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Research Article

Evaluation of The Effects of *Tarantula Cubensis* Alcohol Extract and Sorafenib Treatments on P21 Protein, Total Antioxidant Capacity and Metabolic Profile in Experimental Rats Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is defined as the sixth most common cancer type and the third most common cancer type in terms of cancer-related deaths. *Tarantula cubensis* alcohol extract (TCAE, Theranekron) is a homeopathic medicine frequently used in veterinary medicine in the treatment of papilloma, mammary adenocarcinoma and necrotic disorders. The present study aimed to reveal the treatment effectiveness of TCAE and Sorafenib (S) in HCC induced by Diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR)-induced HCC in rats. Rats were randomly divided into 7 groups: Control (C), Control + TCAE (CT), Control + S (CS), Cancer Control (CC), CC+TCAE (CCT), CC+S (CCS), CC+TCAE+S (CCTS). In the CC group, the values for glucose, triglyceride (TG) and total antioxidant capacity (TAC) values were found to be significantly higher than in all other groups ($p<0.001$), while the p21 levels were found to be significantly lower ($p<0.05$). It determined an increase in serum p21 levels ($p<0.05$) and a significant decrease in glucose and TG levels ($p<0.001$) in the CCT, CCS and CCTS groups compared to the CC group. Histopathological examination revealed that the CC group showed cancer morphology, and the treatment groups caused a decrease in tumor incidence and size. As a result, it can be said that TCAE can be used alone and/or combined with chemotherapy drugs to reveal antiproliferative effects on cancer cells in HCC. Sorafenib and TCAE combination therapy may potentially synergize to improve the magnitude and durability of antitumor responses in patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an important malignancy worldwide and has a high incidence of tumor recurrence and metastasis (Anwanwan et al., 2020). Moreover, HCC is defined as the sixth most common cancer type and the third most common cancer type in terms of cancer-related deaths. The prognosis for HCC is poor in HCC due to the inadequacy of effective systemic and targeted treatments. HCC develops as a result of chronic hepatitis, and etiopathogenesis includes hepatitis B and C infections, diabetes, excessive alcohol consumption, non-alcoholic liver disease and aflatoxicosis (Zhang et al., 2009; Anwanwan et al., 2020; Sagnelli et al., 2020).

Sorafenib is a multikinase inhibitor that is frequently used in HCC treatment. It has an antiproliferative, antiangiogenic and anti-immunosuppressant effects on tumor cells. It triggers antiproliferative and antiangiogenic effects by inhibiting the signaling pathways Ras/Raf/Mitogen-activated kinase (MAPK) and extracellular signal-regulated kinase (ERK) signaling pathways and VEGFR-1,2,3, PDGF-R and FGFR receptors (Huynh et al., 2011; Cervello et al., 2012; Habiba et al., 2022). Long-term treatment with sorafenib often causes decreased sensitivity of cancer cells to chemotherapy in cancer cells, leading to acquired resistance. Therefore, new therapeutic treatments of HCC with better efficacy are urgently needed (Cabral et al., 2020; Xia et al., 2020). *Tarantula cubensis* alcohol extract (TCAE, Theranekron) is a homeopathic medicine obtained from the *Tarantula cubensis* spider species. TCAE is a drug with anticancer and antiproliferative effects that has been used in veterinary medicine for the treatment of papillomas, mammary adenocarcinoma and other different carcinomas and necrotic disorders (Gültiken and Vural, 2007; Ghasemi-Dizgah et al., 2017; Gul Satar et al., 2017; Özdemir et al., 2022; Akcakavak and Ozdemır, 2023; Akcakavak et al., 2024).

p21 is a protein located downstream of p53 that inhibits each member of the cyclin/Cdk family that is required for the transition from G1 phase to S phase of the cell cycle. The p21 protein acts as a potent tumor suppressor in both normal and cancer cells (Abbas and Dutta, 2009; Shamloo and Usluer, 2019). In recent years, the p21 gene has been frequently evaluated in many types of cancer (Huang et al., 2020; Dong et al., 2021; Liu et al., 2021).

In the current study, the effects of TCAE and Sorafenib treatments on p21 protein levels were investigated and their effectiveness in HCC treatment was evaluated. Additionally, total antioxidant capacity (TAC) levels were examined to determine antioxidant capacity, glucose and triglyceride (TG) levels were examined to assess metabolic profile.

MATERIAL AND METHODS

Animal materials

A total of 58 male, 6 weeks old, weighing 140-210 g, Wistar-Albino - rats, obtained from Selçuk University Experimental Medicine Application and Research Center were used. Applications were carried out in accordance with the conditions for the care and use of laboratory animals (12 hours of light: 12 hours dark and 24±3 °C, standard commercial rat food, drinking water ad libitum). Animals were randomly divided into 7 groups. The groups used in the study and the procedures applied are shown in table 1. Diethylnitrosamine (DEN, N0258-1G-Sigma Aldrich) dissolved in dimethylsulfoxide (DMSO) was administered at 120 mg/kg/intraperitoneally(i.p.). Three days after DEN application, N-nitrosomorpholine (NMOR, N0258-1G-Sigma Aldrich) was given at a dose of 50 ppm with drinking water for 21 weeks. Due to the risks of DEN and NMOR application, sorafenib (Mybiosource-MBS655375) dissolved in 200 mg/ml DMSO was applied at 5 mg/kg/gavage to create a minimal toxic level (Sieghart et al., 2012; Yoshiji et al., 2014). TCAE (Richter Pharma) was applied at 0.3 ml/ subcutaneously (s.c). From the beginning to the end of the study, weight changes, feed and drinking water intake, drug side effects and secondary infection controls of the animals in all groups were carried out. In the study, TCAE and Sorafenib treatments started at the 22nd week and were terminated at the 25th week.

Table 1. *Experimental design*

Groups	Applications
C (n;6)	Physiological saline 0.3 ml/sc twice daily/3 days per week.
CT (n;6)	TCAE 0.3 ml/sc 2 times a day/3 days a week.
CS (n;6)	Sorafenib 5 mg/kg/orally 5 days a week
CC (n;10)	There is no treatment
CCT (n;10)	TCAE 0.3 ml/sc 2 times a day/3 days a week.
CCS (n;10)	Sorafenib 5 mg/kg/orally 5 days per week
CCTS (n;10)	TCAE 0.3 ml/sc 2 times a day/3 days a week and Sorafenib 5 mg/kg/orally 5 days a week

(C; Control, CT; Control + TCAE, CS; Control + Sorafenib, CC; Cancer Control, CCT; Cancer Control+TCAE, CCS; Cancer Control+ Sorafenib, CCTS; Cancer Control+ TCAE+ Sorafenib, TCAE; *Tarantula cubensis* alcoholic extract)

Collection of Blood and Tissue Samples

In the 25th week of the study, all rats were anesthetized with intravenous ketamine-xylazine (5-10 mg/kg) and were euthanized by cervical dislocation after their blood was collected intracardiacally. The serum obtained after centrifuging the blood taken at 3000 g +4°C for 15 minutes (Hettich Universal 320R/1406) was stored at -20°C (Kazak et al. 2024a; Kazak et al. 2024b) The livers were removed homogeneously and stored at -80°C until analysis. On the day of analysis, the sera and liver samples were allowed to thaw gradually. Liver tissue samples were homogenized in phosphate buffer solution ((1:10 w/v) adjusted to pH 7.4 in a homogenizer (Heidolph, Silent Crusher M). Afterwards, the homogenates were then centrifuged for 15 minutes at 12,000 rpm at +4°C for 15 minutes and the supernatants were placed in eppendorf tubes.

Biochemical analyzes and liver tissue analyzes

Rat-specific enzyme-linked immunosorbent assay (ELISA) kits p21 (BT-LAB, E1082Ra), TAC (Elabscience, E-BC-K136-M), glucose and TG levels were analyzed from liver tissue and serum samples by the enzyme immunoassays method. All parameters were evaluated as a result of readings made on an ELISA reader (Biotek ELx800, USA).

Histopathological examination

Liver samples of necropsied rats were fixed in 10% neutral formaldehyde solution for 24-48 hours. Afterwards, paraffin blocks were obtained through routine tissue processing procedures. 5-micrometer sections taken from paraffin blocks were stained with Hematoxylin-Eosin (H-E) and examined under a light microscopy (Olympus BX51, Tokyo, Japan)(Akcakavak et al. 2023).

Statistical Analysis

Statistics were evaluated in the SPSS 22 (Inc., Chicago, IL) package program. Comparison between groups was evaluated by one-way analysis of variance and post-hoc Duncan test. $p<0.05$ was accepted as significance value.

RESULTS AND DISCUSSION

Biochemical results

Significant changes were detected in blood biochemistry and p21 values in the CC and CC+ treatment groups (CCT, CCS, CCTS) (Table 2). A significant increase in TAC values ($p<0.001$) was detected in the CC, CCT and CCS groups compared to the C groups. Glucose and TG values were obtained at the highest level in the CC group compared to all groups ($p<0.001$). Especially in the CCTS group, the glucose, TAC and TG results were close to those of the C group values, which was considered an important improvement ($p<0.001$). The lowest p21 level was determined in the CC group compared to the CC+ treatment groups (CCT, CCS, CCTS) ($p<0.05$).

Table 2. *The values of blood glucose, TAC, TG and p21*

Parameters / Groups	Glucose (mg/dl)	TAC (nmol/L)	TG (mg/dl)	P21 (ng/ml)
C	106.40 ± 11.38^{e}	1.43 ± 0.08^{b}	104.18 ± 6.02^{d}	0.68 ± 0.15^{bc}
CT	116.64 ± 11.68^{cde}	1.46 ± 0.27^{b}	106.11 ± 13.77^{d}	0.79 ± 0.15^{bc}
CS	133.37 ± 8.93^{ab}	1.31 ± 0.46^{b}	130.50 ± 17.41^{cd}	1.03 ± 0.30^{ab}
CC	138.99 ± 8.24^{a}	2.45 ± 0.37^{a}	289.55 ± 65.87^{a}	0.53 ± 0.08^{c}
CCT	123.99 ± 4.59^{bc}	2.51 ± 0.55^{a}	195.08 ± 37.44^{b}	0.95 ± 0.40^{ab}
CCS	118.88 ± 9.92^{cd}	2.62 ± 0.40^{a}	206.67 ± 20.88^{b}	1.03 ± 0.12^{ab}
CCTS	107.53 ± 7.53^{de}	1.38 ± 0.36^{b}	153.53 ± 16.10^{c}	1.23 ± 0.33^{a}
p	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.05**

$^{a\text{-}e}$The difference between groups with different letters in the same column is important (p<0.001, p<0.05). Group averages are given as Mean+SE (n;6) (C; Control, CT; Control + TCAE, CS; Control + Sorafenib, CC; Cancer Control, CCT; Cancer Control+TCAE, CCS; Cancer Control + Sorafenib, CCTS; Cancer Control+ TCAE+ Sorafenib, TCAE; *Tarantula cubensis* alcoholic extract)

Liver tissue p21 changes

Changes in p21 levels in the liver tissue remained at a more limited level (p>0.05) and no statistical difference could be detected (Table 3).

Table 3. *Liver tissue p21 values*

Parameter / Groups	p21 (pmol/ml)
C	0.65 ± 0.11
CT	0.72 ± 0.06
CS	0.81 ± 0.13
CC	0.73 ± 0.15
CCT	0.70 ± 0.11
CCS	0.87 ± 0.13
CCTS	0.82 ± 0.08
p	**p>0.05**

Group averages are given as Group averages are given as Mean+SE (n;6) (C; Control, CT; Control + TCAE, CS; Control + Sorafenib, CC; Cancer Control, CCT; Cancer Control+TCAE, CCS; Cancer Control + Sorafenib, CCTS; Cancer Control+ TCAE+ Sorafenib, TCAE; *Tarantula cubensis* alcoholic extract)

Histopathological Results

Histopathological examination revealed that the liver samples of the healthy control groups (C, CT, CS) groups had a normal structure (Figure 1. A). In the cancer groups (CC, CCT, CCS, CCTS), atypical cell features, mitotic figures, steatosis, large clear cells and eosinophilic colored inclusions (Mallory bodies) were observed (Figure 1. B-E). Additionally, it has been observed that trabecular and pseudo-glandular structures are often together in cancerous areas. There was a decrease in the frequency and size of cancerous cells and the structures formed by them in the livers in the CC+ treatment groups (CCT, CCS, CCTS) compared to the CC group (Figure 1. C-E).

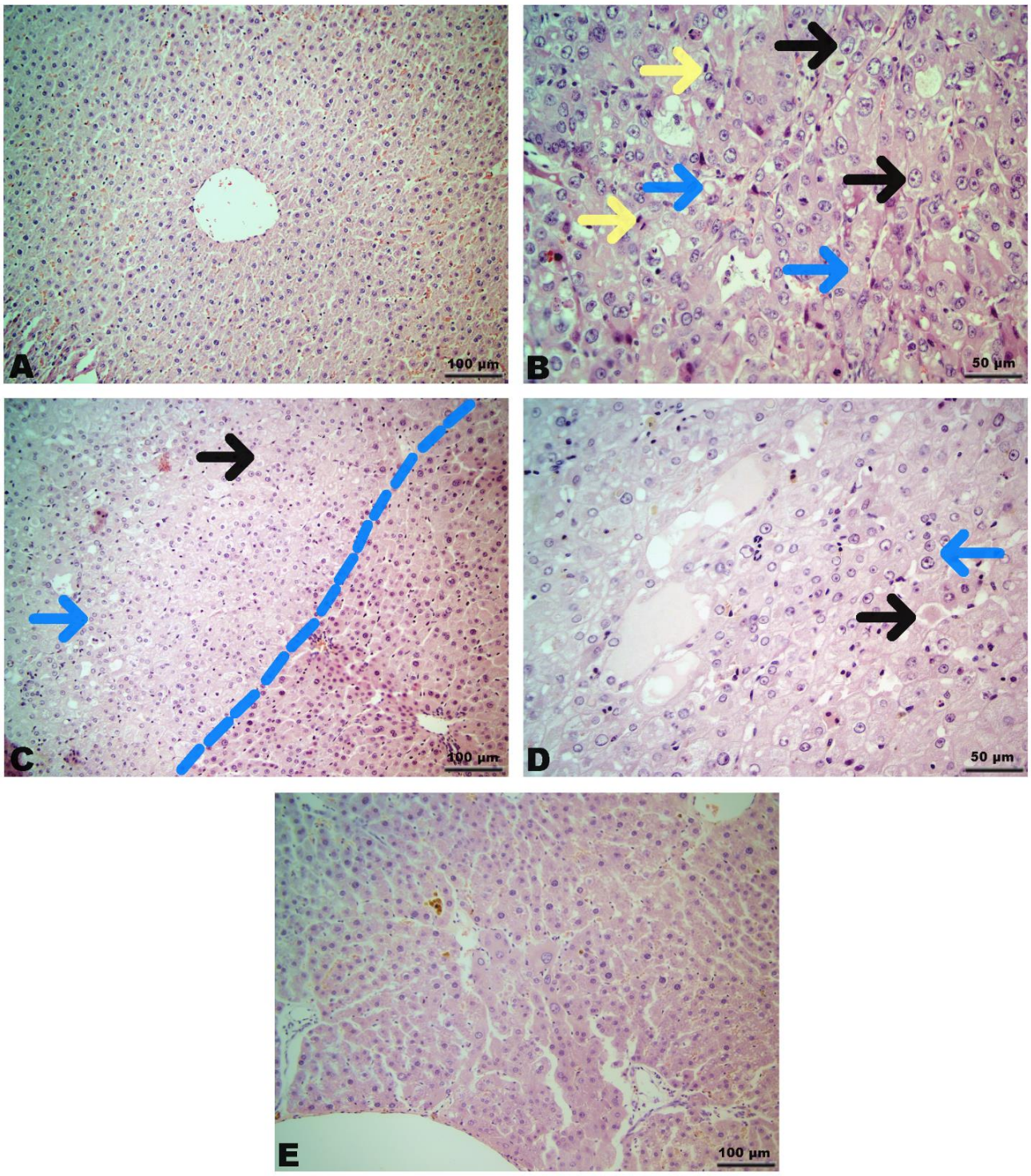

Figure 1. *Histopathological appearance of the groups, H-E, A. Normal histological appearance of livers in the C group, x20. B. Atypical cell characteristics (black arrows), steatosis (blue arrows) and mitotic figure (yellow arrows) appearance in the CC group, x40. C. Appearance of tumoral focus, clear cells (blue arrow) and eosinophilic inclusion (black arrow) in the CCT group, x20. D. Atypical cells (blue arrow) and eosinophilic inclusions (black arrow) in the CCS group, x40. E. Microscopic view of the CCTS group, x20. (C; Control, CT; Control + TCAE, CS; Control + Sorafenib, CC; Cancer Control, CCT; Cancer Control+TCAE, CCS; Cancer Control + Sorafenib, CCTS; Cancer Control+ TCAE+ Sorafenib, TCAE; Tarantula cubensis alcoholic extract)*

DISCUSSION

Supplying adequate energy is vital for cancer cell growth and proliferation of cancer cells. Increases and/or decreases in blood glucose and TG levels may occur in hepatocarcinogenesis. The most important reason for this change is that the cause involved in the etiopathogenesis of HCC stimulates different metabolic pathways. During the HCC process, disruptions occur in liver metabolism, which has an important place in the energy mechanism (Asgari et al. 2015; Boroughs and DeBerardinis, 2015). It has been stated that hyperinsulinemia and/or insulin resistance development due to abnormal glucose and lipid metabolism during the HCC process causes intrahepatic fat accumulation and triggers HCC progression (Shi et al. 2021).

Significant changes in hepatic function that occur in the development of hepatocellular carcinoma can lead to a deterioration of the metabolic lipid profile. Hepatic cellular damage causes abnormalities in serum lipid and lipoprotein levels (Uccello et al., 2011). In the study conducted by Huang et al. (2016), they found in their comprehensive analysis of HCC patients they found that hyperlipidemia increased tumor growth. In a different study, in their study of liver damage study with DEN in rats, they found that ALT, AST, cholesterol and TG levels were significantly increased compared to the healthy control group (Alsahli et al., 2021). Karabacak et al. (2015) reported that TG levels increased in the aflatoxin group in their study of liver damage with aflatoxin. In the current study, higher TG levels were obtained in the CC group compared to all groups, which is consistent with the results of previous the studies. In addition, the high TG levels in the CC group may have been caused by the deterioration of lipid metabolism as a result of severe damage to the liver and chronic inflammation. This situation is supported by the histopathological examination of the CC group, which found more severe glycogen and fat accumulation as well as enlarged hepatocytes, Mallory bodies and deterioration of hepatic lobular structures compared to all groups (Vanli et al., 2024). In a study investigating the effect of aflatoxicosis on metabolic profile in rats, it was found that TCAE treatment in the Aflatoxin+TCAE group reduced TG levels compared to the Aflatoxin group (Karabacak et al., 2015). Similarly, the TG levels obtained in the CCT group were found to be significantly lower than in the CC group. This result shows that TCAE treatment in HCC may be effective in controlling high TG levels.

It was found that low-dose Sorafenib treatment in the early-stage HCC suppressed hyperlipidemia, improved liver fatness, and significantly reduced blood TG levels (Jian et al., 2020). Similarly, it was determined that Sorafenib treatment in the CCS group was found to significantly reduced blood TG levels compared to the CCS group. The fact that the decreases were more pronounced in the CCTS group shows that the combined treatment is more effective in controlling hypertriglyceridemia. In a comprehensive study carried out by Ooi et al. (2005); They found that the plasma TG levels of HCC patients were not significantly different compared to controls and reported that it was difficult to evaluate the occurrence of HCC by measuring lipoprotein fractions alone. Therefore, detection of other lipid fractions is needed for full evaluation.

Chen et al. (2019) reported that blood glucose levels decreased over time in the DEN group compared to the control group in HCC induced by DEN.. In the present study, hyperglycemia occurred in the CC group compared to all groups. Similarly, in the comprehensive analysis examining the metabolic pathway and profile related to HCC, higher serum glucose levels were obtained in patients with advanced stage HCC compared to patients with early stage HCC (Casadei-Gardini et al., 2020). Karabacak et al. (2015) in their study on hepatic damage; They found that in the Aflatoxin+TCAE group, TCAE treatment did not reduce glucose levels and that a decrease in glucose levels occurred in the Aflatoxin group. In this study, it was determined that TCAE treatment led to significant decreases in the CCT group compared to the CC group ($p<0.001$). It has been reported that Sorafenib treatment does not cause any change in blood glucose levels in DEN-induced cirrhosis-associated HCC (Kurma et al., 2022). In the present study, it was determined that Sorafenib treatment significantly reduced blood glucose levels in the CCS group. In the current study, the highest decrease in blood glucose levels occurred in the CCTS group compared to the CC group, and the combined treatment of TCAE and Sorafenib reduced blood glucose levels more effectively in HCC. It is worth noting that additional studies are needed to reveal the relationship between the HCC process and hyperglycemia.

The most important activity for protecting tissues and organs against increasing reactive oxygen species (ROS), inhibiting ROS activity and cellular repair cells is provided by antioxidant capacity. Excessive reactive oxygen species that occur during DEN and NMOR metabolism in the liver appear as an initiating, expanding and progressive cause of HCC (Li et al., 2023). HCC patients have been observed to have higher plasma levels of oxidative stress markers and lower antioxidant capacity compared to healthy controls, hepatitis patients, or

patients with cirrhosis, and this unbalanced state has been implicated as an important cause of the development and progression of HCC (Nishimura et al., 2013; Shimomura et al., 2017). In our study, it was observed that TAC levels increased in the CC, CCT, and CCS groups and was associated with HCC. In one study, it was determined that TCAE reduced oxidant activity and increased TAC values in the polymicrobial sepsis model (Tanyeli et al., 2019). The fact that TAC values in the CCTS group were close to healthy groups was interpreted to be related to the effectiveness of combined treatment. However, in order to fully evaluate TAC levels, total oxidant capacity must be determined.

p21 acts as a potent tumor suppressor protein in both normal and cancer cells (Shamloo and Usluer, 2019). In patients with hepatocellular carcinoma, it has been reported that p21 expression is observed in 37% of HCC tissues following tumor resection and that p21 serves as an independent prognostic factor for survival (Kao et al., 2007). In cases of chronic hepatitis and cirrhosis, activation of the p21 checkpoint activation has been found to be associated with an increased risk of HCC (Plentz et al., 2007). It has been reported that p21 is upregulated by inflammation in chronic liver diseases and is associated with HCC in cirrhotic patients (Wagayama et al. 2002). Unlike these studies that associate the risk of hepatocellular carcinoma with the increase in p21 (Wagayama et al. 2002; Plentz et al., 2007), in our study the lowest p21 levels among the groups were detected in the CC group. It has been reported that TCAE treatment in rats with colorectal cancer causes inhibition of Proliferating cell nuclear antigen (PCNA) in cancer cells and cancer proliferation may be suppressed (Ozdemir et al., 2022; Akcakavak et al., 2024). It has been reported that the spider venom "Macrothele raveni" spider venom (Gao et al., 2007) and Apigenin (Şirin et al., 2020) increase p21 accumulation of p21 in HepG2 cells and show an antiproliferative effect by stopping the cell cycle in HCC cells. Consistent with these studies, blood p21 levels in the CC+treatment groups were statistically similar to the healthy control group and were interpreted as an antiproliferative effect.

The high levels of p21 in the CC+treatment groups did not inhibit apoptosis; on the contrary, the presence of a large number of apoptotic HCC cells in these groups reflects the effectiveness of the treatments applied in the groups. This is supported by Vanli et al. (2024) finding that treatments applied in the CC + treatment groups obtained higher caspase-3 and granzyme B results than the CC group. The molecular behavior of p21 in cancer cells depends on its subcellular localization and has a dual role in some types of cancer, including HCC. While nuclear p21 can be pro-apoptotic by inhibiting cell proliferation, cytoplasmic p21 can have oncogenic and anti-apoptotic functions (Ohkoshi et al., 2015). Therefore, additional research is needed to fully determine the inhibition of p21-mediated proliferation in HCC and its targets in this pathway.

The current study has some limitations. Future studies may provide a more comprehensive perspective on the effects of TCAE on HCC by evaluating the expression of different proteins that play important roles in the cell cycle.

CONCLUSION

Considering the current study results, it has been determined that TCAE may produce antiproliferative effects through the p21 protein. Considering the healing effects on the metabolic profile of using TCAE alone or in combination with Sorafenib, it can be said that it is a drug with potential to be used in the treatment of HCC. In this context, in order to fully reveal the antiproliferative effects of TCAE, there is a need to investigate proteins involved in the cell cycle and proliferation.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTION

All authors contributed equally.

ETHICAL APPROVAL

This study was approved by the Selçuk University Animal Experiments Ethics Committee's Decision dated 25.09.2020 and numbered 2020/33.

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