as gangrene, septicemia, and toxemia [12]. Moreover, it is reported to increase apoptosis in cancer cells *in vitro* via the caspase-3 (Cas-3) pathway [13] and to cause clinically positive effects in caninen (*Canis lupus familiaris*) mammary tumors [14] via apoptotic pathways [15]. It has been successfully used to treat canine oral papilloma [16] and reported to reduce Bcl-2 and Ki-67 gene expression in canine mammary adenocarcinoma [17], as well as reduce aberrant crypt foci (ACF) and polyp formation in colon cancer [18].

In the last stage of apoptosis, caspases, which degrade vital intracellular proteins, are activated. Bcl-2 is antiapoptotic, and Bax is proapoptotic. It has been reported that caspase-9 activates effector caspases [3, 6, 7] that degrade vital cellular proteins and provide cellular destruction [19]. ACF is observed by staining the colon tissue of CRC patients with Methylene blue. It has also been found to be important for early CRC diagnosis [20]. ACF has been identified as an important parameter in experimental CRC studies where it is indicative of colon carcinogenesis [21].

TCAE [13] and Capecitabine [6] are individually effective in some types of cancer. It has been hypothesized that the combined effects of TCAE and Capecitabine on mitochondrial dysfunction in apoptosis and ACF in rats with CRC would increase survival compared with monotherapy. In this study, we determined the effects of combined and single Capecitabine and TCAE treatment on ACF score and expression of Bcl-2, Bax, and Cas-3 in rats with CRC.

MATERIALS AND METHODS

Animal material

This study used 96 male Wistar Albino rats (12–16 weeks old, 220–250 g obtained from Selcuk University Experimental Medicine Application and Research Center, Konya, Turkey. Study protocol was approved by ethic committee (Ethic No:2019-32). The rats were randomly divided into eight groups with 12 animals in each group: Healthy control (C), CRC control (CC), healthy with TCAE (C + TCAE), CRC with TCAE (CRC + TCAE), healthy with Capecitabine (C + Capecitabine), CRC with Capecitabine (CRC + Capecitabine), healthy with TCAE and Capecitabine (C + TCAE+ Capecitabine), and CRC with TCAE and Capecitabine (CRC + TCAE + Capecitabine). Azoxymethane (AOM; 15 $mg{\cdot}kg^{-1}$, intraperitoneal injection (IP), administered twice in a 1-week interval; Sigma-Aldrich, Germany) was administered to all CRC groups [18, 22]. The rats in the TCAE groups were administered TCAE (Theranekron D6 inj; Richter pharma AG, Austria) via IP at a dose of 0,2 mL/rat for 4 weeks, with 3 days intervals, starting from the 15th week [18, 23]. Capecitabine (Kapeda tablet, Kocak Farma, Istanbul, Turkey) groups were orally (PO) administered Capecitabine daily for 30 days at 40 $mg{\cdot}kg^{-1}$ (SID) starting in the 15th week. In the combined treatment groups, both drugs were administered simultaneously with the dose and administration method indicated for the single treatment groups. Rats were sacrificed by cervical dislocation one hour after the last injection using Ketamine (95 $mg{\cdot}kg^{-1}$, subcutaneous -SC-) and Xylazine (5 $mg{\cdot}kg^{-1}$, SC) anesthesia. After the colon tissue was opened longitudinally and washed with physiological saline, tissue samples were taken from the proximal, median, and distal regions and immediately frozen in liquid nitrogen before being stored at -80°C (Haier, DW-86L628, China) until needed for real-time polymerase chain reaction (RT-PCR) analysis. The remaining tissue was fixed with 10% formaldehyde for pathological examination.

Molecular analysis

Tissues from six animals from each group were chosen randomly for RT-PCR analysis. Equal amounts of samples taken from the proximal, distal, and median portions of the colon were used to represent the entire colon. Tissues were isolated with an Ribonucleic acids (RNA) Isolation Kit (Biobasic; Markham, ON, Canada). The A260:A280 ratio was determined with a Total RNA 2000/2000 cycl (c) Nanodrop Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). All RNA samples were treated with DNase I (Thermo Fisher Scientific; Waltham, MA, USA) to remove DNA contamination. Complementary DNA (cDNA) was synthesized using the iScript *cDNA synthesis kit* (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommended protocol and stored at -20°C until required. The mRNA information and sequences of the primers used to amplify the target genes (*Bax*), (*Bcl-2*), and (*Cas-3*) housekeeping gene (*GAPDH*) are presented in TABLES I and II, respectively [30, 45, 46]. In addition, the mRNA and cDNA sequences of the genes were checked with The National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov), and the sequences of their primers were checked with NCBI's Primer Blast (http://www.ncbi.nlm.nih.gov/tools/) and the Oligo7 primer design program. The designed PCR primers were synthesized by Oligomer (Ankara, Turkey).

TABLE I
References of primers used in the study and NCBI Accession number (No.)

Gene names	References	NCBI Accession No.
Bax	[45]	NM_017059.2
Bcl–2	[45]	NM_016993.1
Cas–3	[30]	NM_012922.2
GAPDH	[46]	NM_017008.3

TABLE II
Gene names, symbols, sequences and product sizes (bp) of the primers

Gene names	Symbols	Sequences	bp
Bax	*Bax–F*	GAGAGGTCTTCTTCCGTGTG	133
	Bax–R	ATCAGCTCGGGCACTTTAG	
Bcl–2	*Bcl–2–F*	TGGTACCTGCAGCTTCTTTC	131
	Bcl–2–R	ATCTCCAGTATCCCACTCGTAG	
Cas–3	*Cas–3–F*	GAGACAGACAGTGGAACTGACGATG	176
	Cas–3–R	GGCGCAAAGTGACTGGATGA	
GAPDH	*GAPDH–F*	ACGGCAAGTTCAACGGCACAG	146
	GAPDH–R	GACGCCAGTAGACTCCACGACA	

Primer efficiency was calculated by RT–PCR (Bio–Rad, Hercules, CA, USA) using all cDNAs in the study for each primer

RT–PCR

It was used the iTaq universal SYBR Green kit (Bio–Rad, Hercules, CA, USA) for all RT–PCR. The thermal cyclic conditions were initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation, annealing and amplification (95 °C 15 s, Primer TM 30 s, 72 °C 30 s). The melting curve analysis was performed as follows: 95 °C for 1 min, then fluo–rescence measurements were performed at every 1°C increment between 65°C and 95°C using the CFX Connect Real–Time PCR Detection System (Bio–Rad, Hercules, CA, USA). Nuclease–free water (NFW) was used for the negative control, and optical measurements were made on both cDNA and PCR mixes for RT–PCR control purposes.

ACF analysis

Colon tissue was fixed with 10% formaldehyde and stained with Methylene blue (0.2%). The ACF score was evaluated under a light microscope (Olympus Corporation, Tokyo, Japan). The ACF score was randomly counted and scored at 50 ACFs in each rat colon using the following scheme: Score 1 = 1–3 crypts; score 2 = 4–6 crypts; score 3 = 7–9 crypts; and score 4 = ≥10 crypts [24].

Statistical analysis

Each gene's RT–PCR results were normalized using the GAPDH housekeeping gene. Their fold increase was calculated using the formula $2^{-(\Delta\Delta Ct)}$ [25]. The relationship between fold increases in gene expression was evaluated using ANOVA with Tukey's posthoc test. Crypt counts and scores in ACFs were evaluated with the Mann–Witney U test in SPSS 22 (Chicago, IL, USA). Tests with $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Animal material

During this study, one rat in the CRC+TCAE group died, leaving 11 rats in this group. No cancer or drug–related symptoms were observed during necropsy of this deceased rate.

Determination of primer specificity

A pooled sample was created by mixing 2 µL of cDNA from each sample, and serial dilutions of 1:2, 1:4, 1:8, and 1:16 ratios were prepared. RT–PCR–based primer efficiencies were determined for each primer.

Primer yields were calculated using the formula $10^{-(1/slope)}$ and TABLE III is presented.

TABLE III
Primer activities used in the study

Primer	Slope	R^2	Primer efficacy
GAPDH	-3,010	0,973	2,15
Bax	-3,24	0,9908	2,04
Bcl–2	-3,8202	0,9841	1,83
Cas3	-3,7305	0,9928	1,96

RT–PCR Results with *Bax*, *Bcl–2*, and *Cas–3*

The fold increase results and *Bax*/*Bcl–2* ratios determined with RT–PCR are shown in FIG. 1.

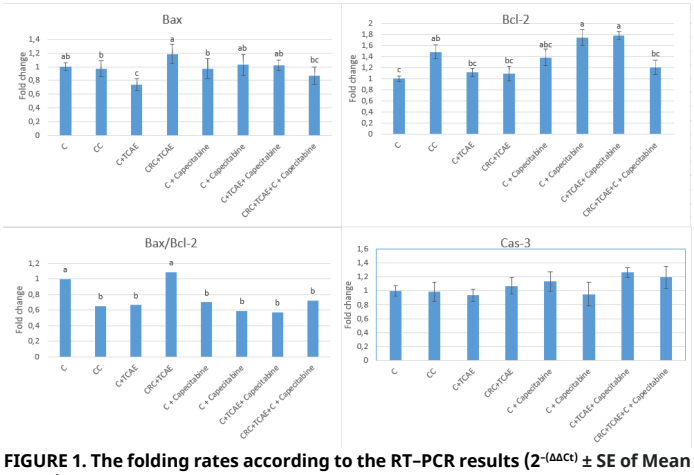

FIGURE 1. The folding rates according to the RT–PCR results ($2^{-(\Delta\Delta Ct)}$ ± SE of Mean of log). C: Healthy control, CC: Cancer control, C + TCAE: Healthy TCAE, CRC + TCAE: Cancer TCAE, C + Capecitabine: Healthy Capecitabine, CRC + Capecitabine: Cancer Capecitabine, C + Capecitabine + TCAE: Healthy combined, CRC + TCAE + Capecitabine: Cancer combined. a,b,c: Different letters in the same column are statistically significant ($P<0.05$)

ACF and Macroscopic Findings

Polyps visible to the naked eye were detected in some animals in the cancer groups in the study (FIG. 2).

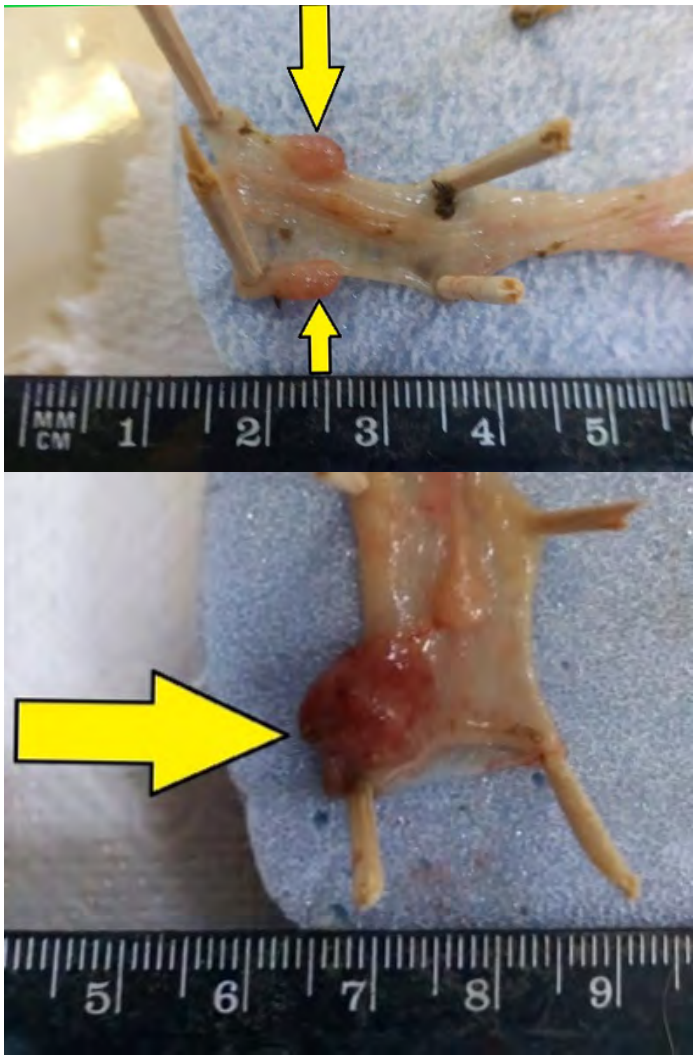

FIGURE 2. Cancer polyps observed in different groups (yellow arrow; cancer polyp)

While ACF was not found in healthy rats, it was found in all groups that were administered AOM. ACF scores and the number of crypts used to score it (FIG. 3) are shown in TABLE IV.

CRC is one of the most common types of cancer and is known to cause mortality [1]. In addition to general treatment, research on alternative treatment options continues [18]. There is limited information about the use of Capecitabine to treat rats with CRC. The dose level used in this study is the predictive treatment dose. It was determined that Capecitabine treatment decreased the ACF score in rats with CRC. Moreover, a decrease in the ACF score was also found with combined TCAE and Capecitabine treatment. However, decreases in the single- and combined-treatment groups were not significantly different ($P>0.05$; TABLE IV).

TABLE IV
ACF numbers between groups

Group	CC	CRC + TCAE	CRC + Capecitabine	CRC + TCAE + Capecitabine
Score median	2[a]	2[b]	2[b]	2[b]
(Mean ± SE)	(2.31 ± 0.05)	(1.79 ± 0.05)	(1.71 ± 0.04)	(1.72 ± 0.04)
Median	5[a]	4[b]	4[b]	4[b]
number of crypts (mean ± SE)	(5.85 ± 0.15)	(4.54 ± 0.15)	(4.30 ± 0.15)	(4.19 ± 0.15)

CC: CANCER control, CRC + TCAE: Cancer TCAE, CRC + Capecitabine: Cancer Capecitabine, CRC + TCAE + Capecitabine: Cancer combined. a,b: Different letters on the same line are statistically significant ($P<0.05$)

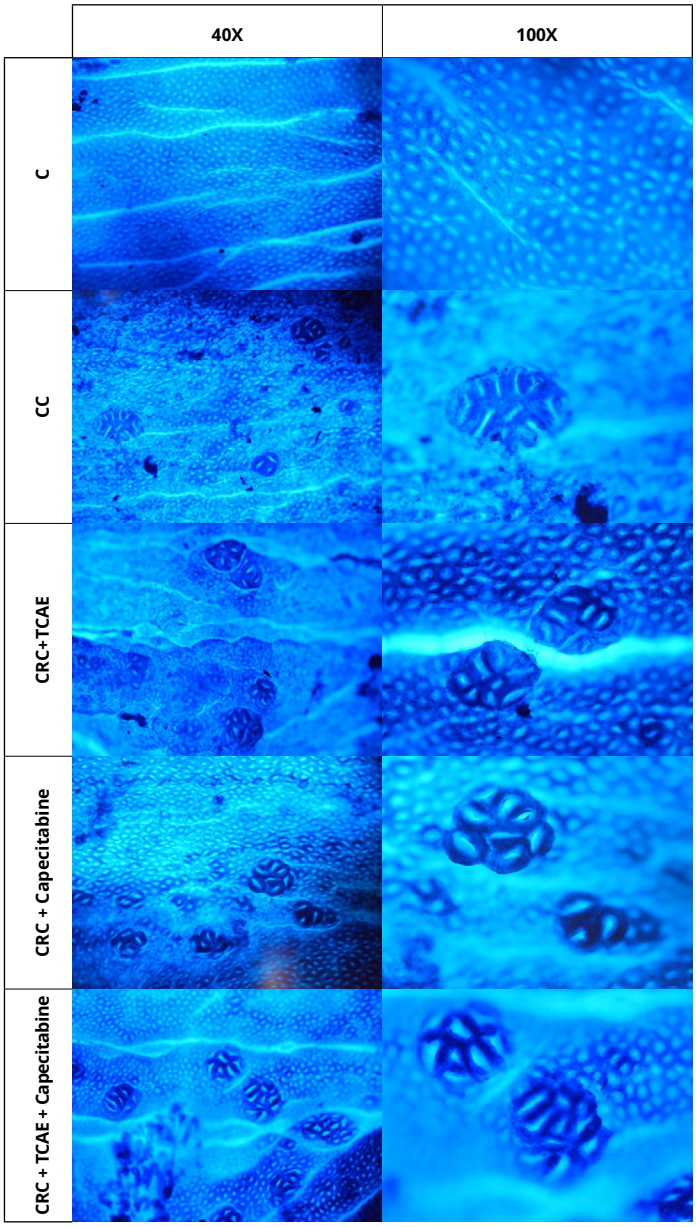

FIGURE 3. ACF and crypt appearance in methylene blue stained colon tissue. C: Healthy control, CC: Cancer control, C + TCAE: Healthy TCAE, CRC + TCAE: Cancer TCAE, C + Capecitabine: Healthy Capecitabine, CRC + Capecitabine: Cancer Capecitabine, C + Capecitabine + TCAE: Healthy combined, CRC + TCAE + Capecitabine: Cancer combined

In addition, there was no significant difference in *Cas–3* expression between groups. However, as a paradoxical or feedback effect, it was observed that the *Bax*/*Bcl–2* expression ratio was significantly lower in the CRC group compared with the control group, which was primarily due to increases in *Bcl–2* expression (P<0.05; FIG. 1). In this study, Capecitabine was administered at a dose of 40 mg·kg^{-1} by gavage once a day for 30 days. However, the antitumor efficacy of Capecitabine may be dose–dependent, and a dose of 500 µmol·kg^{-1} is required to prevent tumor growth [26].

In addition, intraperitoneal Capecitabine administration at a dose of 10 mg·kg^{-1} for two weeks was reported to cause greater weight gains in rats with CRC than those in the control group and to decrease the numbers of ACFs and crypts in ACFs [27]. ACF is a diagnostic marker in CRC, and the findings are similar in humans and experimental cancer models [21]. In rats with CRC, 5–FU has been reported to reduce ACF numbers and colonic mucosa thickness in monotherapy. In addition, it has been reported that when combined with vitamin D3 has a synergistic effect, with the number of ACFs reduced to a greater extent than with 5–FU alone [28].

Administration of metformin or 5–FU in CRC mice has been reported to reduce the number of ACFs [29]. *Bax* is proapoptotic, and *Bcl–2* is antiapoptotic. In addition, *Bax* and *Bcl–2* are active in Cas–3–mediated apoptosis due to mitochondrial dysfunction. Cas–3 is activated by Cas–9 and induces cellular apoptosis [30]. The expression of these genes changes after the exposure of C6 cell cultures to Ru2Cl(Ibp)4, where *Bax* expression increases at the 6th and 24th h but remains similar to its level at the 0th and 72 nd h. The expression of *Bcl–2* is also significantly higher in the 24th h before decreasing to levels similar to the control group [31].

However, *Bax* expression does not change in cancer cells 24 hours after 5–FU administration, but *Bcl–2* expression decreases [32]. *Bax* expression does not change in cancer cells when 5–FU, N–acetylcysteine, and vitamin E are given individually, but it does increase when they are given together [33]. However, while *Bax* protein levels increase over time in the presence of andrographolide, its mRNA levels do not change [34]. In CRC cell lines, the micro–RNA miR–206 is directly associated with Bcl–2 and plays a crucial role in the development of 5–FU resistance [35]. The Cas–3 protein ratio does not change when 5–FU is administered to 5–FU–resistant cells [36]. However, in future studies, it will be important to jointly determine changes in *Cas–3*, *Bax*, and *Bcl–2* mRNA and protein levels at different times in order to evaluate the efficacy of the drugs used. While the prognostic potential of Capecitabine is better than the ACF score, a paradoxical or feedback effect exists at the mRNA level. However, the fact that *Cas–3* mRNA levels did not change in this study may indicate that the paradoxical or feedback effect observed in Capecitabine administered groups was limited to the *Bax* and *Bcl–2* genes.

In this study, a non–statistical synergistic effect was observed with Capecitabine, where TCAE reduced the ACF score in rats with CRC (P<0.05; TABLE IV). In addition, it was determined that TCAE decreased the *Bax*/*Bcl–2* ratio in healthy rats and increased it in rats with CRC. It was determined that the increase in this ratio was due to increased *Bax* expression in rats with CRC. In addition, it was observed that when used in combination with Capecitabine, it increased the Bcl–2 gene in healthy animals. (P<0.05; FIG. 1). TCAE is reported to disrupt cellular adhesion, increase cell death rate depending on the concentration, be more cytotoxic in cancer cells, and trigger apoptosis through cas–3 [13]. It has been shown that the combination of these drugs is effective in slowing down the growth of this type of tumor, however, more studies are required to determine which combination of doses is the most effective and which is the mechanism by which these effects occur. Er *et al*. [37] reported that TCAE causes fluctuations in IL–6 and IL–10 levels and an increase in TNF–α and TGFß levels in cancerous cells. In addition, TCAE reportedly inhibits the proliferation of cancer cells depending on concentration and time. However, cell death due to TCAE is not specific to cancer cells, as it was also observed in healthy cells. It has been suggested that cell death may be related to the Ethanol used in the extraction process [38]. It was found in the study that TCAE inhibited tumor development and proliferation and stimulated non–mutagenic tumor suppressor genes [39].

In addition, *tarantula*–logoplex is reported to be less cytotoxic in healthy cells than in cancerous [40]. Er *et al*. [18] found that TCAE administration in rats with CRC decreased the number of ACFs and increased levels of PGE–2, IL–2, and IL–10. TCAE is reported to suppress increases in midkine, TGF–ß, VEGF, AFP, COX–2, IGF, and Cas–3 levels in the colon [41]. It has been reported that Flavanol can change *Bax* and *Bcl–2* expression levels at different rates in different cell lines [42]. It has also been reported that *Bax* expression is increased in the indigestible part of the bean compared to the cancer group, but *Bcl–2* expression is decreased in rats with cancer in AOM [43]. The application of different types of bacteria to rats with cancer was found to reduce levels of *cas–3* mRNA but prevent cancer development via a different mechanism [44]. It can be concluded that TCAE can act specifically on cancer cells and trigger apoptosis through Bax in rats with cancer, slowing down cancer development through increased *Bax* expression, which is important for cancer development. While TCAE and Capecitabine have similar treatment efficacy in their single and combined uses, a non–statistical synergistic effect is observed in the combined use.

CONCLUSION

In this study, İt was aimed to determine the efficacy and type of effect of Capecitabine and TCAE in rats with CRC induced by AOM. At the molecular level, TCAE activity differed in healthy and CRC rats. However, it can be stated that TCAE can slow down the development of cancer by increasing Bax expression, which generally decreases in cancer. It is remarkable that it affects apoptosis mechanisms in rats with cancer but not in healthy animals. Future studies in this area will improve understanding of its mechanism of action. Capecitabine may have a paradoxical or feedback effect when used as gavage once a day for 30 days at a dose of 40 mg·kg^{-1} in rats. While it was observed to not induce apoptosis, it did reduce ACF scores, which are a diagnostic marker for CRC. A non–statistical increase in efficacy was observed with the combined use of TCAE and Capecitabine, which might be caused by a drug–drug interaction. In addition, more *in vivo* studies are required to explore different dose concentrations for both drugs.

Conflict of interest

The authors declare that they have no conflict of interest.

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