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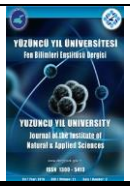

Research Article

Alteration of Apoptosis-Related Gene Expression by *Pistacia khinjuk* Stocks in Rats Exposed to Ethanol-Induced Toxicity

Neşe ERAY VURAN*

Van Yuzuncu Yil University, Science Faculty, Department of Molecular Biology and Genetics, 65080, Van, Türkiye
*Corresponding author e-mail: neseeray@yyu.edu.tr

Abstract: In this study, it was aimed to determine the effect of *Pistacia khinjuk* Stocks (Bıttım) (Pk), administered through different application methods, on apoptosis-related gene expression in rats subjected to experimental oxidative stress induced by ethanol. The study materials were collected from Siirt-Eruh. The ground samples were macerated in water, and the extracts were used in the experiments after lyophilization. Coffee was prepared from the roasted samples, then filtered and used in the experiments. Dried and powdered Bıttım was mixed into the rats' feed. The rats were divided into five groups: Group I: Normal Control (NC) (n=6), Group II: Oxidative Stress Group (EtOH) (n=6), Group III: Pk: 20% EtOH + Bıttım-based feed (400 mg/kg) (n=6), Group IV: PkE: 20% EtOH + Bıttım water extract (400 mg/kg) (n=6), Group V: PkC: 20% EtOH + Bıttım coffee (400 mg/kg) (n=6). RNA was isolated from the liver and blood, and following cDNA synthesis, gene expression was analyzed using quantitative PCR (qPCR). As a result, this study demonstrated that Pk modulates apoptosis-related gene expression in the liver and blood, in response to ethanol-induced toxicity. Ethanol exposure significantly increased apoptotic markers such as p53 and CASP-3, whereas Pk in feed and extract forms exhibited a protective effect by suppressing apoptotic gene expression, with the feed form being more effective. However, the coffee form showed limited protective capacity. Unexpected fluctuations in gene expression suggest that cellular responses to oxidative stress are complex and influenced by multiple factors.

Keywords: Apoptosis, Bıttım, Hepatotoxicity, *Pistacia khinjuk* Stocks

Etanol Toksisitesine Maruz Bırakılan Ratlarda *Pistacia khinjuk* Stocks Tarafından Apoptozla İlişkili Gen Ekspresyonunun Değişimi

Öz: Bu çalışmada, farklı uygulama yöntemleri kullanılarak verilen Bıttım (*Pistacia khinjuk* Stocks) (Pk) bitkisinin, etanol ile indüklenen deneysel oksidatif strese maruz bırakılan sıçanlarda apoptozla ilişkili gen ekspresyonu üzerindeki etkilerini belirlemek amaçlanmıştır. Çalışma materyali Siirt-Eruh'tan toplanmıştır. Öğütülmüş örnekler su ile maserasyona tabi tutulmuş ve ekstreler liyofilizasyon sonrasında deneylerde kullanılmıştır. Kavrulmuş örneklerden kahve hazırlanmış, ardından süzülüp deneylerde kullanılmıştır. Kurutulup toz haline getirilen Bıttım, sıçanların yemine karıştırılmıştır. Sıçanlar beş gruba ayrılmıştır: Grup I: Normal Kontrol (NC) (n=6), Grup II: Oksidatif Stres Grubu (EtOH) (n=6), Grup III: Pk: %20 EtOH + Bıttım içeren yem (400 mg/kg) (n=6), Grup IV: PkE: %20 EtOH + Bıttım su ekstresi (400 mg/kg) (n=6), Grup V: PkC: %20 EtOH + Bıttım kahvesi (400 mg/kg) (n=6). Karaciğer ve kandan RNA izolasyonu yapılmış ve cDNA sentezinden sonra gen ekspresyonu kantitatif PCR ile analiz edilmiştir. Sonuç olarak, bu çalışma Pk'ın etanol kaynaklı toksisiteye yanıt olarak karaciğer ve kanda apoptozla ilişkili gen ekspresyonunu modüle ettiğini göstermiştir. Etanol maruziyeti, p53 ve CASP-3 gibi apoptotik belirteçleri önemli ölçüde artırırken, Pk'ın yem ve ekstre formları apoptotik gen ekspresyonunu baskılayarak koruyucu bir etki göstermiş, yem formunun daha etkili olduğu belirlenmiştir. Ancak, kahve formunun koruyucu etkisi sınırlı kalmıştır. Gen ekspresyonundaki beklenmedik dalgalanmalar, oksidatif strese karşı hücresel yanıtların karmaşık olduğunu ve birden fazla faktörden etkilendiğini düşündürmektedir.

Anahtar Kelimeler: Apoptoz, Bıttım, Hepatotoksisite, *Pistacia khinjuk* Stocks

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1. Introduction

Alcoholic liver disease is one of the leading causes of morbidity and mortality related to liver disorders and constitutes a significant global health concern. It encompasses a range of liver pathologies, including steatosis (fatty liver), steatohepatitis (inflammation of the fatty liver), fibrosis (scarring of the liver), cirrhosis, and even hepatocellular carcinoma (liver cancer). The main pathological processes involved in ethanol-induced liver damage include oxidative stress, inflammation, apoptosis, and fibrosis (Altamirano & Bataller, 2011).

Long-term excessive alcohol consumption has been found to lead to oxidative stress. This phenomenon can result in the production of reactive oxygen species (ROS) and protein and DNA adducts, which induce inflammatory signaling pathways in the liver, leading to the expression of pro-inflammatory mediators. These mediators can promote hepatocyte apoptosis and necrosis. Apoptosis is the process of programmed cell death; it is triggered by the activation of a mechanism that includes a self-destruction program encoded in the genetic system (Palai & Mishra, 2015). The mitochondrial stress within hepatocytes caused by ROS exposure may also lead to structural and functional mitochondrial complications, increasing the frequency of apoptosis (Karkucinska-Wieckowska et al., 2022). Since apoptosis is one of the main pathological processes involved in ethanol-induced liver damage, it is important to examine apoptosis-related gene expression in rats with this condition.

The most studied genes related to apoptosis are the tumor suppressor gene p53, the anti-apoptotic gene Bcl-2, and the pro-apoptotic gene Bax The p53 gene was first recognized as a tumor suppressor in 1989 and is often referred to as the "guardian of the genome" due to its central role in the DNA damage response (Hafner et al., 2019). In response to chemical damage, p53 is activated to drive stress-specific transcriptional response programs, such as slowing cell division or inducing programmed cell death (Aly et al., 2019). While the Bcl-2 gene protects the cell against death by inhibiting the apoptosis pathway (Song et al., 2014), the Bax gene acts as a regulator of apoptosis (Li et al., 2013). The p53 gene regulates apoptosis by interacting with the Bcl-2 family and providing direct transcriptional activation of the Bax promoter, downregulating the Bcl-2 gene and upregulating Bax gene expression (Song et al., 2014). Caspase-3 is regarded as the most crucial among the executioner caspases and can be activated by any of the initiator caspases, including caspase-8, caspase-9, or caspase-10 (Palai & Mishra, 2015).

Throughout history, humans have utilized plants for various purposes. Herbal remedies are regarded as a prudent approach with minimal side effects for disease prevention. The growing popularity of herbal medicines as alternative therapies reflects rising consumer demand for safer and healthier antitoxic agents (Ghasemzadeh Rahbardar & Hosseinzadeh, 2024). *Pistacia* is a genus belonging to the Anacardiaceae family, comprising about 20 different plants (Bozorgi et al., 2013). The main species belonging to this genus, such as *Pistacia lentiscus*, *Pistacia atlantica*, *Pistacia vera*, *Pistacia terebinthus*, and *Pistacia khinjuk* (Pk), among others, are of particular importance; they have been reported for their potential benefits for both medicinal and commercial purposes (Rauf et al., 2021). *Pistacia khinjuk* (Bıttım) is an important cultural product in Türkiye, particularly in much of Southeastern Anatolia. Traditionally, it is consumed as a special type of coffee and is also used in soap production. As a frequently consumed product in Türkiye, Pk is of great importance in health research due to its bioactive compounds and traditional medicinal uses.

Studies have investigated phytopharmaceuticals affecting the expression of apoptosis-related genes in hepatotoxicity models. In a study, it was aimed to investigate the effect of catechin against D-GalN-induced hepatotoxicity. It was reported that pretreatment with catechin was effective in preventing the disruption of mitochondrial membrane potential, upregulation of p53, Bax, and downregulation of Bcl-2 mRNA levels in the liver of rats (Vasanth et al., 2010). Gedikli & Şengül (2019) investigated the protective effect of quercetin on Cyclophosphamide (CYP)-induced hepatotoxicity in rats. They determined that MDA levels were significantly higher in the CYP group compared to the control group and decreased with quercetin administration. While immunopositivity for Bax and Caspase-3 was higher in the CYP group than in the other groups, Bcl-2 immunopositivity was lower in the CYP group (Gedikli & Şengül, 2019). It was determined that MDA levels were significantly higher in the CYP group compared to the control group and decreased with quercetin administration. In addition, while immunopositivity for Bax and Caspase-3 was higher in the CYP group than in the other groups, Bcl-2 immunopositivity was lower in the CYP group than in the other

groups (Gedikli and Şengül, 2019). Data in the literature indicate that members of the *Pistachio* genus contain the chemicals catechin and quercetin (Rajput et al., 2020).

In light of the information from the literature, the aim of the present study is to investigate the effects of different preparations of *Pistacia khinjuk* on the expression of apoptosis-related genes in rats. While previous studies have reported the hepatoprotective effects of various Pistacia species, no study has specifically examined the impact of *Pistacia khinjuk* on apoptosis-related gene expression in an ethanol-induced liver damage model. Therefore, this study contributes to the literature by exploring the potential influence of *Pistacia khinjuk* on apoptotic pathways in liver tissue.

2. Material and Methods

2.1. Animals and experimental design

The study began with approval from Van Yuzuncu Yil University Experimental Animals Unit Ethics Committee dated 23.02.2023, numbered 2023/05-06. The experimental study was performed on 30 female *Wistar albino* rats. The animals were housed at 22±2˚C in daily light/dark cycle, 60% humidity, and were fed ad libitum in stainless cages. Throughout the study, rats received humane care according to criteria outlined in the ‘’Guide for the Care and Use of Laboratory Animals’ ‘prepared by the National Academy of Science and published by the National Institutes of Health.

Rats were randomly divided into 5 groups (n=6). Before dividing the Wistar rats into groups, an acute toxicity test was conducted on 6 rats, and their behavior was monitored. The test followed the Organization for Economic Co-operation and Development (OECD) guideline 425. Three groups were formed, each with 2 rats, and their weights were measured before placing them in separate cages. One group received 2000 mg/kg Bıttım water extract, another received 2000 mg/kg Bıttım coffee, and the third received pellet feed with 2000 mg/kg Bıttım. The rats were observed for 48 hours. As no deaths occurred, the toxicity test was concluded, and the study began. The groups were then formed as follows:

Group I: Normal Control (NC) (n=6): Rats were given pellet feed and drinking water ad libitum.

Group II: Ethanol Control (EtOH) (n=6): Rats were given 20% ethanol in drinking water and fed with a standard pellet diet ad libitum.

Group III: 20% EtOH + Pk (n=6): Rats were given 20% ethanol in drinking water and 400 mg/kg of Bıttım-based pellet feed.

Group IV: 20% EtOH + PkE (n=6): Rats were given 20% ethanol in drinking water, 400 mg/kg of Bıttım extract via oral gavage, and fed with a standard pellet diet ad libitum.

Group V: 20% EtOH + PkC (n=6): Rats were given 20% ethanol in drinking water, 400 mg/kg of Bıttım coffee via oral gavage, and fed with a standard pellet diet ad libitum.

The experiment was conducted for 60 days. At the end of the study, the animals were anesthetized, blood and tissue samples were taken, and the study was concluded.

2.2. Preparation of *Pistacia khinjuk* (Bıttım)

Pistacia khinjuk Stocks (Pk) was collected from Siirt, Türkiye, and identified by Assoc. Prof. Dr. Süleyman Mesut Pınar. A voucher specimen was deposited at Van Yuzuncu Yil University’s herbarium (VANF) (Herbarium number: 164257). To prepare Bıttım-containing feeds, standard rat feed and Bıttım were powdered. The mixture was moistened with a small amount of water, turned into dough, and shaped into pellets. The prepared pellets were dried in a dark, dry, and cool environment and made ready to be given to experimental animals.

To make water extracts of Pk, 200 grams of the ground plant sample were weighed, placed in a glass beaker, and extracted with 1000 ml of pure water. The beaker was covered with aluminum foil. The mixture, which was homogenized in a shaker at 25 °C for 24 hours, was then placed in a centrifuge. The homogenized mixture was centrifuged at 8000 rpm for 10 minutes. All the supernatants obtained were placed in the same container. Then, the supernatants were partially purified from their solvent at +37 °C with the help of an evaporator. The concentrated extract was stored at -80

°C overnight and then kept in a lyophilization device for 3 days. The lyophilized extracts obtained were stored at –20 °C until the analysis process began.

To prepare the coffee of Pk, the plant was roasted in a pan with continuous stirring until it slightly changed color. The roasted seeds were ground, and coffee was prepared with hot water at a concentration of 400 mg/kg. After cooling and filtering, it was made ready to be administered to animals.

2.3. Gene expression analyses

During dissection, small pieces of liver were taken and placed into RiboSaver solution. The blood was collected into a sterile tube containing heparin. Total RNA from the liver was isolated using the GeneJET RNA Purification Kit (Thermo) according to the manufacturer's instructions. Blood RNA isolation was carried out using the Hydra RNA isolation kit according to the manufacturer's instructions. The concentration of each total RNA sample was quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) at 260 and 280 nm. Nuclease-free water (DEPC) was used as a blank, and RNA concentration was calculated from the equation shown below (1):

$$\text{RNA concentration (ng/µL)} = 40 \text{ X } A_{260} \text{ X dilution factor} \quad (1)$$

cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Thermo) according to the manufacturer's instructions. The quantitative PCR (qPCR) studies in the tissues were performed in a 96-well plate in triplicate, using the Roche LightCycler® 480 real-time thermal cycler. The total reaction volume was 10 µL containing 5 µL SYBR Green, 0.1 µL forward primer, 0.1 µL reverse primer, and 4.8 µL DEPC-treated water. The sequences of the primers are shown in Table 1. The amplification conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 50 cycles of 15 s at 95 °C, 1 min at 50 °C, and 10 s at 72 °C. The expressions of p53, Bax, Bcl-2, and CASP-3 genes in each sample were normalized with the housekeeping gene (β-actin). The expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) and expressed as relative expression compared to the control group (NC).

Table 1. Primer sequences used in the experiment

Gene	Forward Primer	Reverse Primer
β-aktin (Housekeeping)	5'-TCTTCCAGCCTTCCTTCCTG-3'	5'-CACACAGAGTACTTGCGCTC-3'
P53 (Housekeeping)	5'-CTCCTCTCCCCAGCAAAAGA-3'	5'-GTAGACTGGCCCTTCTTGGT-3'
Bax (Ramezani et al., 2019)	5'-ACAGGGTTTCATCCAGGATCGAG-3'	5'-AGCTCCATGTTGTTGTCCAGTTC-3'
Bcl-2(Ramezani et al., 2019)	5'-GGATTGTGGCCTTCTTTGAGTTC-3'	5'-AGAGCGATGTTGTCCACCAG-3'
Casp3 (Yardim et al., 2020)	5'-GGAGCTTGGAACGCGAAGAA-3'	5'-ACACAAGCCCATTTCAGGGT-3'

3. Results

Firstly, RNA was isolated from liver and blood samples. After RNA isolation, purity and concentration analyses of the isolated RNAs were conducted (Table 2). The concentrations of RNAs isolated from the liver were considerably higher than those isolated from the blood.

Table 2. Purity and concentration analyses of RNAs

	The Groups	RNA concentration ng/µl		The Groups	RNA concentration ng/µl
Liver	NC	978	Blood	NC	147.62
	ETOH	907.8		ETOH	182.77
	EtOH+Pk	827.8		EtOH+Pk	126.75
	EtOH+PkE	764.63		EtOH+PkE	142,9
	EtOH+PkC	854.47		EtOH+PkC	124.96

In gene expression analysis, β-actin was used as a housekeeping gene. The C_T (cycle threshold) values obtained from the qPCR reactions for β-actin are given in Table 3. The C_T value is the minimum number of cycles at which the fluorescent signal can be detected while the PCR reaction is ongoing (Figure 1-3).

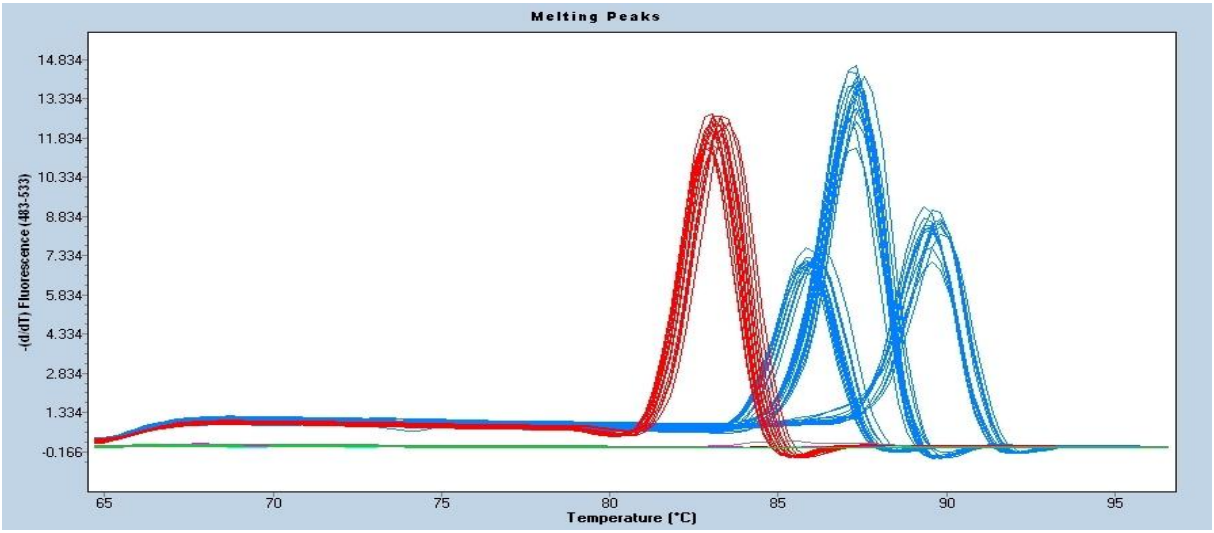

Figure 1. Melting curve analysis of primers by qPCR (1). The figure presents the melting peak analysis, where distinct peaks correspond to specific amplicons. The sharp, well-defined peaks indicate a homogeneous PCR product, while the presence of additional peaks may suggest non-specific amplification or contamination. The temperature at which the peak occurs represents the melting temperature (Tm) of the amplified product, which is influenced by the GC content and sequence composition.

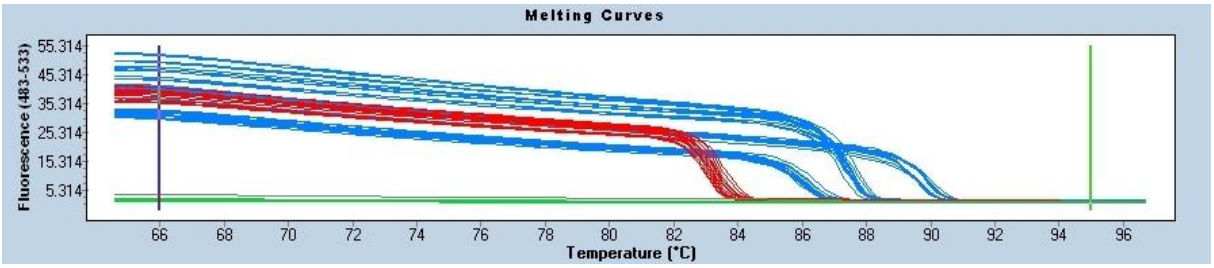

Figure 2. Melting curve analysis of primers by qPCR (2). The figure displays the full melting curve profile, illustrating the gradual denaturation of the PCR product as the temperature increases. A smooth, single transition from a high fluorescence signal to baseline confirms the presence of a single, specific amplicon. Multiple inflection points or irregularities in the curve may indicate primer-dimer formations or non-specific amplifications.

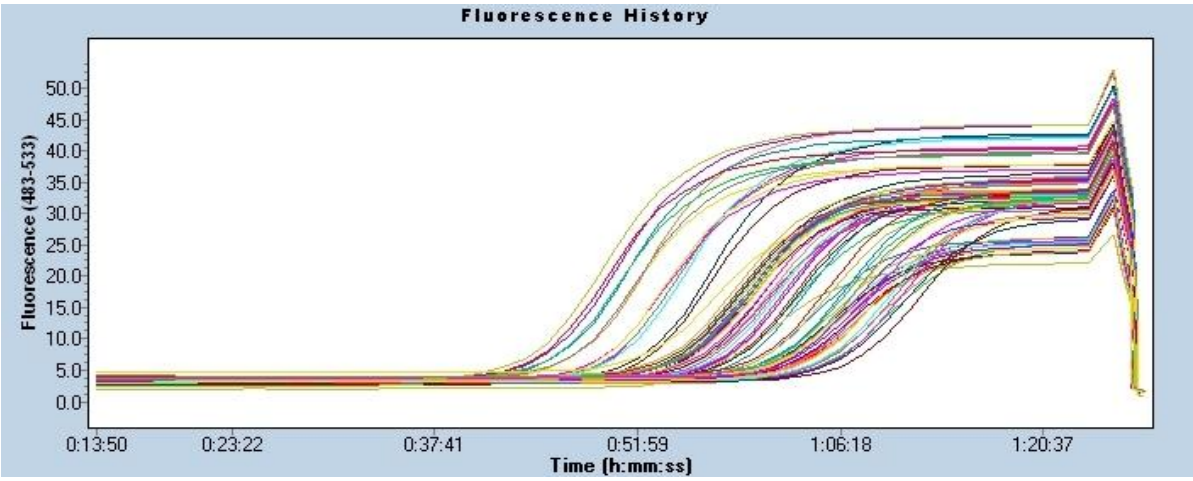

Figure 3. qPCR amplification of all samples. **Baseline Phase (Initial Time Period: 0:13:50 - ~0:50:00):** During the early cycles, fluorescence remains low and stable, indicating that the amount of target DNA is below the detection threshold. **Exponential Amplification Phase (~0:50:00 - 1:10:00)**: A sharp increase in fluorescence occurs as the target DNA undergoes exponential amplification. **Plateau Phase (~1:10:00 - 1:20:37):** The fluorescence signal levels off, indicating that the reaction has reached saturation.

Table 3. C_T Values of β-actin in liver and blood samples from qPCR results

	The Groups	β-aktin C_T±SD		The Groups	β-aktin C_T±SD
Liver	NC	22.89±0.55	Blood	NC	23.25±0.25
	ETOH	24.6±0.64		ETOH	20.44±0.59
	EtOH+Pk	21.04±0.23		EtOH+Pk	20.61±0.66
	EtOH+PkE	20.85±0.49		EtOH+PkE	20.77±0.65
	EtOH+PkC	22.58±0.71		EtOH+PkC	20.12±0.08

After gene expression analysis, $2^{\wedge-\Delta\Delta CT}$ values of p53, Bax, Bcl-2 and CASP-3 genes in the liver are shown in Table 4. As shown in Table 4, values less than 1 indicate a decrease in gene expression compared to the NC group, while values greater than 1 indicate an increase in gene expression compared to the NK group. The expressions of p53, Bax, Bcl-2 and CASP-3 genes in the liver were increased in the EtOH and PkC groups, while they decreased in the EtOH+Pk and EtOH+PkE groups (Table 4, Figure 4).

The $2^{\wedge-\Delta\Delta Ct}$ values for the p53, Bax, Bcl-2, and CASP-3 genes in the blood after gene expression analysis are shown in Table 5. The expressions of the p53 and CASP-3 genes in blood increased in all groups compared to the NC group. Expressions of Bax and Bcl-2 decreased in all groups compared to the NC group (Table 5, Figure 5).

Table 4. Apoptosis-related gene expression in liver tissue

		$2^{-\Delta\Delta Ct}$			
	The Groups	**p53**	**Bax**	**Bcl-2**	**CASP-3**
Liver	NC	1	1	1	1
	ETOH	1.58±0.72	2.32±0.69	3.70±0.97	1.87±0.91
	EtOH+Pk	0.84±0.31	0.51±0.09	0.42±0.09	0.77±0.17
	EtOH+PkE	0.63±0.44	0.47±0.2	0.42±0.13	0.99±0.4
	EtOH+PkC	1.22±0.42	1.18±0.9	1.17±0.51	1.39±0.35

Table 5. Apoptosis-related gene expression levels in blood samples

		$2^{-\Delta\Delta Ct}$			
	The Groups	**p53**	**Bax**	**Bcl-2**	**CASP-3**
Blood	NC	1	1	1	1
	ETOH	2.03±0.85	0.27±0.08	0,43±0.21	1,99±0.32
	EtOH+Pk	3.12±0.72	0.29±0.043	0,61±0.29	1,77±0.45
	EtOH+PkE	3.23±0.6	0.35±0.084	0,54±0.076	2,58±0.98
	EtOH+PkC	4.14±1.04	0.48±0.078	0,63±0.19	2,09±0.78

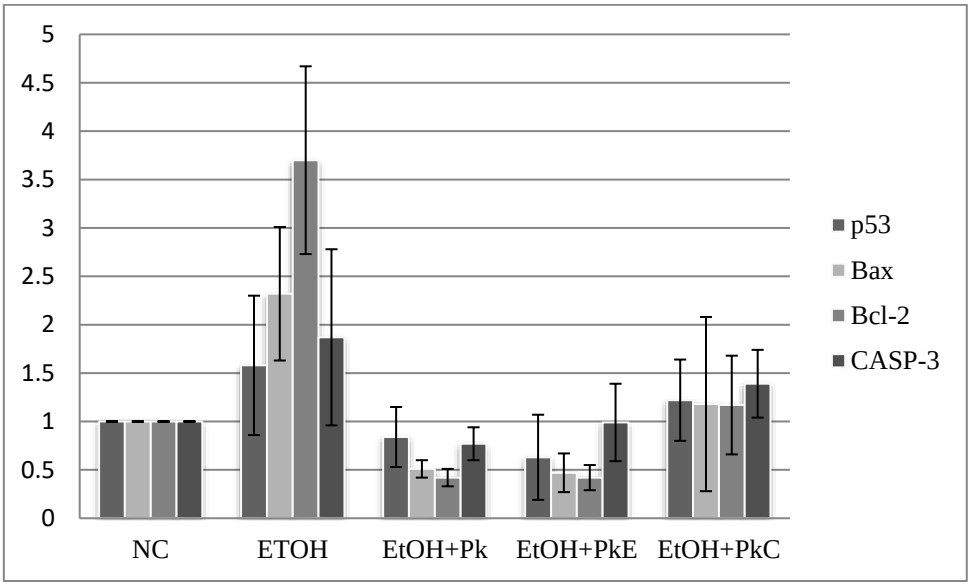

Figure 4. Expressions of apoptosis-related genes in the liver.

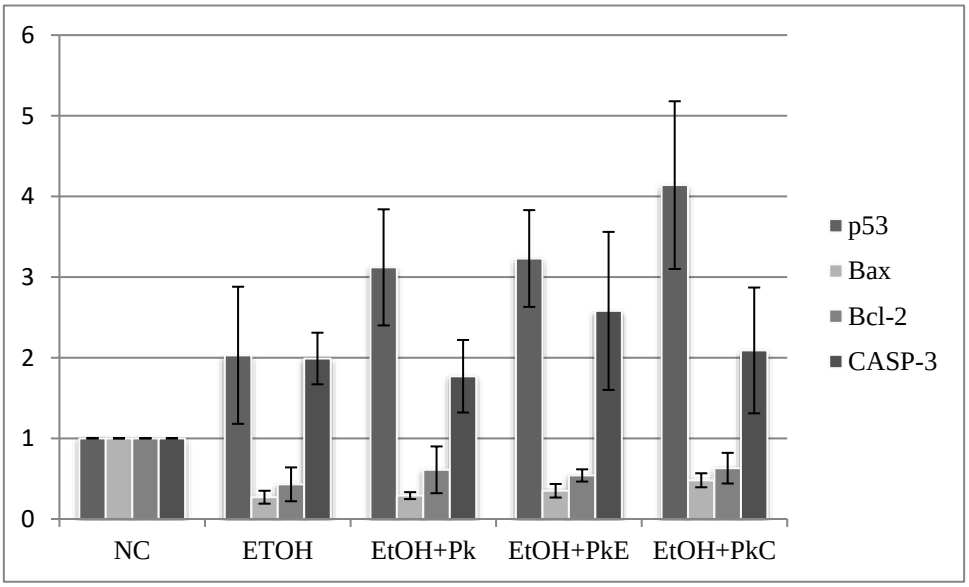

Figure 5. Expressions of apoptosis-related genes in the blood.

4. Discussion

Ethanol is a systemic toxin that can cause damage to various tissues depending on the duration and dosage of exposure. It leads to redox changes in the alcohol dehydrogenase pathway, affecting the metabolism of lipids, carbohydrates, proteins, and purines (Lieber, 2017). The liver is the primary organ responsible for metabolizing ethanol. Oxidative stress plays a significant role in the development of ethanol-related liver disease. This process involves an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates or repair the resulting damage (Liu et al., 2021). Chronic alcohol consumption leads to increased oxidative stress, which can cause inflammation, apoptosis, and fibrosis in the liver (Ramos-Tovar & Muriel, 2020). Therefore, in a study that induces liver damage through ethanol exposure, the genes whose expression should be examined are typically associated with pathophysiological processes such as oxidative stress, inflammation, apoptosis, and fibrosis resulting from ethanol. Thus, the aim of the present study was to investigate the effects of oxidative stress that may occur as a result of ethanol metabolism on the expression of apoptosis-related genes, and how these effects could change with the administration of *Pistacia khinjuk* in different forms to rats.

To assess the expression of a specific gene using reverse transcription polymerase chain reaction (RT-PCR or qPCR), the isolation of total RNA and the measurement of ribonucleic acid (RNA) concentration are required. Therefore, the isolation of total RNA represents one of the most critical steps that can significantly influence the outcomes of subsequent experiments. In the present study, RNA concentration obtained from liver tissue was found to be substantially higher compared to that from blood tissue. The samples collected from the liver during dissection were immediately placed in an RNA stabilization solution. In contrast, blood samples were collected in heparinized tubes and stored in liquid nitrogen. Since the 2'OH group in RNA can interact with phosphate groups, RNA is more susceptible to hydrolysis than DNA (Becskei & Rahaman, 2022). These results highlight the importance of using RNA stabilization solutions. There was a significant loss of RNA in the blood samples, likely exacerbated by freeze-thaw cycles, which led to further degradation of RNA. The results showed that specialized collection tubes were necessary for RNA stabilization for blood (Duale et al., 2012).

A melt curve analysis evaluates the separation of double-stranded DNA at high temperatures. When a single DNA type is generated from a particular primer pair, it produces one distinct peak. Conversely, if multiple DNA types or primer dimers are present, the analysis will show two or more peaks which suggest the use of non-specific primers (Adams, 2020). Melt curve analysis is a tool that not only confirms the specificity of the amplified product but also provides valuable information about amplification efficiency and product quality. Researchers can optimize the PCR conditions. For example, if a melt curve indicates the presence of multiple products, adjustments in primer design or annealing temperatures may be warranted (Miao et al., 2020). In the present study, a distinct peak was observed for each pair of primers, indicating that primer specificity had been achieved.

Housekeeping genes, also known as reference genes, are typically selected based on their stable expression levels across various experimental conditions and tissue types (Bustin et al., 2009). The best known housekeeping genes in the literature are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal proteins (RPL), ubiquitin (UBQ), β-tubulin, 18S ribosomal protein (18S rRNA) and phosphoglycerate kinase (PGK) (Rebouças et al., 2013). In the present study, β-actin was used as a housekeeping gene. The use of β-actin as a housekeeping gene is prevalent due to its stable expression across different cell types; however, it is crucial to validate its stability in specific experimental contexts to avoid misleading conclusions (Rebouças et al., 2013).

The C_T (cycle threshold) value is the minimum number of cycles at which the amount of fluorescent signal can be observed while the PCR reaction is ongoing. A high C_T value indicates that the number of copies is low. This is because the production of enough nucleic acid to give a signal was achieved in later cycles (Gunay et al., 2016). The implications of C_T values extend beyond mere quantification; they also influence the reproducibility and reliability of gene expression analyses. Studies have shown that factors such as primer efficiency, template quality, and the presence of inhibitors can impact C_T values, necessitating rigorous experimental design and controls to ensure accurate data interpretation (Vandesompele et al., 2002; Bustin et al., 2009). Additionally, employing

multiple housekeeping genes for normalization can enhance the robustness of results, particularly in heterogeneous tissues or under varying physiological conditions.

The method of administration of substances to laboratory animals is a critical component of experimental design, as it directly influences the pharmacokinetics of the compounds, including their absorption, distribution, metabolism, and excretion (Turner et al., 2011). Phytopharmaceuticals are naturally present in plants at specific concentrations; however, their levels tend to decrease to some extent after processing. There is limited information on how much of these diminished plant-derived compounds remain available in the body to perform their biological functions. The health benefits of phytopharmaceuticals largely depend on their bioavailability, which includes their absorption, distribution, metabolism, and excretion within the digestive and circulatory systems (Aqil et al., 2013). Based on these considerations, Pk was administered to experimental animals in three different forms: coffee, extract, and feed. In the liver, the results indicate that ethanol exposure significantly upregulated apoptosis-related genes due to oxidative stress. However, this increase was markedly suppressed in the EtOH+Pk and EtOH+PkE groups, suggesting a protective effect of Pk in feed and extract forms. In contrast, the EtOH+PkC group exhibited relatively higher apoptotic gene expression, which may be attributed to changes in bioavailability following the roasting process. These findings highlight that differences in bioavailability play a crucial role in determining the efficacy of Pk in modulating apoptosis.

In the blood, the results suggest changes in apoptotic gene regulation across the different experimental groups, particularly in response to Pk treatments. Ethanol significantly induces apoptosis in blood cells, as evidenced by increased p53 and CASP-3 expression. The feed and extract forms of Pk moderately suppress apoptosis, with the feed form showing slightly greater protective effects. The coffee form unexpectedly exhibits higher apoptotic gene expression, potentially due to alterations in its bioavailability after processing.

In the current study, the expressions of p53, Bax, Bcl-2 and CASP-3 genes in the liver increased in the EtOH and PkC groups, while they decreased in the EtOH+Pk and EtOH+PkE groups compared to the normal control (NC) group. Normally, p53 activation enhances the expression of Bax, which triggers the apoptotic process, while suppressing Bcl-2 expression (Song et al., 2014). However, in this study, all three genes were upregulated. The increased expression of p53, Bax, and Bcl-2 genes together in the experiment could be due to several possible causes. Cells under stress conditions can activate both apoptotic and anti-apoptotic mechanisms. This may reflect an attempt by the cells to survive despite the stress. The increased expression of p53 and Bax may drive apoptosis, but the upregulation of Bcl-2 suggests that the cells are attempting to counteract this process and avoid cell death. This phenomenon has been observed and documented in the literature, where Bcl-2 expression is elevated as a defense mechanism under extreme stress (Vousden & Lane, 2007). High Bcl-2 expression often correlates with an effort to inhibit apoptosis and promote cell survival. Another explanation is timing. Gene expression profiles can change over time as cells respond to damage or stress. In the early stages, p53 and Bax expression increase to initiate apoptosis, but as the process progresses, anti-apoptotic proteins like Bcl-2 may become upregulated to help cells resist apoptosis (Miyashita & Reed, 1995). Also, the liver is an organ composed of heterogeneous cell types. Different cell populations (hepatocytes, stellate cells, Kupffer cells, etc.) may respond differently to ethanol-induced stress. While some cells undergo apoptosis, others may increase Bcl-2 expression to survive (Ramos-Tovar & Muriel, 2020). These aspects could explain the simultaneous increase in all three genes observed in the study, depending on the timing of the experiment.

Expressions of p53 and CASP-3 genes in the blood increased in all groups compared to NC, while Bax and Bcl-2 expressions decreased. Increased CASP-3 expression is commonly observed alongside p53 elevation, as p53 directly or indirectly facilitates CASP-3 activation, marking the final stages of apoptosis. This relationship is well-supported in the literature, where stress-induced activation of p53 often leads to caspase-mediated cell death (Vousden & Lane, 2007). Where p53 is upregulated and apoptosis is actively occurring (as suggested by increased CASP-3 expression), it's common to observe downregulation of Bcl-2 since its anti-apoptotic role is inhibited to allow apoptosis to proceed. But Bax is usually upregulated by p53. One explanation for the downregulation of Bax where p53 is upregulated could be that apoptosis has progressed to a stage where Bax is no longer required or that other pro-apoptotic pathways are taking precedence in inducing caspase activity (Miyashita & Reed, 1995). Another explanation is that apoptosis may be proceeding through

alternative pathways that do not require Bax activation. Previous studies have shown that p53 can trigger apoptosis via caspase-dependent mechanisms without directly inducing Bax expression (Murray-Zmijewski et al., 2006; Vousden & Lane, 2007). Furthermore, the rapid clearance of apoptotic cells in circulation may contribute to the observed downregulation of Bax (Elmore, 2007).

Pistacia khinjuk contains key phytochemicals such as gallic acid, caffeic acid, ellagic acid, quercetin, and catechin (Mohammadi et al., 2019; Merlin et al., 2021). It has been demonstrated that caffeic acid, quercetin, and ellagic acid modulate apoptotic gene expression in human cervical cancer cell lines, with all three compounds increasing p53 expression. Additionally, quercetin upregulates caspase-3, caspase-9, and Bax, while both caffeic acid and quercetin downregulate Bcl-2 (Chang et al., 2010). A study has demonstrated that gallic acid significantly increases the mRNA expression of p53, Bax, and caspase-3 in the MCF-7 breast cancer cell line (Çakır et al., 2023). In the current study, the effects of Pk on gene expressions may be attributed to its bioactive compounds supporting the potential role of *P. khinjuk* as a natural modulator of apoptosis. Similarly, the significant up-regulation of p53 observed in a study suggests that *Pistacia khinjuk* hydroalcoholic extract may activate the mitochondrial (intrinsic) apoptotic pathway by increasing Bax gene expression while decreasing Bcl-2 gene expression, thereby exhibiting potent apoptotic activity against HepG2 cells (Harandi et al., 2022). *Pistacia vera* hull extracts have been shown to inhibit breast cancer cell proliferation through increased expression of Caspase-3, Caspase-8, Bax, and SOD, while downregulating Bcl-2 (Seifaddinipour et al., 2020).

5. Conclusion

This study demonstrated that *Pistacia khinjuk* (Stocks) modulates apoptosis-related gene expression in the liver and blood, in response to ethanol-induced toxicity. Ethanol exposure significantly increased apoptotic markers such as p53 and CASP-3, whereas Pkin feed and extract forms exhibited a protective effect by suppressing apoptotic gene expression, with the feed form being more effective. However, the coffee form showed limited protective capacity. Unexpected fluctuations in gene expression suggest that cellular responses to oxidative stress are complex and influenced by multiple factors.

These findings highlight the potential of Pk-derived bioactive compounds in modulating apoptosis-related pathways, supporting its role as a natural antioxidant with therapeutic potential in ethanol-induced liver damage. However, further mechanistic studies, protein-level validations, and in vivo models are required to confirm these effects and explore their clinical relevance.

This study has certain limitations. The use of six rats per group may limit the statistical power of the findings. The sample size could not be increased due to ethical and financial constraints, which should be considered when interpreting the generalizability of the results. Gene expression analyses in this study relied solely on β-actin as the reference gene. Given that β-actin expression may vary under different experimental conditions, the inclusion of multiple reference genes, such as GAPDH or 18S rRNA, is recommended in future research to enhance the reliability of gene expression normalization. In this study, gene expression analyses were performed using qPCR and significant changes were detected in the relevant genes. However, changes in gene expression levels may not always be directly correlated with protein. Therefore, in future studies, verification of changes in protein levels using techniques such as Western blot or ELISA will provide stronger clinical and translational support for the findings. While this study provides new insights into the role of Pk in oxidative stress regulation, future research should address these limitations to enhance the robustness and applicability of the results.

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