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Research article

Investigation of the effects of *Eremurus spectabilis* Bieb. lyophilized and nanoparticle extracts on the cellular and enzymatic immune system in experimentally-induced hepatocellular carcinogenesis in rats

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Abstract

Cancer is the leading cause of death after cardiovascular diseases. Hepatocellular carcinoma (HCC) constitutes the majority of primary malignancies of the liver. In this study, the effects of *Eremurus spectabilis* lyophilize and nanoparticle plant leaves extracts (LPLE-NPLE) were carried out on cellular and enzymatic immune system of hepatocellular carcinoma experimentally induced with diethylnitrosamine (DEN) in rats. The aims of study, it is to investigate the plant leaf extracts on T lymphocyte subsets mature T lymphocyte (CD3+), helper T lymphocyte (CD4+), suppressor-cytotoxic T lymphocyte (CD8+) and the CD4+/CD8+ as cellular immune systems. Further, it is aim to determinate activity of myeloperoxidase (MPO) and adenosine deaminase (ADA) activities in lung and spleen tissues of rats as enzymatic immune systems too. The study was conducted on six groups in each group 6 rats as normal control (NC), cancer control (CC), cancer+50 mg LPLE/kg (CLPLE1), cancer+100 mg LPLE/kg (CLPLE2), cancer+50 mg NPLE/kg (CNPLE1) and cancer+100 mg NPLE/kg (CNPLE2). To reveal the effects of the plant extracts in rats treated with two doses on cellular and enzymatic constituents of immune systems, the blood, spleen and lung samples were taken from rats at the end experiment. CD3+, CD4+, CD8+ cells and CD4+/CD8+ ratio was analysed by flow cytometry in blood samples. Furthermore, MPO and ADA enzyme activities were analyzed in supernatants of the lung and spleen tissues. According to the obtained results; $CD3^+$ and $CD8^+$ T cells of CLPLE1 bases were statistically reduced compare with NC and CC groups. Again, a significant decrease was found statistically the CNPLE2 group compared to the NC and CC groups. $CD4^+$ T cells were significantly decreased compared to NC. On the other hand, ADA enzyme, which is an enzyme of the immune system, decreased in CC compared to NC, while it increased in lung and spleen tissues in CNPLE1, CNPLE2, CLPLE1 and CLPLE2 groups. Regarding the MPO; In the groups supplemented with plant extract, MPO enzyme activity increased in both lung and spleen tissues compared to NC and CC. According to these results, the manuscript results present some new data and original theory about healing effects of *E. spectabilis* LPLE and NPLE on experimentally induced cancer complications as constituent of immune system with DEN. But it was concluded that more studies are needed to reveal the mechanism of action of *E. spectabilis* in cancer treatment and its therapeutic use.

Keywords: *Cellular and enzymatic immune system; Eremurus spectabilis; hepatocellular carcinoma*

1. Introduction

In recent years, medical and economic problems caused by the occurrence of serious side effects from synthetic drugs have led to the idea that natural is always effective and side-effect free. Add to this the threat posed by many chronic diseases for which no cure is yet possible, and these concerns have become considerable. Plant-derived compounds are very important due to their various biological effects such as anti-obesity, anti-hypertension, anti-cancer, anti-inflammatory, anti-diabetic, antimicrobial, antioxidant and anti-Alzheimer (Sharifi-Rad et al., 2018; Salehi et al., 2019; Islam et al., 2020; Gulcin, 2020).

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In addition, ecological approaches resulting from environmental pollution in industrialized countries reinforce this idea. Due to many factors, herbal treatment has become popular again. Cancer herbal treatment, also known as phytotherapy, is used as a complementary therapy alongside or after medical treatment, especially to reduce the side effects of medical treatment, correct and support the damaged immune system, increase the benefit of treatment, reduce the ability of cancer cells to metastasize, and kill cancer stem cells (Choudhari et al., 2020).

Globally, hepatocellular carcinoma (HCC) ratio is one of the most common types of cancer and is the second leading cause of cancer-related deaths. (Bray et al., 2018). Notably, 83% of liver cancer cases are concentrated in underdeveloped regions, particularly in Asia and Africa (Ferlay et al., 2015). The majority of HCC patients have a medical history involving hepatitis B virus (HBV), chronic liver disease or cirrhosis hepatitis C virus (HCV). The incidence of liver cancer varies depending on the underlying cause of cirrhosis, with hepatitis B infection being the primary cause in our country. Furthermore, cirrhosis and hepatitis C can also result from alcohol consumption (El-Serag and Rudolph, 2007).

Wild perennial herbaceous *Eremurus spectabilis* belongs to the *Eremurus* genus within the Liliaceae family. Asphodel, an extinct wild plant species, once served as both a nutritious vegetable and an ornamental soil plant in various industries. Approximately 50 species comprise this genus, predominantly found in southern and Asian regions, including the Middle East, the Caucasus, and Turkey (Tuzlaci, 1985). Notably, *E. spectabilis* and *E. cappadocus* have been documented in Anatolia (Gungor, 2002). *E. spectabilis* has recently gained popularity as an ornamental geophyte plant, particularly in mild climate zones, and is extensively used in cut-flower production practices (Schiappacasse et al., 2013).

The shoots and leaves of these plants are commonly consumed as a vegetable and are valued for their medicinal properties. In traditional medicine, various parts of the plant are utilized to address ailments such as fungal diseases, diabetes, jaundice, and liver disorders (Baytop, 1984; Tuzlaci and Dogan, 2010). Also, traditionally, the leaves of *E. spectabilis* are used for gastrointestinal disorders. In a study conducted on rats experimentally induced with indomethacin, stomach ulcers were formed, and as a result of the analyzes performed at the end of the study, it was determined that *E. spectabilis* had an antiulcer effect (Karaoglan et al., 2018). On the other hands, plants, which are also very rich in minerals, are good sources of vitamin C and antioxidants. It had been found that *E. spectabilis* is rich such as K, Ca, Na P, Mg, Fe, Zn, Cu mineral constituent and also very rich in vitamin C. (Tuzlaci, 1987).

In during years, there has been a rising interest in examining on novel hepatoprotective drugs derived from natural sources, offering promising avenues for therapeutic advancement. Among these, *E. spectabilis* stands out as a particularly promising medicinal plant due to its antioxidant properties and pharmaceutical potential. However, despite its potential benefits, there has been a notable absence of experimental studies investigating the medicinal effects of this plant on hepatocellular carcinoma and its immunological impacts in rats. The aims of study to assess the therapeutic effects of different extracts from the plant's leaves on immune cellular biomarkers including CD3+, CD4+, CD8+, and the CD4+/CD8+ ratio, using flow cytometry analysis of blood samples. Additionally, the enzymatic immune system, specifically myeloperoxidase (MPO) and adenosine deaminase (ADA) enzyme activities will be evaluated in lung and spleen tissues during the experimental induction of hepatocellular carcinogenesis in rats using diethylnitrosamine.

2. Materials and methods

2.1. Chemicals

The chemicals used in the study were supplied from Sigma Chemical Co. The kits for cellular elements of the immune system supplied from Elabscience Ltd.

2.2. Plant materials

The plant material was collected in Çınardı (Pizongan) village of Korkut district of Muş on 22.04.2021. The plant authenticity was confirmed by Prof. Dr. Fevzi ÖZGÖKÇE from the Department of Molecular Biology and Genetics at Van Yuzuncu Yil University. The herbarium number of *E. spectabilis* was determined to be 16270 and a sample of the plant was stored at the Van Yuzuncu Yil University Van Herbarium (VANF).

2.3. Preparation of plant extracts

In the preparation of alcohol extracts, the alcohol concentration used affects the biochemical profile of the extract (Vizzoto et al., 2018). They prepared *E. spectabilis* extracts with different alcohol concentrations and found that the antioxidant influence of the different extracts was higher when 80:20 (%) ethyl alcohol: water was used compared to 70% ethanol and pure ethanol. Therefore, in the study, ethanol extracts were prepared using 80% ethanol. 100 g of *E. spectabilis* leaves were blended with 500 ml of 80% ethanol solution and homogenized. The samples were extracted overnight in a magnetic stirrer at room temperature, surrounded by foil. Afterwards, the extracts were first filtered with a coarse strainer and the coarse pulp was removed. The resulting filtrate was pipetted into tubes and centrifuged at 3500 rpm for 5 minutes. Samples removed from the centrifuge were passed through coarse filter paper and collected in a screw-cap bottle protected from light. The filtrate solvents were firstly removed by the evaporation at 60 atm pressure at 37°C. Then, the pressure of the device was lowered to 18 atm and the water was removed to obtain a concentrated extract. The concentrate plant extract has divided to the tubes as 15 ml. Tubes were stored at -80°C overnight for nanoparticles and lyophilized process.

2.4. Preparation of the lyophilized plant extract

Water and ethanol extract was prepared using a revised method based on Dalar and Konczak (2013) approach. Following the extraction process, the concentrated extract sample was frozen at -50°C and subjected to lyophilization under 50 millitorrs pressure conditions for 7 days.

2.5. Characterization of plant extract and synthesis of nanoparticle

After the plant extracts were centrifuged and filtered, the density of the supernatant obtained was doubled with a rotary evaporator. 10 ml of the concentrated extract was taken and

placed in the vial. While this vial was mixed in the magnetic stirrer at 5000 rpm, ammonium persulfate $(NH4)_2S_2O_8$ as an initiator, glutaraldehyde solution $[OHC(CH_2)_3CHO]$ as a cross-linker and as an accelerator N,N,N',N'-Tetramethyl ethylenediamine $[(CH_3)_2NCH_2CH_2N(CH_3)_2]$ were added respectively. The mixture was centrifuged at 10000 rpm for 4 hours. It was accepted that nanoparticles were synthesized when there was a color change in the mixture. After centrifugation, the remaining filtrate was dried (Sahiner and Sengel, 2016).

2.6. Animals and experimental design

Wistar albino rats were employed in this research. Thirty-six Wistar albino rats, aged between 3 and 4 months and weighing an average of 181-258 g, were procured from the Van Yuzuncu Yil University Experimental Animal Research Center. The study was conducted in accordance with the approval obtained from the Van Yuzuncu Yil University Experimental Animals Unit Ethics Committee on September 29, 2022, under the reference number 2022/09-06. The rats were housed at a temperature of 22 ± 2°C with a 12-hour light/12-hour dark cycle and provided ad libitum access to food and water.

For this investigation, the 36 rats were divided into six groups, each comprising six rats, one of which served as a control. The study duration spanned 10 weeks. The grouping of the rats for the study was as follows:

2.6.1. Normal control (NC)

Rats in this group, which would not be exposed to any treatment, were fed with standard rat chow and tap water as ad libitum.

2.6.2. Cancer control (CC)

Rats in this group were received to 150 mg EN/kg body weight (bw) once a month and 200 mg Tiyoasetamid (TAA)/kg bw once a week as intraperitoneal (ip) (Hassan et al., 2017).

2.6.3. Cancer+50 mg/kg lyophilized plant extract (CLPE1)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg bw once a week as ip. Additionally, the rats received 50 mg CLPE/kg bw per day orally by gavage.

2.6.4. Cancer+100 mg/kg lyophilized plant extract (CLPE2)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg body weight once a week as ip. Additionally, the rats received 100 mg CLPE/kg bw per day orally by gavage.

2.6.5. Cancer+50 mg/kg nanoparticle plant extract (CNPE1)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg body weight once a week as intraperitoneal (ip). Additionally, the rats received 50 mg CNPE/kg bw per day orally by gavage.

2.6.6. Cancer+100 mg/kg nanoparticle plant extract (CNPE2)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg body weight once a week as intraperitoneal (ip). Additionally, the rats received 100 mg CNPE/kg bw per day orally by gavage.

2.7. Preparation of tissues supernatant

At the end of the 10 weeks, the experimental phase was finalized by sacrificing the rats and taken the tissues such as spleen, lung and blood, samples. To achieve this, the rats were anesthetized with ketamine (5 mg/100 g body weight) as intraperitoneal. Intracardiac blood samples using injectors were taken for the analysis of CD3+, CD4+, CD8+ cells, and the CD4+/CD8+ ratio via flow cytometry.

Following the dissection of spleen and lung tissues, the samples were transferred to petri dishes and washed with 0.9% NaCl solution before being preserved at -78°C until analysis. Subsequently, the tissues were homogenized for 5 minutes using a stainless-steel probe homogenizer (20 KHz frequency ultrasonic, Jencons Scientific Co.) after the addition of 50 mM ice-cold KH2PO4 (1:5 w/v) solution. The resulting mixture was then centrifuged at 10,000xg for 30 minutes at 4°C. The supernatants obtained were utilized for assessing MPO and ADA activities (Celik et al., 2009).

2.8. Biochemical analysis

ADA activity determination was made according to Bergmeyer. According to this method, ADA catalyzes the formation of deoxyinosine from adenosine. The ammonia released at this time, together with sodium hypochlorite and phenol/nitroprusside, forms dark blue indophenol in alkaline solution. The resulting dark blue indophenol was measured colorimetrically at 630 nm. (Bergmeyer, 1974).

MPO activity determination was performed according to the method described by Bradley et al. This method is based on measuring the absorbance at 460 nm of the product formed because of the reduction of H_2O_2 oxidized by MPO to O-dianisidine. (Bradley et al., 1982).

2.9. Flow cytometric examination

Blood samples for flow cytometric analysis of blood samples taken into standard blood count tubes with K3 EDTA and it was first passed through a Coulter Immunoprep Leucocyte Preparation System (Q prep) device. Monoclonal test for lymphocyte subgroups was added to 100 µl of the cell preparation passed through the Q prep device.

20 µl of antibodies (Immunotech Kid No: 8546859/ISOTON) were added and incubated for 15 minutes in the dark at room temperature. Monoclonal antibodies were then conjugated with ECD (Extracellular Domain) and PE (phycoerythrin). Among the conjugated monoclonal antibodies, CD3-ECD is specific for circulating mature T lymphocytes, and CD4-ECD/CD8-PE is specific for helper and cytotoxic T cells. Cells labeled with antibodies were analyzed using a Coulter Epics XL II flow cytometer.

2.10. Statistical analysis

The statistical analysis was conducted using the Minitab 14 software package for Windows, and all data were presented as mean ± standard deviation (SD). To assess differences between the means of the experimental groups, paired t-tests were

Table 1
Effects of the lyophilized and nanoparticle plant extracts on T Lymphocyte subsets (X̄±SD).

Groups	T Lymphocyte subsets.			
	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
NC	81.57±8.99	35.79±6.95	17.97±3.53	2.05±0.58
CC	80.29±6.53	34.59±5.69	17.34±5.62	2.21±0.88
CNPLE1	74.03±9.33	28.7±7.48[b]	13.25±3.80	2.16±0.55
CNPLE2	61.79±11.17[a,b]	27.67±6.09[a,b]	12.9±6.24[a,b]	2.58±1.23
CLPLE1	63.77±6.31[a,b]	35.19±3.40	13.34±2.64[a,b]	2.60±0.50
CLPLE2	78.03±8.57	37.31±5.60	16.76±5.82	2.50±0.93

a: There is a significant difference compared to the NC group (p≤0.05).
b: There is a significant difference compared to the CC group (p≤0.05).

employed, with a significance level set at $p \leq 0.05$.

3. Results and discussion

Flavonoids and polyphenolic molecules in plants have been studied for their various effective biological activities. However, although these plant molecules are used for different purposes, relatively little is known about their healing properties.

Table 2
Effects of the lyophilized and nanoparticle plant extracts on MPO activities of some tissues (X̄±SD).

Groups	Tissues	
	Spleen (U/g)	Lung (U/g)
NC	1443.1±237	1190.3±290.4
CC	1570.5±229.2[a]	1442.8±218
CNPLE1	1804±334	1374.1±118[a]
CNPLE2	1854.3±255[b]	1481±308[a]
CLPLE1	1872.7±137.5[b]	1763.3±153.7[b]
CLPLE2	1820±361	1348.2±237.4[a]

a: There is a significant difference compared to the NC group (p≤0.05).
b: There is a significant difference compared to the CC group (p≤0.05).

Table 3
Effects of the lyophilized and nanoparticle plant extracts on ADA activities of some tissues (X̄±SD).

Groups	Tissues	
	Spleen (U/g)	Lung (U/g)
NC	3.80±0.48	4.3±1.7
CC	1.75±0.46	1.01±0.47[a]
CNPLE1	5.80±1.60[b]	6.67±1.8[a]
CNPLE2	7.30±1.90[b]	7.0±1.8[a]
CLPLE1	3.30±0.60[a]	3.70±8.0
CLPLE2	4.05±1.01	4.04±2.0

a: There is a significant difference compared to the NC group (p≤0.05).
b: There is a significant difference compared to the CC group (p≤0.05).

This study designed to investigate the potential protective effects of *E. spectabilis* lyophilized and nanoparticle plant extracts on cellular and enzymatic immune system against in experimental Diethylnitrosamine-induced hepatocellular carcinogenesis in rats. It is very important to scientifically reveal the therapeutic properties of these plants, especially in various diseases. In recent scientific studies, it is known that *E. spectabilis* extracts have antioxidant, gastro protective, cytotoxic, antifungal and apoptotic effects. Asphodel has been found to be an important source of reducing power, free radical scavenging activity, phenolic substance content and metal chelating activity. It has been found that the abundance of vitamin C in asphodel is very important for immunity and that it quickly heals eczema, acne and urinary tract infection.

Diethylnitrosamine (DEN) is a compound used for tumor formation in experimental animals (Tolba et al., 2015). The dose applied, the age and gender of the rats are very important to cause liver cancer after DEN administration (Heindryckx et al., 2009). The reason why mice were selected at a younger age is that liver cancer occurs more rapidly because of rapid division of liver cells (Vesselinovitch and Mihailovich, 1983; Vesselinovitch et al., 1984; Hassan et al., 2017; Romualdo et al., 2017). Also, the reasons for choosing rats as experimental animals in liver cancer studies are their genetic similarities with humans, their short lifespan and reproductive characteristics (Tolba et al., 2015).

Many studies are carried out to follow the causes of cancer and the results of treatment methods. Quite long periods of time are required for experimental studies in cancer research. For example; While Karakurt (2018) was conducted for 21 weeks, Vuran (2021) was conducted for 24 weeks. Also, Moreira et al. (2015) carried out over a 10-week period. In this study, the experimental treatment period of the study lasted 10 weeks, considering the duration of studies in the literature. We think this is a reasonable period for experimental treatment.

According to the obtained results, CD3+ and CD8+ T cells of CLPLE1 bases were statistically reduced compare with NC and CC groups. In addition, a significant decrease was found in the CNPLE2 group compared to the NC and CC groups. While CD4+ T cells of CNPLE1 and CNPLE2 groups were significantly decreased compared to CC, CNPLE2 decreased compared to only NC (Table 1). Regarding with the MPO; in the groups supplemented with plant extracts, MPO enzyme activity increased in both lung and spleen tissues compared to NC and CC (Table 2). On the other hand, ADA enzyme, which is an enzymatic biomarker of the immune system, decreased in CC compared to NC, while it increased in lung and spleen tissues in CNPLE1, CNPLE2, CLPLE1 and CLPLE2 groups (Table 3).

Cytotoxic T lymphocytes consistently exhibit surface antigens and play a pivotal role in the anti-tumor immune response. Recent studies have highlighted a reduction in CD3+, CD4+, and CD8+ TIL (Tumor Infiltrating Lymphocytes) expression within HCC tumor tissues. Yarchoan et al. (2017) conducted immunohistochemistry staining on 29 HCC cases, revealing significantly lower T cell expression within tumor tissue compared to background liver tissue, with even lower expression observed on the tumor side than the non-tumor side. Moreover, the central tumor zone exhibited lower T lymphocyte expression and a smaller area of infiltration compared to the border zone (Zhao et al., 2019).

There are many studies examining ADA enzyme activity in cancerous tissues and cells. During of these studies, quite different results were obtained. Some researchers have concluded that cancerous cells increase ADA enzyme activity

(Durak et al., 1993; Koizumi et al., 1993). Some studies have suggested that ADA activity increases in direct proportion to the cancer stage. Sufrin et al. (1978) found that the increase seen in patients with bladder cancer was directly proportional to the stage of the cancer. They also observed a decrease in the ADA enzyme activity level of lung cancer patients after surgery. In another study, it was found that the ADA enzyme activities of patients given radiotherapy decreased significantly (Hiromu et al., 1970). Furthermore, the study performed by Uchida et al. (2017) where they investigated paracetamol toxicity on mice, an increase in MPO level was observed in the toxic groups. The study concluded that the increase in liver MPO is related to the damage occurring in the liver. In a toxicity experiment involving the use of fig seed as a treatment group, it was observed that MPO levels increased in the toxicity group but decreased in the group treated with fig seed oil, suggesting a potential reduction in neutrophil migration (Uchida et al., 2017).

Previous studies have proposed that the inhibitory effects on neutrophils, ADA, and MPO are associated with the suppression of cell activation. This is because MPO is abundantly expressed in neutrophils and directly influences their phagocytic activity (Nussbaum et al., 2013). Furthermore, it has been suggested that an increase in ADA may be related to immune system activation, implying a correlation between changes in immunity and ADA activity (Ozok and Celik, 2019). However, MPO is primarily linked to inflammation and neutrophils, serving as a marker of infiltration (Ozkol et al., 2012). According to these theories, during stimulation of the cellular immune system, other substances disrupting tissue and MPO are released from cells (e.g., reactive oxygen species and cytotoxic proteins) into the extracellular space.

The study results indicate an increase in ADA and MPO levels in HCC groups, consistent with findings in the literature. This may be attributed to carcinoma complications, with the elevation of liver MPO levels being closely associated with liver damage. Additionally, ADA activity is crucial in stimulating receptors that regulate extracellular adenosine concentrations, thereby modulating the inflammatory response (Antonioli et al., 2012). Furthermore, it is impossible to compare the results obtained in this study and generalize about the changes, since there is no study that shows changes in ADA enzyme activity in rats with experimental HCC in this model. However, such changes in ADA and MPO activity across cancer types may also provide good information about the course of treatments.

4. Conclusion

The observations made at the end of our research led us to summarize that the induced experimental hepatocellular carcinoma and the plant extract administration not only leads to decrease in cellular T lymphocyte subsets and causes generally an increase in the immune defense systems enzymes as MPO and ADA. Therefore, based on these results, it cannot be said with certainty that plant extracts contribute to cellular and enzymatic immune system elements in experimental HCC. Nevertheless, the results suggest that regular consumption of this functional food may prove beneficial in preventing chronic degenerative diseases. Furthermore, it was determined that further research is necessary to elucidate the mechanism by which *E. spectabilis* operates in cancer treatment and its potential therapeutic applications.

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