

Preclinical Study on Hepatoprotective Effect of Pollen Extract of *Pinus brutia* Ten. (Red Pine) in Mice and Phenolic Acid Analysis

***Pinus brutia* Ten. (Kızılçam) Polen Ekstresinin Karaciğer Koruyucu Etkisinin Preklinik Olarak Araştırılması ve Fenolik Asit Analizleri**

Hepatoprotective Effect of Pollen Extract of *Pinus brutia* Ten

***Pinus brutia* (Kızılçam) Polen Ekstresinin Hepatoprotektif Etkisi**

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Abbreviations: ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; ApoE: Apolipoprotein E; AST: Aspartate transaminase; b.w.: Body weight; CAT: Catalase; CCl₄: Carbon tetrachloride; ConA: Concanavalin A; GSH-Px: Glutathione peroxidase; HDL: High density lipoprotein; HPLC: High performance column chromatography; LC: Liquid chromatography; LDH: Lactic dehydrogenase; LDL: Low density lipoprotein; MDA: Malondialdehyde; MO: Missouri; USA; RBC: Red blood cell count; SD: Standard deviation; SOD: Superoxide dismutase; United States of America; WBC: White blood cell count.

Abstract

Objective: Many agents including from herbal sources have been searched to prevent or cure hepatotoxicity. Pollens of *Pinus brutia* Ten. known as red pine (Pinaceae) are used against liver diseases in Anatolian folk medicine.

Materials and Methods: In the current work, the pollen ethanol extract of *P. brutia* was investigated for its possible hepatoprotective activity using CCl₄-induced hepatotoxicity model in mice. Swiss albino mice were inserted in 5 groups and compared with silymarin as the reference. The extract was tested at 100, 200, and 300 mg/kg (b.w.). Phenolic acids were analyzed using HPLC in the extracts as pollens are usually known to be rich in phenolics.

Results: Our data revealed that the extract displayed a better hepatoprotection at dose of 100 mg/kg when compared with silymarin (Legalon®), the reference drug. HPLC analysis indicated presence of *i.e.* protocatechuic acid (0.176 mg/g extract), *p*-hydroxybenzoic acid (0.001 mg/g extract), vanillic acid (0.537 mg/g extract), syringic acid (0.050 mg/g extract), and *tr*-cinnamic acid (0.310 mg/g extract), while the major phenolic acid was vanillic acid.

Conclusion: The outcomes of this study conclude that the red pine pollen extract can serve as a promising hepatoprotective agent. Among the phenolic acids analyzed in the pollen extract, vanillic acid as the major one besides some other phenolic acids detected seems to be responsible for its remarkable hepatoprotective effect.

Key words: *Pinus brutia*, red pine, pollen, hepatoprotective activity, HPLC

Özet

Amaç: Hepatotoksisteyi önlemek veya iyileştirmek için bitkisel kaynaklar dahil olmak üzere birçok bileşik araştırılmaktadır. Kızılçam olarak bilinen *Pinus brutia* Ten. bitkisinin polenleri Anadolu'da halk arasında karaciğer rahatsızlıklarında kullanılmaktadır.

Gereç ve Yöntemler: Bu çalışmada, farelerde CCl₄-nedenli hepatotoksiste modeli ile *P. brutia* polen ekstresinin muhtemel etkisi araştırılmıştır. Swiss albino fareler 5 gruba ayrılmış ve referans olarak silimarin (Legalon®) ile karşılaştırılmıştır. Ekstreler 100, 200, ve 300 mg/kg (v.a.) konsantrasyonlarında çalışılmıştır. Polenlerin genellikle fenolik açıdan zengin olduğu bilindiğinden, ekstrede bulunan fenolik asitler HPLC kullanılarak analiz edilmiştir.

Bulgular: Sonuç olarak, ekstre'nin referans ilaç olan silimarin ile karşılaştırıldığında, 100 mg/kg'lık dozda daha yüksek hepatoprotektif etki gösterdiği belirlenmiştir. HPLC analizi ekstrede protokatesik asit (0.176 mg/g ekstre), *p*-hidroksibenzoik asit (0.001 mg/g ekstre), vanilik asit (0.537 mg/g ekstre), sirinik asit (0.050 mg/g ekstre), ve *tr*-sinnamik asit (0.310 mg/g ekstre) bulunduğunu, en yüksek miktardaki fenolik asidin ise vanilik asit olduğunu ortaya koymuştur.

Sonuç: Çalışmamız neticesinde, kızılçam polen ekstresinin umut verici bir hepatoprotektif ajan olarak kullanılabileceği sonucuna varılmıştır. Polen ekstresinde analiz edilen fenolik asitler arasında, majör olan vanilik asidin yanısıra bazı diğer fenolik asitlerin ekstre'nin gösterdiği hepatoprotektif etkiden sorumlu olduğu düşünülmektedir.

Anahtar kelimeler: *Pinus brutia*, kızılçam, polen, hepatoprotektif aktivite, HPLC

INTRODUCTION

Liver is one of the most important organs that regulate metabolic functions, hormones, and defense mechanisms in the body. On the other hand, liver is exposed to many threats such as alcohol, viruses, and xenobiotics; so the protection of the liver is significant for maintaining liver functions.^{1,2} The genus *Pinus* (Pinaceae) contains approximately 80 species all over the world.³ In Turkey, *Pinus* contains 7 species, which are *Pinus pinaster* Aiton, *P. brutia* Ten., *P. halepensis* Mill., *P. pinea* L., *P. sylvestris* L., *P. nigra* J.F. Arnold, and *P. radiata* D. Don.⁴ *Pinus brutia* Ten. (red pine) spread around Eastern Mediterranean countries such as Turkey,

Greece, Cyprus; Black Sea countries such as Ukraine, Georgia; and in the Caucasus countries.⁵ Different parts of *P. brutia* such as barks, resins, tar, and cones are used for asthma, bronchitis, cancer, diabetes, diarrhea, pneumonia, and tuberculosis in Turkish folk medicine.⁶⁻⁹

Pollens from many kinds of plants have been used as food traditionally for many years even since pre-historic times.¹⁰⁻¹⁶ Pine pollens, which is the male spores of *Pinus*, have been used for liver protection, anti-senility, anti-fatigue, gastrointestinal dysfunction, improvement of sexual function, and cerebral-cardiac blood vessel for many years.^{17,18} The relevant literature survey shows that most of the previous studies on *P. brutia* have been so far conducted on its barks. On the other hand, *P. brutia* barks contain some phenolic compounds such as 4-hydroxybenzoic acid, resveratrol, gentisic acid, vanillin, vanillic acid, catechin hydrate, *p*-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, myricetin, naringenin, caffeic acid, luteolin, and kaempferol.¹⁹ On the other hand, there have been a few studies on phytochemistry and biological activity on the pollens of *P. brutia*.²⁰⁻²⁶

There have been several studies describing hepatoprotective effect of various pollens such as bee pollen, chestnut, rape, *Pinus massoniana*, *Schisandra chinensis*, etc.²⁷⁻³² However, any report on hepatoprotective effect of pollen of *P. brutia* is not available up to date, while it is used against liver diseases around Muğla province in the Aegean region of Turkey (personal communication). Taking this information into account, we aimed to perform the present study in order to determine the claimed hepatoprotective use of the red pine pollen ethanol extract using carbon tetrachloride (CCl₄)-induced liver damage in mice and to identify major phenolic acids in the extract using high performance column chromatography (HPLC).

EXPERIMENTAL

Plant material

The pollens of *P. brutia* trees were collected from the forest area, belonging to General Directorate of Forestry, Ministry of Agriculture and Forestry, in vicinity of Antalya province, Turkey in June, 2015.

Preparation of the extract

The air-dried pollens (51.15 g) of *P. brutia* were macerated with 800 mL ethanol (80 %) for 24 h two times at room temperature. The ethanol macerate was filtrated and evaporated until dryness *in vacuo*. The yield of the crude extract was 24.98 % (w/w).

High-performance liquid chromatography analysis

Chemicals used for HPLC (methanol and formic acid) analysis were of chromatographic grade (Sigma Aldrich, St. Louis, MO, USA). Standards of the phenolic acids, *e.g.* gallic acid (GA), *proCA* (protocatechuic acid), *p*-OHBA (4-hydroxybenzoic acid), vanillic acid (VA), caffeic acid (CA), chlorogenic acid (CA), syringic acid (SA), *p*-coumaric acid (*p*-COU), ferulic acid (FA), *o*-coumaric acid (*o*-COU), rosmarinic acid (RA), and *trans*-cinnamic acid (*tr*-CIN) used in HPLC analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (GmbH, Darmstadt, Germany). Analysis of phenolic acids in the extract was carried out with Agilent 1100 series auto sampler system from Agilent, GL Sciences Inc.

(Waldbronn, Germany) equipped with a system controller, DAD detector (G 1315B, 280 nm), a quaternary LC pump (G1311A). The separation was carried out with a Zorbax Eclipse XDB-C18 column (150 mm, 4.6 mm i.d. and 5 µm particle size) (Agilent, Waldbronn, Germany) with the column temperature set at 25°C. Chromatographic separation was carried out using two solvents system: A) methanol:water:formic acid (10:88:2, v/v/v); B) methanol:water:formic acid (90:8:2, v/v/v), as reported elsewhere.³³ The analyses were performed by using a linear gradient program. Initial condition was 100% A; 0-15 min,

changed to 100% A; 15-20 min, to 85% A; 20-30 min, to 50% A; 30-35 min to 0% A; 36-42 min, went back to 100% A. The flow-rate was 1 mL/min and the injection volume was 10 μ L. Signals were detected at 280 nm. The extract was dissolved in a mixture of methanol and water (1:1, v/v) and injected into the HPLC.

Each compound was identified by its retention time and by spiking with the standards under the same conditions. The identities of phenolic acids were also confirmed with a photodiode array (PDA) detector by comparison with ultraviolet (UV) spectra of standards in the wavelength range of 220–320 nm. Each compound was quantified according to the peak area measurements, which were reported in calibration curves of the corresponding standards. Data are reported as means \pm standard deviations of three independent analyses.

Animals

Swiss albino mice of either sex (50 to 70 g) were maintained under standard animal house conditions fed with commercial mice chow and allowed water *ad libitum*. The experimental protocol was approved by Institutional Ethic Committee constituted by PMAS Arid Agriculture University Rawalpindi for the animal study.

Hepatoprotective activity

Forty mice were divided into eight groups of five each (n=5). Group 1; control group received 0.5 mL of saline (0.9%, v/v) in water. Group 2; animals of this group received 0.5 mL of olive oil (0.5%). Group 3; animals of this group received ethanol (0.2%, v/v). All of these animals received doses once *per* day for the entire period (7 days) by *i.p.* injection, respectively.

Animals of group 4 were administrated *i.p* with CCl₄ dissolved in olive oil at a dose of 0.5 mL/kg/day body weight. Animals of group 4 to 8 were administrated *i.p* with CCl₄ dissolved in olive oil at a dose of 0.5 mL/kg/day body weight (b.w.).

Animals of group 5 were fed with silymarin dissolved in ethanol at a dose of 50 mg/kg/day. Animals of group 6 were fed with the pollen ethanol extract at dose of 100 mg/kg once *per* day by gavage, while animals of group 7 and 8 were fed with the extract at 200 and 300 mg/kg of doses, respectively, once *per* day by gavage. At the end of the experiments, all mice were sacrificed, serum was collected, the livers were removed, and washed with ice-cold physiological saline.

Acute oral toxicity study

An acute toxicity study was conducted for a selected suitable dose of plant extracts.³⁴ About 100 to 300 mg of the dried pollen extract was dissolved each in 5 ml of ethanol and one ml of the dose was given to animals by gavage.

Biochemical analysis

Organs were homogenized in 0.1 M Tris HCl buffer (pH 7.4) to give a 10% homogenate. This homogenate was used for the estimation of, triglyceride, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol by using commercial kits (Randox Laboratory) and enzymatic method of Bierman.³⁵ The enzymes; *e.g.* alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin, red blood cell count (RBC), white blood cell count (WBC), and platelet levels were estimated by using their respective diagnostic kits and Auto-analyzer (Merck). The body weights of animals were calculated by measuring weight before and after treatment with the extract.

Statistical analysis

All values are expressed as mean \pm standard deviation (SD). One-way analysis of variance was used to find out the consequences of different treatments by using computer software graph pad prism 5.0.

RESULTS AND DISCUSSION

HPLC analysis

The pollen ethanol extract was analyzed by HPLC, which led to identification and quantification of following phenolic acids, *i.e.* protocatechuic acid (0.176 mg/g extract), *p*-hydroxybenzoic acid (0.001 mg/g extract), vanillic acid (0.537 mg/g extract), syringic acid (0.050 mg/g extract), and *tr*-cinnamic acid (0.310 mg/g extract). The major phenolic acid was found to be vanillic acid (Fig. 1).

Results on liver enzymes, lipid profile, and blood cells

When the results of the extract on lipid profile of the mice were examined, the pollen extract reduced triglyceride and total cholesterol level significantly at 100 mg/kg dose. Nevertheless, the extract also reduced HDL level (Table 1). Although the extract applied at 200 and 300 mg/kg of doses decreased the triglyceride and cholesterol levels, the activities are lower than the activity of 100 mg/kg extract. The extract at dose of 100 mg/kg exhibited a better reducing effect than that of silymarin.

Our data indicated that plasma levels of AST and ALT enzymes were notably raised in rats treated with CCl₄. The ALT, AST, alkaline phosphatase (ALP) and bilirubin levels diminished drastically with extract at the 100 mg/kg, 200 mg/kg, and 300 mg/kg doses (Table 2). The activity of the extract on these enzymes and proteins was better than that of the reference drug; silymarin. Considering the pollen extract treatment on blood cells, the counts of reduced RBC and WBC was increased, so the extract presented similar effect with silymarin (Table 3).

Histopathologic findings

Histopathologic data displayed that the liver from healthy mice group showed normal hepatocyte structures. However, after administration of CCl₄, results in complete loss of liver architecture was observed, whereas the damaging effects of CCl₄ were reversed by treatment with the pollen extract (Fig. 2). The recovery of tissue was significant, when it was treated with 300 mg/kg dose of the pollen extract which indicated that regeneration of the tissues was dose-dependent (Fig. 2e), while similar results have been led by the hepatoprotective agent used, *e.g.* silymarin (Fig. 2b).

Liver damage induced by CCl₄ in rats is one of the most preferable experimental models to study hepatoprotection. Several studies have been performed to determine hepatoprotective or lipid-lowering effects of various pollen extracts as aforementioned since the liver is known to play the foremost role in lipid transformations.²⁷⁻³² Pollen grains are the male tiny particles which are released from trees, weeds and grasses. Main function of pollen grains is to fertilize other parts of plants. An early study on a flower pollen extract (0.4 ml/100 g b.w.), in which name of the extract was mentioned as cernitins, was described to possess hepatoprotective effect in liver damaged by alcohol by reducing serum AST and ALT levels.

Another pollen extract from a flower, whose scientific or local name was not indicated, was reported to exert hepatoprotective effect *via* normalization of AST, ALT, and ALP levels as

well as hypolipidemic and hypocholesterolemic activity in testosterone-androgenized rats.³⁶ The same extract was also shown to have a protective effect in paracetamol-induced hepatotoxicity model in mice along with hypolipidemic effect.²⁷

Bee pollen from China was earlier demonstrated to be effective in decreasing lipofuscin (fine yellow-brown pigment granules composed of lipid-containing residues of lysosomal digestion) amount in cardiac muscle, liver, brain as well as adrenal gland cells in NIH mice.³⁷ Besides, the bee pollen extract with rich polyphenol content from Poland was tested for its anti-atherogenic effect in apolipoprotein E (ApoE)-knockout mice at two doses of 0.1 and 1 g/kg body weight (b.w.) during 16 weeks.³² The extract led to decrease in triglyceride and low density lipoprotein (LDL) levels and displayed a complete protection for coronary arteries at 1 g/kg b.w. The effect was speculated to correlate with polyphenol content of the pollen extract, which was supported by the histopathological data on cardiac vessels. In another study, strong hepatoprotective effect of the pollen ethanol (70%) extract prepared from *Phoenix canariensis* hort. ex Chabaud as one of the palm species was shown in adult male Wistar albino rats.³⁸ The pollen extract was found to contain isorhamnetin-3-O-rutinoside and rutin as the phenolic compounds, which were concluded to contribute to its hepatoprotective effect. Yildiz, Can, Saral, Yulug, Ozturk, Aliyazicioglu, Canpolat, Kolayli²⁹ studied hepatoprotective effect of chestnut bee pollens collected from western Black Sea region of Turkey at doses of 200 and 400 mg/kg/day through CCl₄-induced liver damage in Sprague-Dawley rats. Particularly, bee pollen extract led to a significant decrease in AST and ALT levels at 400 mg/kg of dose, whereas silybinin administered at 50 mg/kg dose in rats revealed a better hepatoprotective effect as compared to that of the bee pollen extract at 200 mg/kg. Phytochemical analysis of the chestnut pollen pointed out to presence of total phenolic compounds (28.87 mg gallic acid equivalent/g), total flavonoids (8.07 mg quercetin equivalent/g), total anthocyanins (92.71 mg cyanidin-3-glucose equivalent/kg) as well as total carotenoids (29 mg β -carotene equivalent/100 g). Since antioxidant activity of the extract in that study was also consistent with its hepatoprotective effect, the phenolic compounds analyzed in the extract were commented to contribute to its antioxidant and hepatoprotective effect. Similarly, the pollen extract of *Schisandra chinensis* of Chinese origin was reported to exert strong antioxidant and hepatoprotective effects against hepatotoxicity induced by CCl₄, which is consistent with our data.²⁸ Recently, Taishan *Pinus massoniana* pollen extract was shown to have a marked hepatoprotection in CCl₄-induced oxidative stress in the liver of rats tested at the doses of 100, 200, and 400 mg/kg b.w., where AST, ALT, ALP, lactic dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels were significantly reduced.³¹ The strong hepatoprotective action of this pollen extract was concluded to be resulted from its polysaccharide content, which was described as an acidic heteropolysaccharide with glucose and arabinose as the key constituent monosaccharides. On the other hand, Rzepecka-Stojko, Kabala-Dzik, Kubina, Jasik, Kajor, Wrzesniok, Stojko³² mentioned a positive correlation between polyphenols present in bee pollen and its hepatoprotective and other biological activities.

In another study on honeybee products including chestnut honey, pollen, propolis, and royal jelly, their hepatoprotective activity was investigated using CCl₄-induced model in rats.³⁹ Recovery of hepatotoxicity was observed by measuring AST and ALT levels as well as oxidative stress parameters such as MDA, SOD, and catalase (CAT). Use of bee pollen due to its discernible bioactivities was also suggested to be beneficial not only for human health, but also animal health (up to 20 g/kg diet) for production and health patterns of livestock.⁴⁰

On the other hand, vanillic acid was detected as the major phenolic compound in the extract along with some other phenolic acids. In fact, vanillic acid was reported to have a strong hepatoprotective activity in a number of plant or mushroom extracts.⁴¹ For instance; vanillic and syringic acids were reported to be the active constituents in edible mushroom *Lentinula edodes* (shiitake) in concanavalin A (ConA)-induced liver injury in mice.⁴² In another study, *L. edodes*, rich in vanillic and syringic acids, was shown to possess a strong hepatoprotection in mice with acute and chronic liver injury induced by CCl₄, which is in good agreement with our findings.⁴³ Phenolic composition of a Taiwanese mushroom species, *Xylaria nigripes* with high amount of epicatechin, catechin, and *p*-coumaric acid, was interpreted to be related to its activity against *in vivo* CCl₄-induced hepatotoxicity by Song, Ko, Lai, Ng.⁴⁴

Consistently, the leaf methanol extract of *Capparis spinosa* of Tunisian origin, found to contain rutin, resveratrol, coumarin, epicatechin, luteolin, catechin, kaempferol, vanillic acid, and gallic acid led to a notable decrease in serum ALT, AST, and LDH levels in CCl₄-induced acute liver damage as well as in amount of hepatic malondialdehyde (MDA) formation, whereas it raised the activities of SOD, CAT, and GPx, and repaired injury occurred in the liver.⁴⁵ In a similar study, a strong hepatoprotective effect was observed with the hot aqueous extract prepared from the leaves of *Asparagus albus* in male Wistar rats by Serairi-Beji, Wannes, Hamdi, Tej, Ksouri, Saidani-Tounsi, Lachaal, Karray-Bouraoui⁴⁶, where some phenolic acids; *e.g.* gallic acid, vanillic acid, and 3,4-dimethoxybenzoic acid along with several flavonoids; *e.g.* catechin, rutin, and quercetin were identified through HPLC. The authors commented that hepatoprotective effect of the extract was correlated with its polyphenolic content. A remarkable *in vivo* hepatoprotection was caused by *Artocarpus lakoocha* fruits which contain chromatotropic, gallic, vanillic, cinnamic, and ferulic acids as well as quercetin and kaempferol, which is consistent with our study.⁴⁷ A *in vivo* study parallel to ours was conducted on hepatoprotective effect induced by thioacetamide of the ethanol extract of *Prunus amygdalus* stem and leaves from Egypt.⁴⁸ Analysis of the extract using LC-DAD-ESI-MS in the negative ion mode indicated presence of a number of phenolics including vanillic and homovanillic acids, which were correlated to hepatoprotection by the plant. Actually, all these previous studies have underlined a considerable contribution of vanillic acid to hepatoprotective activity of a number of plants, which may also lead a comment by us that vanillic acid might be the main compound responsible for hepatoprotective effect of the red pine pollen extract as the major compound.

CONCLUSION

In conclusion, red pine pollen extract exhibited a remarkable and dose-dependent hepatoprotection against CCl₄-induced liver damage in mice. Phenolic compounds, vanillic acid in particular, existed in the pollen extract could be responsible for its notable hepatoprotective effect. It can be commented that red pine pollen extract could serve as a promising hepatoprotective agent.

Conflicts of interest: No conflict of interest was declared by the authors.

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Table 1. Effects of the pollen extract on lipid profile of mice

Group	Treatment	Triglyceride (mg/dL)	Cholesterol (mg/dL)	HDL (mg/dL)
1	Normal (vehicle)	81.15 ± 1.22	65.38 ± 2.34	59.24 ± 2.36
2	Olive oil group	75.27 ± 2.51	71.34 ± 1.34	45.43 ± 1.23
3	Ethanol group	82.35 ± 2.37*	66.24 ± 2.14*	54.26 ± 1.45*
4	CCl ₄ + Olive oil	142.35 ± 2.37	132.35 ± 0.39	112.35 ± 1.34
5	Silymarin + Olive oil	89.31 ± 1.32	74.12 ± 1.25	62.17 ± 0.38
6	Pollen extract at 100 mg/kg	82.31 ± 1.27*	59.23 ± 2.14*	47.26 ± 1.45*
7	Pollen extract at 200 mg/kg	91.38 ± 2.76	88.34 ± 1.32	51.24 ± 2.35
8	Pollen extract at 300 mg/kg	98.35 ± 1.52	89.65 ± 0.57	52.78 ± 1.45

*Significant ($p < 0.05$) values vs control/normal and expressed as mean ± SD, n=5

Table 2. Effects of the pollen extract on liver enzyme and proteins

Group	Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirubin (mg/dL)
1	Normal (vehicle)	81.15 ± 1.21	65.38 ± 2.34	59.24 ± 2.36	0.146 ± 0.028
2	Olive oil group	75.27 ± 1.51	62.54 ± 1.33	45.43 ± 1.25	0.245 ± 0.051
3	Ethanol group	62.35 ± 2.37*	56.24 ± 2.14*	44.26 ± 1.45*	0.691 ± 0.596
4	CCl ₄ + Olive oil	162.35 ± 2.37	152.35 ± 0.39	142.35 ± 1.34	1.289 ± 0.19
5	Silymarin + Olive oil	89.31 ± 1.32	84.12 ± 1.25	72.17 ± 0.38	0.571 ± 0.22
6	Pollen extract at 100 mg/kg	68.31 ± 1.27*	69.23 ± 2.14*	67.26 ± 1.45*	0.169 ± 0.33
7	Pollen extract at 200 mg/kg	61.38 ± 2.76	58.34 ± 1.32	61.24 ± 2.35	0.186 ± 0.02
8	Pollen extract at 300 mg/kg	57.48 ± 1.53	61.54 ± 0.78	62.35 ± 2.57	0.192 ± 0.01

*Significant ($p < 0.05$) values vs control/normal and expressed as mean ± SD, n=5

Table 3. Effects of the pollen extract on blood cells

Group	Treatment	RBCs ($10^6/\text{mm}^3$)	WBCs ($10^3/\text{mm}^3$)	Platelets ($10^3/\text{mm}^3$)
1	Normal (vehicle)	4.65 ± 0.31	5.38 ± 1.34	259.24 ± 1.36
2	Olive oil group	4.87 ± 0.51	6.24 ± 0.33	245.23 ± 1.26
3	Ethanol group	3.25 ± 0.37	3.64 ± 0.14	234.26 ± 1.45
4	CCl_4 + Olive oil	1.25 ± 0.37	1.55 ± 0.39	132.35 ± 1.54
5	Silymarin + Olive oil	3.91 ± 0.32	4.42 ± 0.25	242.15 ± 1.38
6	Pollen extract at 100 mg/kg	$4.81 \pm 0.27^*$	5.93 ± 0.14	243.56 ± 2.15
7	Pollen extract at 200 mg/kg	4.68 ± 0.76	4.84 ± 0.32	241.22 ± 1.37
8	Pollen extract at 300 mg/kg	4.98 ± 0.53	4.91 ± 0.35	248.12 ± 0.58

*Significant ($p < 0.05$) values vs control/normal and expressed as mean \pm SD, n=5

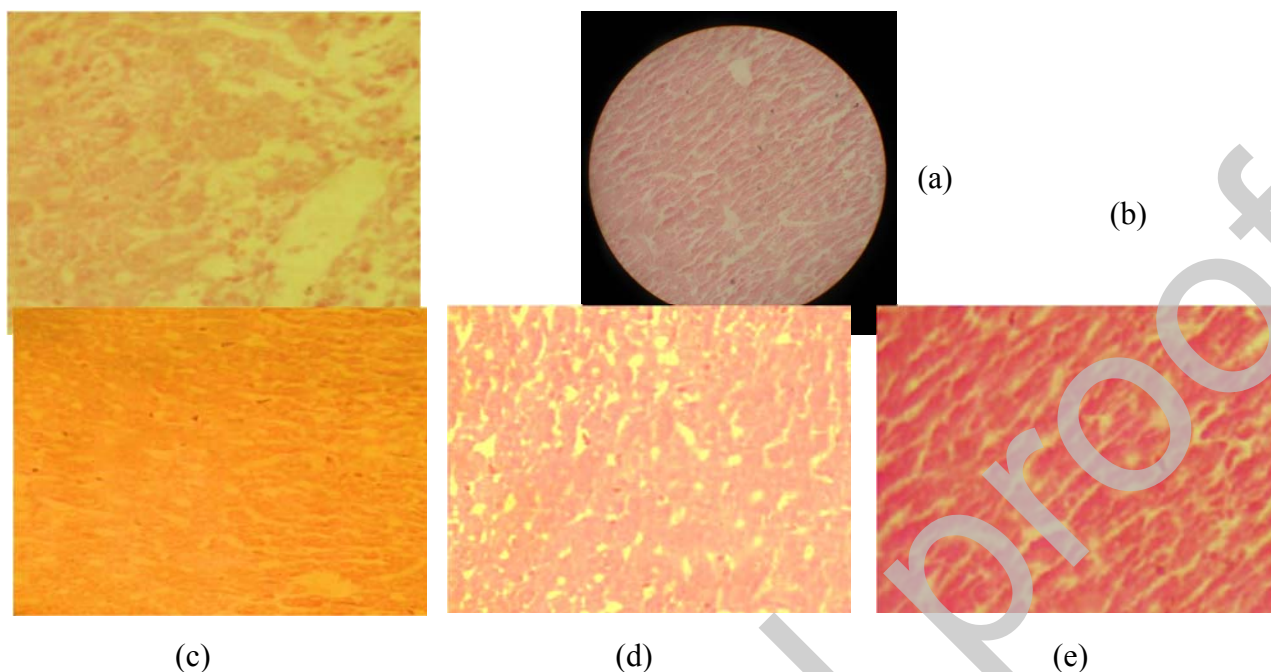


Figure 2. Histopathological results with cellular organization by red pine pollen extract (a) in CCl₄-induced liver damage; (b) by silymarin (c) pollen extract at 100 mg/kg; (d) pollen extract at 200 mg/kg; (e) pollen extract at 300 mg/kg

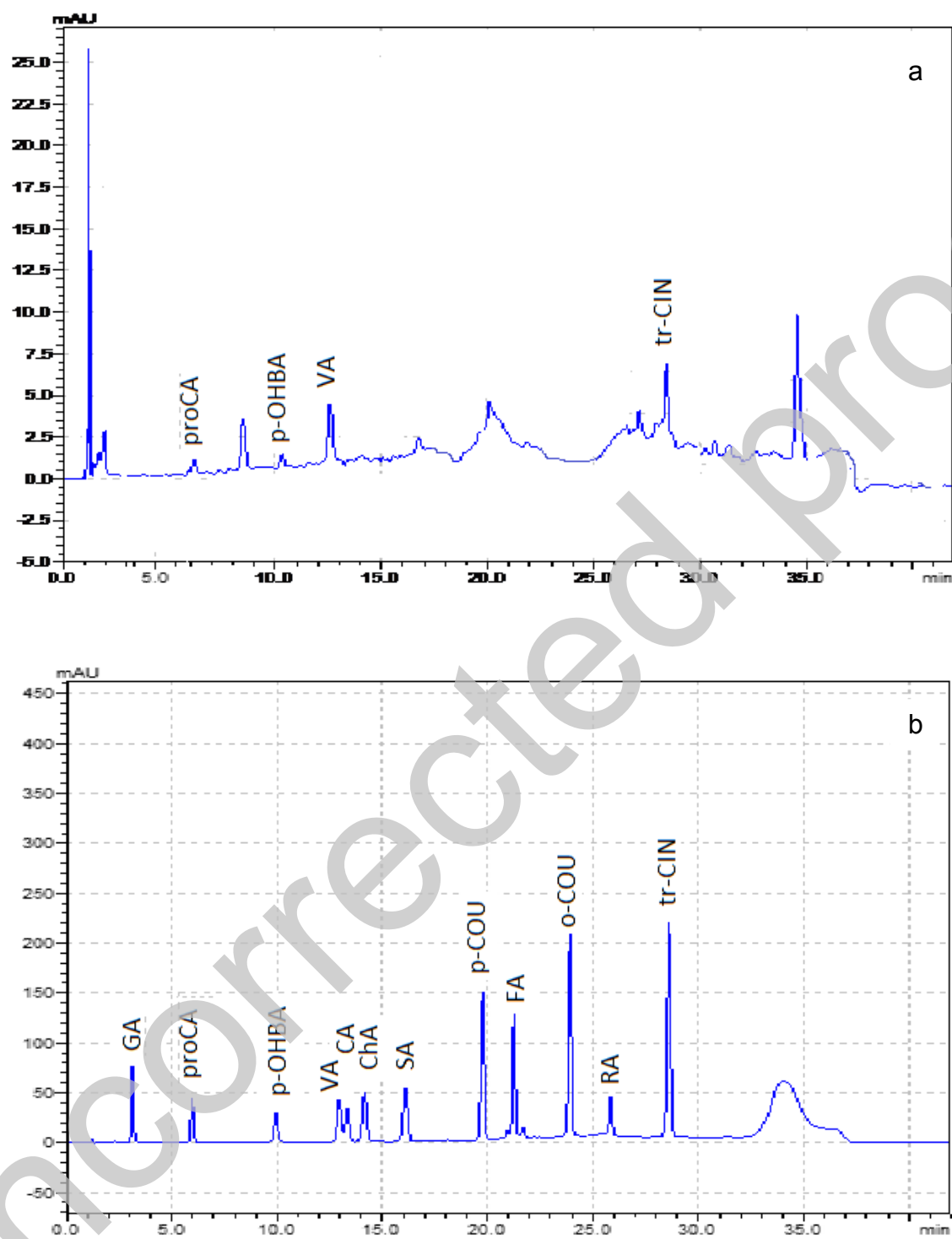


Figure 1. HPLC chromatograms of the pollen extract (a) and standard phenolic acids (b)