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Investigation of the Effects of Resveratrol on Paracetamol Toxicity Established in Hep3B Cells

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ABSTRACT

We therefore wanted to investigate acetaminophen hepatotoxicity by using Hep3B human hepatoma cells exposed to acetaminophen and resveratrol, used as a protective agent. Specifically, we studied the role of some proinflammatory markers and oxidative damage as possible mechanisms of acetaminophen-associated cytotoxicity. The Hep3B human hepatoma cell line was used for this study. In vitro studies (GSH, SOD, CAT, AST, ALT, TNF-alpha, IL-6 and cell viability) were performed by using different methods such as Biochemical analyzer, RT-PCR, ELISA and MTT. Acetaminophen and resveratrol were applied to cells in a different time and doses. Only acetaminophen treatment decreased SOD, CAT and GSH levels in Hep3B cells whereas acetaminophen and resveratrol co-treatment increased these enzymes levels. On the other hand, acetaminophen and resveratrol co-treatment (especially 160 µM dose of resveratrol) lead a severe increase in TNF-alpha and IL-6 levels. In the current study, it was shown that acetaminophen caused liver toxicity and, interestingly, resveratrol applications seriously affected the levels of the above-mentioned parameters. Only, acetaminophen administration may cause abnormal decreases and/or increases in antioxidant enzymes and proinflammatory cytokines levels. Additionally, acetaminophen and high dose resveratrol co-treatment triggered the inflammation and oxidative stress. These results showed that resveratrol have a potential to be an effective agent on the treatment and protection of hepatic damage.

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Introduction

Paracetamol

Paracetamol (Acetaminophen, N-acetyl-p-aminophenol; APAP) is a drug with analgesic (pain reliever) and antipyretic (fever-reducing) effects. It is metabolized primarily in the liver, kidney and intestine [1]. Exceeding therapeutic doses also increases the amount of released NAPQI, and when glutathione exceeds its NAPQI binding capacity, free NAPQI

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causes liver damage, which can lead to hepatic necrosis by making covalent bonds with molecules in the liver [2]. This damage causes an increase in the number of catabolic enzymes that cause the cell death of Ca^{+2}, which accumulates in the cell with the disruption of the intracellular balance in the liver cells [3]. Apoptosis, nitric oxide, reactive oxygen species and lipid peroxidation are also known to cause toxicity in the liver [4].

Substances that cause liver damage are called hepatotoxins. Hepatotoxins are classified in two ways: "intrinsic" and "idiocentral". Intrinsic hepatotoxins act in a dose-dependent manner, and hepatotoxicity that occurs in this way is called intrinsic hepatotoxicity. Hepatotoxins that act in a non-dose dependent manner are idicentric. Hepatotoxicity, which causes hepatic failure to a large extent, is intrinsic and the best example of this is acetaminophen (APAP or paracetamol). The number of patients exposed to idiocentral hepatotoxins is 1 in 10000. More than 1000 drugs and herbal products are idiocentral hepatotoxic and constitute more than 10% of acute liver failure [5,6].

Resveratrol

Resveratrol is produced from grapes, black mulberry and peanuts in response to pathogenic attack and environmental conditions. Resveratrol, which has a strong antioxidant effect, both scavenges free radicals and prevents the damage caused by these radicals. Resveratrol, which is determined to inhibit lipid peroxidation caused by OH radical, also prevents DNA damage caused by OH and H_2O_2 [7]. Resveratrol is also known to inhibit the oxidation of membrane lipids and reduce oxidative stress. [8,9]. Resveratrol is a substance that shows anti-inflammatory properties by inhibiting the formation of substances that cause inflammation [10]. In addition, resveratrol significantly reduces lipopolysaccharide-induced airway inflammation, chronic edema, and osteoarthritis [11].

Resveratrol also has a cardioprotective effect on the ischemic heart thanks to its anti-inflammatory properties. In studies, when the control group and the group given resveratrol were compared, it was determined that resveratrol reduced myocardial infarction and caused improvements that can be observed in the ventricles after ischemia [12]. In recent years, therapeutic-based scientific studies generally focus on alternative active substances. Resveratrol, a natural polyphenol compound that has many biological activities, is easy and cheap to obtain today, is only one of these active ingredients. Many

scientific studies have shown that resveratrol has many anti-carcinogenic, anti-diabetic, apoptotic, anti-angiogenic, anti-microbial, anti-viral, anti-neurotoxic and similar effects [13,14]. Both *in-vivo* and *in-vitro* studies have been carried out in this area, but there is not enough information in the literature about the changes in various immunological and biochemical parameters and the different dose amounts applied in our *in-vitro* study of paracetamol toxicity and resveratrol efficacy. In the current study, it was aimed to investigate the hepatotoxicity of acetaminophen induced hepatotoxicity in Hep3B human hepatoma cells, where acetamonifen and resveratrol, which we think may have a possible protective effect, were applied. In this study, the role of some proinflammatory parameters and oxidative stress, which is one of the possible causes of acetaminophen-mediated cytotoxicity, were investigated.

Material and Methods

In the present study, the Hep3B (ATCC-HB-8064) Human hepatoma cell line obtained commercially from the American Cell Culture Collection (ATCC) cell bank was used. DMSO, Penicillin, Resveratrol and MTT Cellular viability analysis kit sigma brand, Paracetamol Laboratories UPSA brand, Glutathioneperoxidase (GSH), Superoxide Dismutase (SOD) Assay and Catalase (CAT) Assay ELISA Kit Elabscience brand used in our study were used.

Experimental method

In the present study, it was aimed to investigate the preventive effects of resveratrol, a natural polyphenol compound, on inflammatory and oxidative stress parameters in the paracetamol-induced *in vitro* hepatotoxicity model. For this purpose, liver damage was induced by high-dose paracetamol in Hep3B human hepatoma cells, and then changes in immunological and biochemical parameters such as TNF-alpha, IL-6, SOD, CAT and GSH, which were activated in this damage, were observed with resveratrol application.

Experimental Groups Study;

1. Control group
2. Group treated with 160 µM Resveratrol (R160)
3. 80 µM Resveratrol administered group (R80)
4. Group administered 40 µM Resveratrol (R40)
5. 20 µM Resveratrol administered group (R20)
6. 20 µM APAP +160 µM Resveratrol administered group (APAP+R160)

7. 20 µM APAP + 80 µM Resveratrol administered group (APAP+R80)
8. 20 µM APAP + 40 µM Resveratrol administered group (APAP+R40)
9. 20 µM APAP + 20 µM Resveratrol administered group (APAP+R20)
10. 20 µM of APAP was administered in the administered group.

Application of resveratrol and paracetamol doses

After passage of the Hep3B (ATCC-HB-8064) Human hepatoma cell line obtained commercially from the American Cell Culture Collection (ATCC) cell bank, 1,000,000 Hep3B cells were seeded in 6-well culture dishes (6-well plate). Then, commercially available acetaminophen (APAP-Paracetamol) was dissolved in the medium and added to all experimental groups except the control well as 20 µl. After 1 hour of incubation, resveratrol, a natural polyphenol compound, 20; 40; 80; Fresh solutions dissolved in medium at 160 µM concentrations were given to the cells in equal volumes of 100 µl. For each resveratrol dose, sowing was carried out in 3 different wells. Cells were followed by seeding for 24 and 48 hours for all groups. At the 24th and 48th hours following the cultivation, the cells in the wells were collected by treatment with trypsin again and centrifuged. After discarding the supernatant, it was suspended with 1 ml of medium and counted.

Biochemical analysis

Following the incubation procedures, the cell suspension was collected in a 15 ml centrifuge tube and centrifuged at 1500 rpm for 3 minutes and the supernatant was discarded. Alanineaminotransferase (ALT) and aspartataminotransferase (AST) liver enzyme levels were measured by an automatic bioanalyzer device from the cell suspension diluted with 5 ml of fresh medium. Then, evaluations were made with Glutathioneperoxidase (GSH), SuperoxideDismutase (SOD) Assay and Catalase (CAT) Assay ELISA Kit according to the manufacturer's recommendation (Elabscience).

The expression levels of the pro-inflammatory cytokines TNF-alpha and IL-6 cytokines were investigated (performed by applying the Real-time PCR method with the primer shown in Table 1).

Table 1 Primer sequences used for the PCR study

Primers	Forward	Reverse
TNF-alfa	5'-CCAGGAGAAAGTCAGCCTCCT-3'	5'-TCATACCAGGGCTTGAGCTCA-3'
IL-6	5'-CGAAAGTCAACTCCATCTGCC-3'	5'-GGCAACTGGCTGGAAGTCTCT-3'
β-aktin	5'- GCAAGCAGGAGTATGACGAG -3'	5'- CAAATAAAGCCATGCCAATC-3'

Statistical method

SPSS 20.0 (IBM, New York, USA) statistical program was used to evaluate the data obtained from the experiments. One-way ANOVA, Tukey and Duncan's Multiplerange analysis tests were used to evaluate the significance of the difference between groups. The limit of significance was accepted as $p< 0.05$.

Results and Discussion

The effect of resveratrol and paracetamol on liver enzymes

When the data obtained was examined, a statistically significant difference emerged between the liver ALT activity of the APAP+R160 group and all other groups ($p<0.05$). However, there was no statistical difference when compared both between the groups and the control group ($p>0.05$). These data gave similar results in AST activities. The letters and numbers in the figure indicate the significance between the groups (Figure 1).

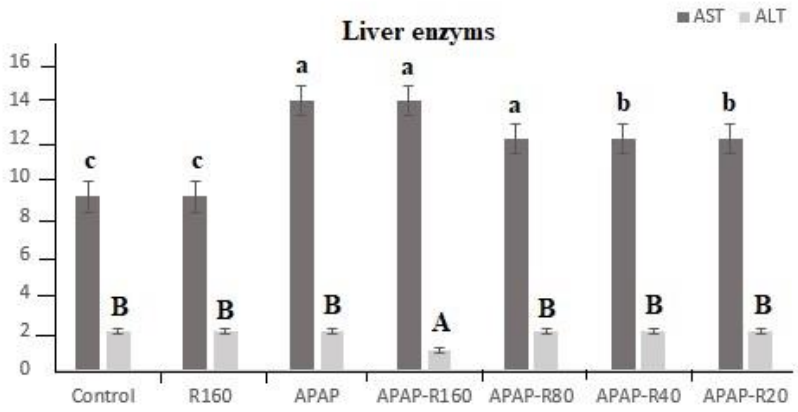

Fig 1 Effects of Resveratrol and Paracetamol on AST and ALT activities (a-c; Shows statistical difference in AST Activity between all experimental groups, A-B; Shows statistical difference in ALT Activity between all experimental groups).

In vitro effect of resveratrol and paracetamol on Hep3B cells

The cellular viability analysis results obtained from the cell culture studies we carried out are shown in Figure 2. When the results obtained were examined, statistically significant

differences were observed between the experimental groups in terms of cell viability. According to the 24th hour MTT results; No significant difference was observed in the control group alone and the groups administered only resveratrol. In other experimental groups, it was determined that paracetamol application significantly reduced the number of viable cells compared to the control and resveratrol-only groups ($p<0.05$). Additionally, the lowest amount of viability was detected in the APAP+R160 experimental group in the groups administered resveratrol together with paracetamol ($p<0.05$). When the 48-hour data obtained from the MTT cell viability analysis test is evaluated; Results parallel to the 24-hour data were determined. In addition to paracetamol having a toxic effect on living cells, high dose resveratrol application also causes serious decreases in the number of living cells.

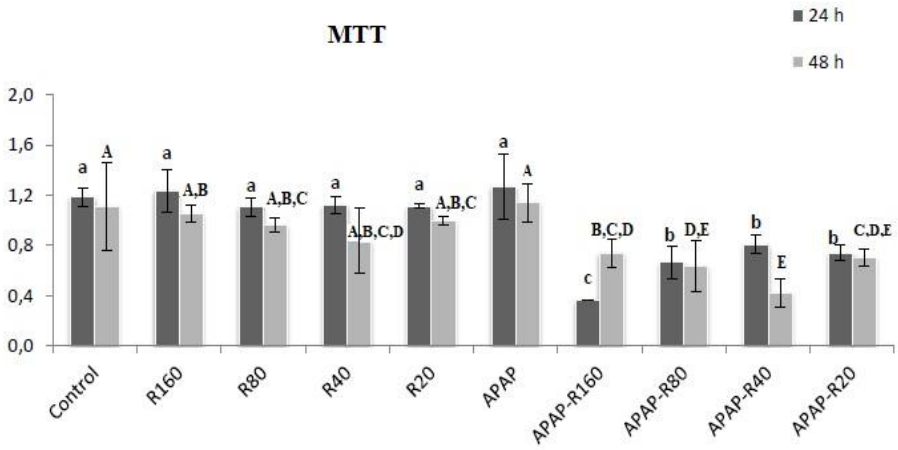

Fig 2 Viability analysis test results of all experimental groups (a-c; shows statistical difference between 24-hour MTT results, A-E; shows statistical difference between 48-hour MTT results).

Effects of resveratrol administration on SOD, CAT and GSH activity

When Figures 3 were examined, it was observed that paracetamol application SOD, CAT and GSH activities were significantly different and at the lowest level in all experimental groups ($p<0.05$). While a significant increase was detected in SOD and CAT activities of high dose resveratrol compared to the control group ($p<0.05$), on the contrary, no increase was detected in GSH activity ($p>0.05$).

In Figure 3B, there was no statistically significant difference in CAT activity between the Control group and APAP+R80 ($p>0.05$). However, all other experimental groups increased or decreased statistically significantly both within themselves and when

compared to the control group. In Figure 3C, GSH levels in the paracetamol applied group were found to be significantly lower compared to the control group ($p<0.05$).

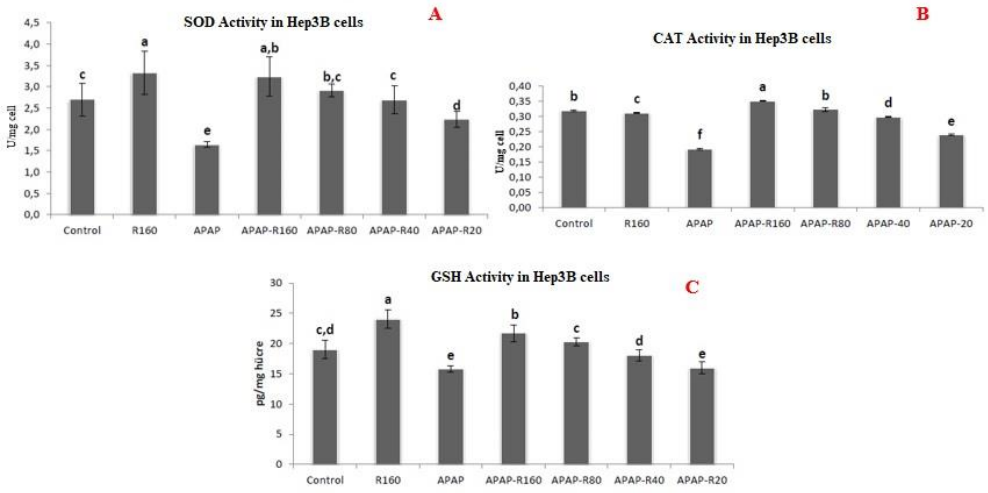

Fig 3 Effects of Resveratrol Administration on SOD, CAT and GSH Activity

Effects of Resveratrol Administration on the Gene Expression levels of Pro-Inflammatory Cytokines

When Figure 4 was examined, a significant increase was observed in the expression levels of pro-inflammatory cytokines IL-6 and TNF-alpha in both paracetamol and high dose resveratrol administered groups ($p<0.05$). This situation seriously triggered inflammation. However, although other doses of resveratrol reduced the amount of IL-6 and TNF-alpha in the APAP applied groups, they could not reduce it to the level of the control group. Partial increases and decreases were observed in other experimental groups. As a result, the data obtained reflects the data we expected in our study.

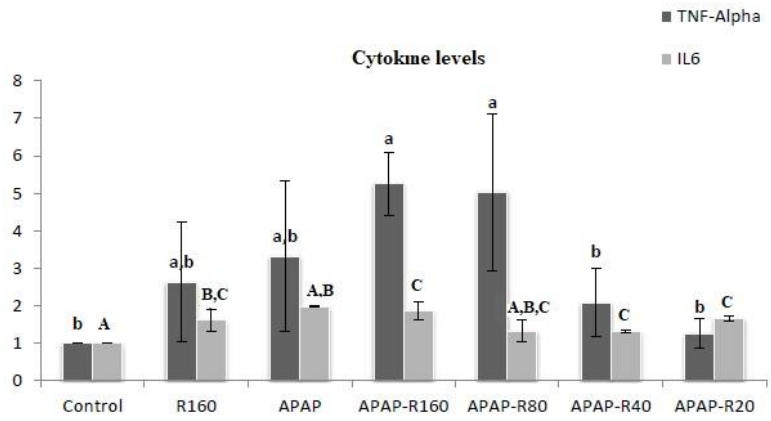

Fig 4 Effects of Paracetamol and Resveratrol applications on expression of the TNF-alpha and IL6 a-b; Shows the statistical difference between the experimental groups in terms of TNF-alpha, 1-3; It shows the statistical difference between the experimental groups in terms of IL-6.

Discussion

Paracetamol is an analgesic and antipyretic drug that is widely used worldwide and has few side effects when taken in appropriate doses [15]. Although it is recommended that the annual paracetamol consumption amount is less than 8 grams per person in some countries (USA, UK, Canada, Australia and New Zealand), this rate can exceed 20 grams per person [16,17]. Due to its high consumption and being an easily accessible drug, the risk of organ toxicity is also quite high. It is an ideal medicine when consumed in sufficient amounts, but when used in high amounts it is very likely to cause fatal liver failure. 2% of the paracetamol taken into the body is excreted unchanged in the urine. However, a small amount of paracetamol is metabolized to N-Acetyl-p-benzoquinone imine (NAPQI) by the cytochrome P-450 enzyme. This metabolite first depletes GSH and then binds to a number of cellular proteins, including mitochondrial proteins, and these bindings initiate liver damage [18]. As a result of these cellular events, consumption of ATP and mitochondrial oxidative stress events are observed. ATP consumption leads to cellularoncotic necrosis in hepatocytes and sinusoidal endothelial cells [19].

In general, fulminant liver toxicity occurs after ingestion of high doses of paracetamol. As an indicator of this damage, elevated liver transaminases (AST and ALT) can be shown. Elevated levels of these enzymes indicate paracetamol-induced liver damage. At the same time, this elevation indicates that the functional integrity of the cell membrane is impaired [20]. Other scientific studies related to the subject have also shown that paracetamol causes elevations in liver AST and ALT enzyme activities [21,22]. It has been found that paracetamol causes significant increases in liver enzyme activities in both experimental animals and *in vitro* studies [23]. In our current study, liver AST and ALT enzyme activities were investigated in order to understand whether paracetamol administration causes toxic effects on liver cells. The data obtained showed that it was statistically higher in the paracetamol administered groups compared to the control group, and this showed us that the hepatotoxicity model we planned in our study was formed. As stated in the findings section, it was determined in the current study that AST and ALT levels in the resveratrol applied groups decreased compared to the paracetamol applied group and approached the control group. In conclusion, this increase in AST and ALT activities is a sign that the toxic dose of paracetamol causes P-450-induced hepatotoxicity [24]. The inhibition of the increase in the activities of these enzymes is the primary

evidence of the hepatoprotective effect of resveratrol at the enzymatic level. In parallel with this, Anundi, et al.. [25] observed periportal and perivenous hepatotoxicity in cell culture with high-dose paracetamol administration.

GSH is one of the very important molecules that play a role in cellular defense against reactive toxic compounds or oxidative stress. Glutathione exists in two forms, oxidized and reduced, and there is a balance between the two forms. In its reduced form, the cysteinethiol group is capable of donating reducing equivalents to unstable molecules such as reactive oxygen products. This mechanism can prevent tissue damage by removing free radical species such as hydrogen peroxide and superoxide radicals. It is known that NAPQI as a mediator of oxidative stress at sufficiently high doses of paracetamol causes a decrease in GSH levels and an increase in lipid peroxidation due to this decrease [26]. In our current study, GSH values were determined in parallel with the known literature. While it was observed to be low in the paracetamol administered groups, significant increases were observed in the resveratrol administered groups. Some scientific studies in which different active ingredients are used as therapeutic agents support our current data. In a study by Manda, et al.; GSH values were found to be decreased in the group with paracetamol-induced liver toxicity compared to the control group, and these values were increased with the applied beta-carotene treatment [27]. Similarly, in another study, GSH level was found to be low in the paracetamol toxicity group, while an increase in GSH level was observed in the L-carnitine administered group [28].

One of the parameters responsible for oxidative stress in paracetamol toxicity is the superoxide radical, and these radicals are a family of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. It protects cells that metabolize oxygen against the harmful effects of superoxide free radicals. Therefore, it is an important antioxidant defense mechanism in almost all cells exposed to oxygen [29]. Some studies have mentioned that the increase of superoxide level in all of the mechanisms that cause the increase of free oxygen radicals has a very important role in the formation of oxidative stress [30]. Antioxidant enzymes such as SOD are easily inactivated by lipid peroxidases or reactive oxygen products, and therefore, decreases in these enzyme activities are detected in paracetamol toxicity. In our current study, it was determined that SOD activity decreased significantly in the groups treated with

paracetamol. In addition, resveratrol administration increased the SOD activities of these groups in a dose-dependent manner. When the literature is scanned, it is determined that there are scientific studies with similar mechanisms. In one of these studies, SOD levels were low in acute liver toxicity induced by paracetamol, but Picroside II given at increasing doses caused an increase in SOD levels in experimental groups [31]. Similarly, in a similar study, it was observed that while the amount of SOD decreased in the toxicity group, the level of SOD increased in the treatment group [32]. Likewise, many scientific studies with similar effects have been conducted and SOD activity has been shown to decrease in acute liver injury [33,34].

Catalase is an antioxidant enzyme commonly found in all prokaryotic and eukaryotic organisms. Catalase catalyzes the conversion of hydrogen peroxide, a strong and stable oxidant, into water and molecular oxygen. Catalase enzyme is also affected by oxidative stress, as are important antioxidant enzymes such as SOD, glutathione peroxidase (GPOX), and glutathione reductase (GR). Antioxidant enzymes such as catalase and SOD are easily inactivated by lipid peroxidases or reactive oxygen products, and therefore, decreases in these enzyme activities are detected in paracetamol toxicity [35]. In our study, parallel to this information, CAT level was found to be decreased in the paracetamol group, and statistically significant increases were observed in CAT levels after resveratrol treatment. It was in agreement with our current findings in other scientific studies, just as in other antioxidant enzymes. In a study performed by Awolede, et al., it was shown that CAT activity decreased in paracetamol-induced acute liver toxicity, but Carica papaya Linn application caused an increase in CAT level [36]. Similarly, in another study conducted in our country, it was stated that CAT levels decreased in the hepatotoxicity model created with paracetamol, but thiamine pyrophosphate application caused improvements in these levels [37].

Considering the data of our own study and the existing literature, we can say that resveratrol reduces oxidative stress in liver cells and increases hepatic antioxidant enzyme activities.

In addition to oxidative stress, the role of proinflammatory cytokines and inflammation precursors TNF-alpha and IL-6 in acute liver injury due to paracetamol toxicity has been demonstrated in scientific studies. TNF-alpha, produced from macrophages in the liver, plays an active role in many liver injuries such as fulminant liver failure [38]. TNF-alpha

can aid tissue repair through its central regulatory role. When scientific studies on this subject were examined, it was observed that the expression level of TNF-alpha increased significantly in acute liver toxicity due to paracetamol [39,40]. Studies have shown that TNF-alpha, which plays a role in the initiation of inflammation, increases significantly in hepatotoxicity, and these levels are significantly reduced with the use of agents with anti-inflammatory properties [41]. In our study, in parallel with the existing literature, the amount of TNF-alpha in the paracetamol-administered group was found to be 3 times higher than the control group, while statistically significant decreases were detected in the resveratrol-administered group.

Similarly, IL-6, which is a pro-inflammatory, has a protective effect on liver damage. It arises through the prevention of mitochondrial dysfunction and suppression of oxidative stress [42]. Similarly, scientific studies carried out in recent years have shown that IL-6 level increases in paracetamol-induced acute liver toxicity, and the therapeutic agents administered following this decrease the IL-6 level [43,44]. In the current study, it was shown that the paracetamol group caused an increase in the IL-6 level, but resveratrol administration showed efficacy even at different doses, as in TNF-alpha, and caused a decrease.

Conclusion

In the current study, it was shown that acetaminophen caused liver toxicity and, interestingly, resveratrol applications seriously affected the levels of the above-mentioned parameters. Only, acetaminophen administration may cause abnormal decreases and/or increases in antioxidant enzymes and proinflammatory cytokines levels. Additionally, acetaminophen and high dose resveratrol co-treatment triggered the inflammation and oxidative stress. These results showed that resveratrol have a potential to be an effective agent on the treatment and protection of hepatic damage.

Abbreviations

ALT: Alanin Aminotrenferaz, APAP: Asetominofen, GSH: Glutatyon, IL-6: İnterlökin-6, INF-α: İnterferon-alfa, KAT: Katalaz, MTT: Hücresel Canlılık Analizi, SOD: Süper Oksit Dismutaz, TNF – α: Tümör Nekroz Faktör – Alfa.

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Data Availability statement

The author confirms that the data supporting this study are cited in the article.

Compliance with ethical standards
Conflict of interest
The authors declare no conflict of interest.

Ethical standards
The study is proper with ethical standards.

Authors' contributions
In this work, these laboratory studies were carried out by Dr Alpgiray TURGUT, Dr Tubanur Aslan ENGİN and Dr Muhammet TURGUT. While Prof. Dr. Mesut Bünyami HALICI supervised the coordination, the article was organized and finalized by Dr Alpgiray TURGUT.

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