



Evaluation of propolis hepatotoxicity in male rats

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ABSTRACT

Background: Propolis is a natural resinous combination that honeybees produce from the materials they gather from plant parts, buds, and exudates. Numerous favorable pharmacological qualities have been demonstrated for propolis.

Objectives: This study aimed to investigate the hepatotoxic effects of ethanolic propolis extract on the liver of male rats.

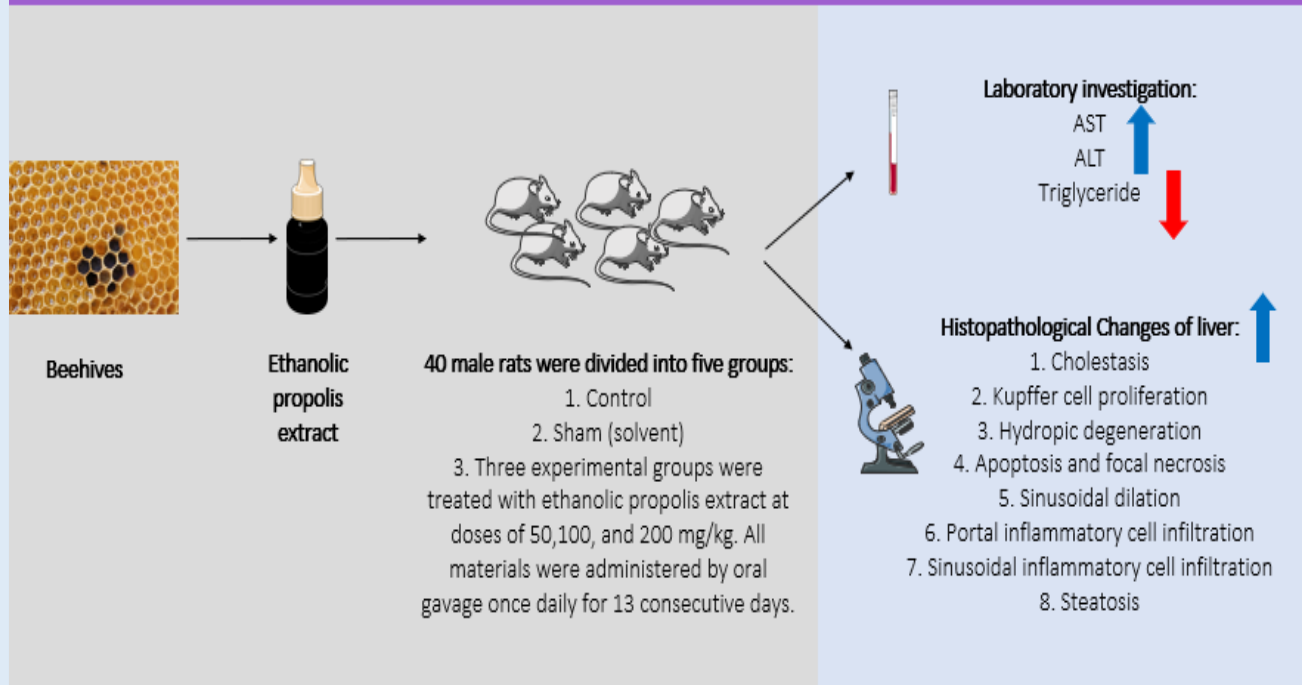
Methods: In this research, 40 male rats were divided randomly into five groups: 1. Control 2. Sham (solvent), and 3. Three experimental groups (ethanolic propolis extract at doses of 50,100 and 200 mg/kg). All materials were administered by oral gavage once daily for 13 consecutive days. On the 14th day, blood sampling was performed to measure serum levels of liver function enzymes and triglycerides. After deep induction of anesthesia, the liver of the rats was removed for histopathological studies. Data were analyzed using ANOVA and Tukey's test ($p < 0.05$).

Results: The data showed that the administration of propolis significantly increased the serum levels of aminotransferases in a dose-dependent manner and decreased triglycerides, accompanied by pathological changes.

Conclusions: The results of this study indicate that the administration of propolis is associated with liver toxicity, and it seems that it should be consumed more carefully.

Keywords: Alanine aminotransferase, Aspartate aminotransferase, Propolis, Hepatotoxicity Triglycerides

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INTRODUCTION

Propolis, also known as "bee glue," is a sticky, resinous substance produced by bees. It can be found in various plant sources, including bud exudates, flowers, and leaves [1]. Biochemical analyses have shown that propolis bumblebee consists of a variety of compounds, including aliphatic acids, esters, aromatic acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins, inorganic substances, and flavonoids, each of which has a high value in the pharmaceutical industry [2]. In the last few decades, numerous studies have been

conducted on the properties of propolis, providing significant evidence of its antibacterial, antifungal, antiprotozoal, antiviral, antioxidant, antitumoral, cytostatic, and wound healing properties. Propolis has also demonstrated anti-inflammatory, immunomodulatory, antihypertensive, and antiatherosclerotic activities. Moreover, its constituents possess medicinal properties that enable tissue regeneration and repair after damage [3-6].

The body's largest gland, the liver, is necessary for metabolism, bile generation, detoxification, and water and electrolyte regulation [7]. Cytochrome P450 (CYP), which is primarily expressed in the liver, mediates the

metabolism of many endogenous and exogenous substances [8-9]. Propolis can induce apoptosis in hepatocellular carcinoma (HCC) liver hepatocytes through mitochondrial dysfunction and reactive oxygen species generation [10].

Triglycerides (TG) are the primary means of fatty acid storage and transportation. The primary organ for the metabolism of fatty acids is the liver. Through both de novo production and hepatic absorption from the plasma, fatty acids accumulate in the liver. Triglyceride-rich, very-low-density lipoproteins are eliminated by being either secreted into the plasma or oxidized within the cell. Under normal circumstances, only a small quantity of fatty acids is stored in the liver as triglycerides. Under abnormal conditions, the metabolism of fatty acids in the liver is altered, which can lead to the accumulation of triglycerides in the liver cells [11-12]. An investigation revealed that the level of serum TG correlated with the stage of liver disease, especially depending on the presence of steatosis and cirrhosis [13-14].

Liver damage, which varies in severity from fulminant hepatic failure to asymptomatic increase in liver tests, can present with a wide array of phenotypes that resemble other liver illnesses. When a threshold dose or exposure is reached, hepatotoxicity can be induced by drugs or substances that cause toxicity to liver cells [15]. Abnormal results from biochemical tests such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are typically used to diagnose and confirm liver injury [16]. The liver can repair and regenerate itself after injury, a function that is vital for survival after acute liver failure [17]. Numerous immune and non-immune cells in the liver are involved in mechanisms that can lead to either regeneration or fibrosis in both acute and chronic inflammation [18]. Due to an increasing tendency to use natural materials and functional foods in the general medical and pharmaceutical industry, growing health consciousness

among people, consumers' belief in the non-hazardous nature of natural products [19-22], and side effects and toxicity of natural products, further research on the hepatotoxicity of natural products should be considered. Studies on the liver mechanisms and stages of propolis detoxification are limited, and by assessing the mechanisms of interaction, choosing appropriate methods for tracking the toxic effects, it is possible to take measures to limit the toxic effects of propolis. Based on the therapeutic effects of propolis and its increasing consumption, this study examined the hepatotoxicity of propolis in the liver of male rats.

MATERIALS AND METHODS

Experimental Design: This experimental study used 40 male rats weighing 200 -230 gr (Razi Company, Karaj-Iran). Every animal was kept in neat, conventional cages with free access to food and water, a controlled temperature of 22 ± 2 °C, and a light-dark cycle of 12 hours each day. All experiments were carried out under approved ethical guidelines (IR-QUMS-REC-1395.237) by the Qazvin University of Medical Sciences Ethics Committee. The rats were randomly divided into five groups of eight members, including 1. Control (untreated); 2. Sham (propolis solvent), 3. All three experimental groups, received one of the extract concentrations of 50, 100, and 200 mg/kg body weight. The rats received 1 ml of the extract or solvent via a nasogastric tube once daily for 13 consecutive days.

Propolis Preparation: Propolis was taken from hives in the mountainous region of northern Qazvin, Iran. Using the procedure outlined by Moreno et al., ethanol extract was created. First, 100 ml of 80% ethanol (Merck, Germany) was combined with 25 grams of propolis (Merck, Germany). After a 48-h period at room temperature and a horizontal shake at 150 rpm in the dark, the mixture was twice passed through grade 4 filter paper, and the alcohol was extracted using a rotary evaporator. Ultimately, the obtained pure

ethanolic extract was weighed and stored in a dark glass container in the refrigerator. The dried extract was dissolved at the intended dosage of propylene glycol (German-Merck) prior to oral administration [23].

Laboratory Investigations: One day following the final gavage, blood was drawn from the rat tail vein to assess the serum concentrations of triglycerides (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). After being separated by centrifugation for 15 minutes at 3000 rpm, the serum was kept at -70 °C until analysis. The calibration Selectra analyzer and kit Bionik Co- Iran were employed for serum analysis. The colorimetric and enzymatic methods were used to measure ALT and AST (Bionik kit, Iran) and TG levels, respectively [24].

Histopathological Studies: On the last day of treatment, the rats were anesthetized using ketamine/xylazine (60/6 mg/kg) (Merck, Germany), followed by laparotomy to remove the liver, which was then fixed using formalin 10%. The paraffin-embedded tissue blocks were then serially sectioned at a thickness of 3 µm using a Leica (Leica, Germany) microtome, and the tissue was stained with Hematoxylin and Eosin methods [25]. The histopathological evaluation criteria included 1. cholestasis (accumulation of bilirubin in hepatocytes), 2. Kupffer cell proliferation (stellate sinusoidal macrophages in the linings of the liver sinusoids), 3. Hydropic degeneration (cytoplasmic clearing and cell

swelling), 4. Apoptosis and focal necrosis (replacement of hepatocytes by inflammatory cells), 5. Sinusoidal dilation (hepatic capillary enlargement), 6. Portal inflammatory cell infiltration 7. Sinusoidal inflammatory cell infiltration (lymphocytes, plasma cells, and eosinophils), and 8. Steatosis (fat deposition in the hepatocytes). The grading of changes was negative to mild (\pm), mild (+), moderate (++) , and severe (+++) [26].

Statistical Analysis: SPSS V.20 software was used to analyze the data. For the statistical analysis, Fisher's exact test, Tukey test, and one-way ANOVA were used. A P-value of less than 0.05 was deemed significant.

RESULTS

Serum Aspartate Aminotransferase (AST): The data collection and analysis revealed the mean and standard deviation of AST serum levels to be (193.1 \pm 5.5) in the control group and (166.9 \pm 4.3), (235.5 \pm 7.53), and (317.9 \pm 8.9) IU / L in the propolis group at concentrations of 50, 100, and 200 mg/kg, respectively.

The results indicated a significant increase in the serum level of AST in groups receiving propolis at doses of 100 mg/kg (P <0.05) and 200 mg/kg (P <0.01) compared to that in the control group. The serum AST level decreased in rats receiving propolis at a 50 mg/kg dose, but this reduction was not statistically significant. The mean difference in AST between the groups receiving 200 mg/kg and 50 mg/kg propolis was significant (P<0.05). (Figure 1)

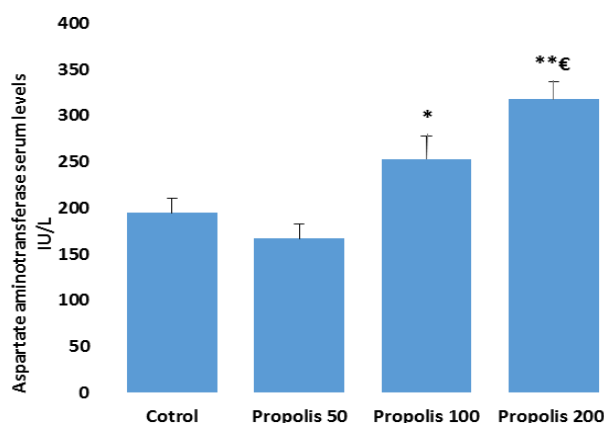


Figure 1. Comparison (Mean \pm SD) of the AST serum levels in male rats (n=8). *p<0.05, **p< 0.01 compared to control; € p<0.05 compared to propolis 50 mg/kg.

Serum Alanine Aminotransferase (ALT): The mean and standard deviation of ALT enzyme serum levels were (61.9 ± 3.9) in the control group and (51.3±3.2), (80.5±5.25), and (88.5 ± 5.8) IU / L in the rats' gavage by different concentrations of propolis, including 50, 100, and 200 mg/kg, respectively. The results showed that the consumption of propolis at doses of 100 and 200 mg/kg significantly increased the serum levels of ALT (P<0.01).

The serum level of ALT difference between the group receiving propolis 100 and 200 mg/kg was significant in the group receiving propolis 50 mg/kg (P<0.05 and P<0.01, respectively) (Figure 2). These results indicate that increased concentrations of propolis elevate serum levels of ALT and AST, resulting in higher levels of liver cell damage.

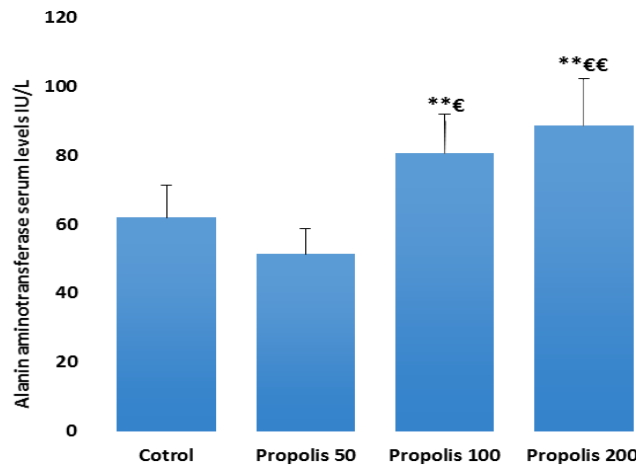


Figure 2. Comparison (Mean ± SD) of the serum levels of ALT in experimental groups (n=8). * p<0.05, **p< 0.01 compared to control; € p<0.05, €€ p<0.01 compared to propolis 50 mg/kg.

Serum Triglycerides (TG): The results showed that the consumption of propolis at all concentrations decreased

serum levels of TG, which was significant in the groups receiving 100 and 200 mg/kg (P<0.01 and P<0.05, respectively) compared to the control group.

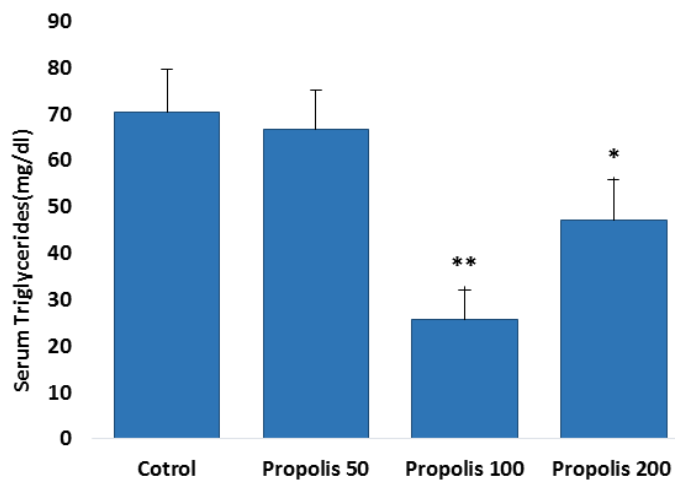


Figure 3. Comparison (Mean ± SD) of the serum levels of triglycerides (TG), of serum in experimental groups (n=8). * p<0.05, **p< 0.01 compared to control.

Histomorphologic studies: The histomorphologic changes in the control group were rare, and those in the sham group included negative to mild Kupffer cell proliferation, sinusoidal dilation, and sinusoidal inflammatory cell infiltration (Table 1).

The rats that received the ethanolic extract at a concentration of 50 mg/kg showed mild cholestasis, mild Kupffer cell proliferation, mild hydropic degeneration, negative to mild apoptosis and focal necrosis, mild sinusoidal dilation, mild portal inflammatory cell infiltration, mild sinusoidal inflammatory cell infiltration in zone 3, and mild steatosis (periportal hepatocytes) (Table 1).

The rats treated with 100 mg/kg of the extract presented mild cholestasis, moderate Kupffer cell

proliferation, moderate hydropic degeneration, mild apoptosis and focal necrosis, moderate sinusoidal dilation, mild portal inflammatory cell infiltration, mild sinusoidal inflammatory cell infiltration in zone 3, and mild steatosis (periportal hepatocytes) (Table 1).

The rats treated with 200 mg/kg of the extract exhibited significant histomorphological changes, including moderate cholestasis, moderate Kupffer cell proliferation, severe hydropic degeneration, moderate apoptosis and focal necrosis, severe sinusoidal dilation, moderate portal inflammatory cell infiltration, moderate sinusoidal inflammatory cell infiltration in zone 3, and moderate steatosis (periportal hepatocytes) (Table 1) (Figure 4).

Table 1. The effect of propolis on liver histomorphologic presentations after oral administration of propolis for thirteen days in different groups. - Negative; ±Negative to mild; + Mild; ++ Moderate; +++ Severe

Group Histomorphologic presentations	Control	Sham	Propolis 50 mg/kg	Propolis 100 mg/kg	Propolis 200 mg/kg	P-value
Cholestasis	-	-	+	+	++	0.00
Kupff cells proliferation	-	±	+	++	++	0.00
Hydropic degeneration	-	-	+	++	+++	0.00
Apoptosis and focal necrosis	-	-	±	+	++	0.00
Sinusoidal Dilation	-	±	+	++	+++	0.00
Portal inflammatory cells infiltration	-	-	+	+	++	0.00
Sinusoidal inflammatory cells infiltration	-	±	+	+	++	0.00
Steatosis	-	-	+	+	++	0.00

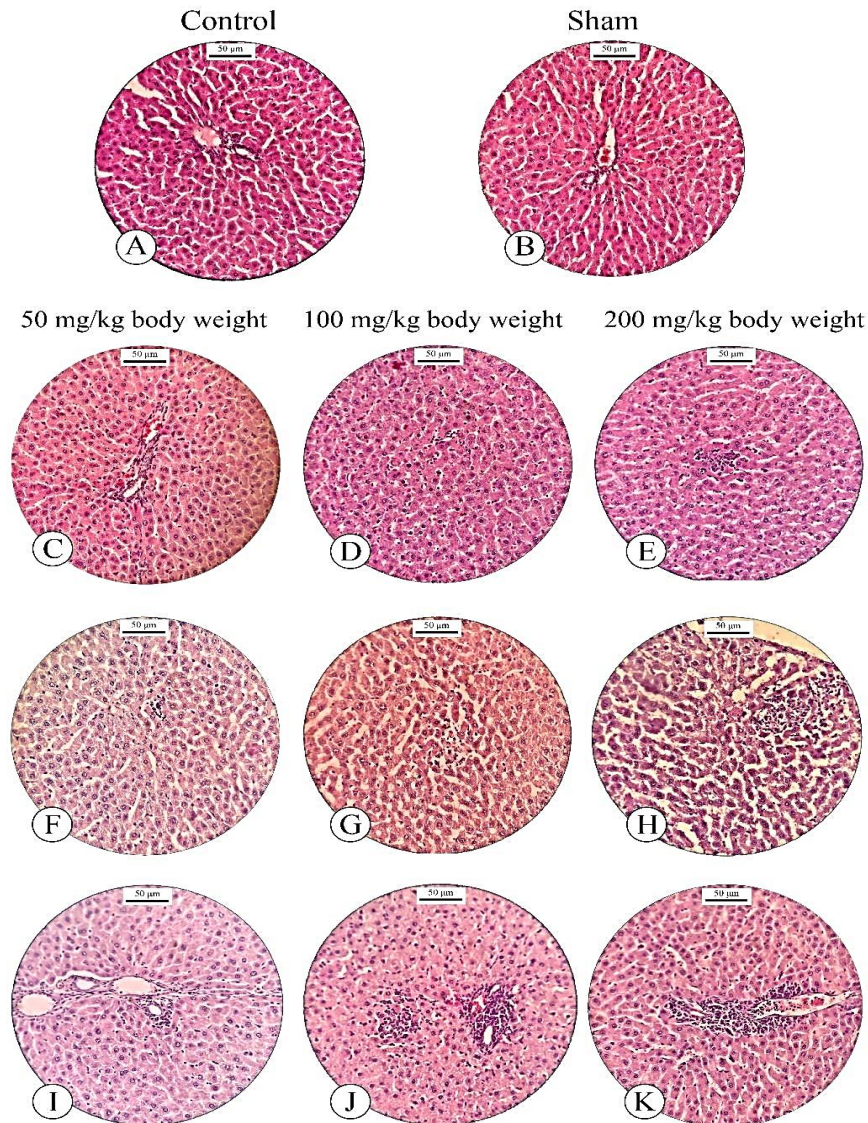


Figure 4. The various histopathologic findings of liver tissue in experimental groups.

Control group: Hepatocytes and portal space (A). Sham group: Congestion, sinusoidal dilation, and increase of Kupffer cells (negative to mild) (B). C: Propolis 50 mg/kg: Sinusoidal dilation, Kupffer cells proliferation, Sinusoidal inflammatory cells infiltration (mild) (C). Propolis 100 mg/kg: Sinusoidal dilation, Kupffer cells proliferation, Sinusoidal inflammatory cells infiltration (moderate) (D). Propolis 200 mg/kg: Sinusoidal dilation, Kupffer cells proliferation, Sinusoidal inflammatory cells infiltration (severe) (E). Propolis 50 mg/kg: Cholestasis and focal necrosis (negative to mild and mild) (F). Propolis 100 mg/kg: Cholestasis and focal necrosis (mild) (G). Propolis 200 mg/kg: Cholestasis and focal necrosis (moderate) (H). Propolis 50 mg/kg: Portal inflammatory cells infiltration (mild) (I). Propolis 100 mg/kg: Portal inflammatory cells infiltration (mild) (J). Propolis 200 mg/kg: Portal inflammatory cells infiltration (moderate) (K). Magnification x400, scale bars 50 µm, Hematoxylin and Eosin.

DISCUSSION

Propolis (a natural resin) is a multifunctional substance used by bees in the production and reinforcement of their hives [27]. Since ancient times, propolis has been used to treat many diseases, and there has been much

research on its therapeutic properties. According to previous studies, propolis appears to be relatively non-toxic; however, recent studies have suggested the possibility of liver toxicity of propolis [28-30]. In this study, we examined the hepatotoxicity of propolis in

adult male rats. The findings of this study showed that increasing the concentration of propolis led to an increase in serum levels of AST and ALT. In addition, the data showed a correlation between propolis-induced liver toxicity and TG levels. There was a significant reduction in serum TG levels in the propolis-treated rats. Increasing the dose of propolis also induced histomorphological variation in the hepatocytes, vessels, portal spaces, and bile ductules. As a result, increasing the concentration of propolis resulted in more liver cell damage.

Several studies have examined potential interactions with conditions such as viral infection, liver damage, and dyslipidemia. Hyperlipidemia has been observed in patients with HBV cirrhosis. The abnormal aggregation of fat in the liver is caused by molecular pathways that are not entirely understood. The possible mechanism for these changes may be due to alterations in fatty acid metabolism by enzymatic elongation and desaturation [14,31,32]. The creation of new fatty acids, their export, and subsequent redistribution to other tissues are all coordinated by the liver, an organ crucial to lipid metabolism that maintains lipid homeostasis [33]. The complex interactions between hormones, nuclear receptors, transcription factors, and feeding behaviors closely regulate these processes [34]. Hypolipidemia may arise due to the fat retention in the liver caused by disturbances in one or more of these variables [35]. Many studies have stated that lipid export is enriched in early disease stages but decreases with the severity of the disease because of increasing impairment in hepatocyte metabolism. Lipid retention in hepatocytes may induce oxidative stress and lead to the progression of liver disease [36]. Increased production of reactive oxygen species, mitochondrial malfunction, endoplasmic reticulum stress, and excessive hepatic fat accumulation cause inflammation, cell death, and ultimately fibrosis [34,36].

Cytochrome P450 isoenzymes are among the most critical enzymes involved in the metabolism of substances. Although this system is present in all cells, it is more active in the endoplasmic reticulum of hepatocytes. CYP isozymes are responsible for the detoxification of endogenous and exogenous compounds. However, they can cause hepatotoxicity via the production of active interstitial metabolites [20,37]. Various foods or plant supplements can inhibit or induce CYP [37-38]. Many studies have reported evidence of the hepatotoxic effects of these superfluid materials, including propolis [39]. However, the mechanism underlying liver damage remains unclear. The liver toxicity spectrum of this substance is vast, including the elevation of liver enzymes, acute and chronic hepatitis, cholestasis, liver necrosis, fibrosis, cirrhosis, liver failure, liver transplantation, and autoimmune hepatitis. These effects can be dose-dependent and idiosyncratic [40]. Chang et al. (2016) conducted a study investigating the inhibitory effects of a purified honeybee propolis extract on CYP human liver microsomes. The pure propolis extract of the honeybee could inhibit some of the CYP isoenzymes. This substance inhibited the CYP1A2, CYP 2B6, CYP 2C9, CYP 2D6, and CYP 3A4 isoenzymes with IC₅₀, 6.9, 16.8, and 43.1 µg / ml, respectively [39].

Concerning general health and the increase in the age of the population, the use of natural substances has increased, especially in people with chronic illnesses. In patients with chronic illness, drug interactions may occur during the simultaneous use of a variety of supplements with each other or with other drugs. Drug interactions can occur in patients who simultaneously use chemical drugs and drugs such as propolis. For example, propolis interacts with duloxetine, a drug that metabolizes CYP isozymes and inhibits its function [39].

Herra et al. (2014) showed that the effects and toxicity of propolis depend primarily on its physical and chemical properties, mechanism of action and

biochemical characteristics, site of production, collection, maintenance, and removal method. The effects appear to be higher when ethanol extraction is used [41].

In examining the reasons for this lack of conformity, the results of this study show that propolis may have protective effects against liver toxicity or other toxic substances. However, its high dose and long-term use can lead to complications such as liver toxicity. Since the amount and type of propolis compounds vary according to location, collection time, and production method, this seems to be one of the reasons for the difference in the data obtained in different studies and the discrepancy between the results of some studies and the review of the present study [42]. In general, the protective properties of propolis appear to be due to the presence of antioxidant substances and their toxic effects due to the presence of some polyphenolic compounds (such as Caffeic Acid-Caffeic Phenyletherester), Phenyl Compounds or diploids occur in phenolic compounds or diterpenoids [43-44].

Seydi et al. (2016) reported an increase in AST, ALT, and Alkaline phosphatase in three doses of propolis extract (75, 150, and 300 µg / ml), which significantly increased the serum concentrations of enzymes associated with an increase in the alphaphethoprotein content due to the higher propolis levels. Their results showed that propolis extract reduced the mitochondrial membrane potential, mitochondrial swelling, and cytochrome C release with increasing ROS levels and activating caspase 3 in hepatocytes and mitochondria isolated from HCC liver from normal but untreated rats, suggesting that propolis selectively poisoning on HCC of the rat hepatocytes by directly targeting mitochondria and inducing apoptosis [10]. In two similar studies, propolis was found to have cytotoxic activity against liver cells, the severity of which is altered in a different type of propolis depending on the chemical

composition, the place of production, and the extraction method [30,39].

Reports have shown that more than 350 drugs in modern pharmacopeia can induce liver injury. In addition to many compounds, herbal medicines and dietary supplements can also induce liver damage, such as hepatitis, cholestasis, ductopenia, phospholipidosis, and steatosis [45]. Our results confirmed that continuous consumption of propolis may produce these side effects.

CONCLUSION

In general, the results of the present study showed that the use of propolis is associated with dose-dependent liver toxicity, as measured by increasing the levels of AST and ALT. In addition, TG levels decreased in treated rats, which could be a marker of liver damage through propolis consumption. Due to histological differences between liver cells and intracellular organelles, especially mitochondria, in humans and rats, the results of this experimental study are not entirely generalizable to human conditions and require cautious interpretation. Higher concentrations of propolis led to higher levels of liver damage. Moreover, we recommend the use of biochemical techniques and measuring cytokines in the prospective studies.

Abbreviations: CYP: cytochrome P450, HCC: hepatocellular carcinoma, AST: aspartate aminotransferase, ALT: alanine transaminase, TG: Triglycerides, SD: standard deviation, ANOVA: one-way analysis of variance

Conflict of Interests: The authors declare that they have no conflict of interest.

Author contributions: Study concept and design (FS, NG), acquisition of data (FS, NG), analysis and interpretation of data (FS, MS, MM), drafting of the

manuscript (FS, MS, EB), critical revision of the manuscript for important intellectual content (FS, MS), administrative, technical, or material support (FS, NG), and study supervision (FS, NG). All authors have made a significant contribution to this study and have approved the final manuscript.

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