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RESEARCH PAPER

The optimal dose of *Protaetia brevitarsis seulensis* (Kolbe) for providing protective effects against CCl_4-induced hepatic damage in rats

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Abstract

Larvae of edible insects *Protaetia brevitarsis seulensis* (Kolbe) is well known in Korean traditional medicine for improvement of blood circulation. This study focused on the optimal dose of *P. brevitarsis* larvae for protection against CCl_4-induced hepatic injury in rats. The optimal dose of *P. brevitarsis* larvae was estimated by intraperitoneal administration of 100, 300, 1,000, or 3,000 mg/kg, once daily for 5 days, to CCl_4-induced hepatotoxicity rats. CCl_4 administration showed much higher levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, compared to control groups. Also, histopathological results showed extensive liver lesions, characterized by substantial necrosis, steatosis, and hemorrhage. In addition, an increased prothrombin time (PT) and activated partial thromboplastin time (aPTT) level and decreased fibrinogen level was observed in the CCl_4 groups. In contrast, the treatment of *P. brevitarsis* larvae showed significant protection against the CCl_4-induced hepatotoxicity reducing the serum AST and ALT activities ($P < 0.05$); the lowest level of serum AST and ALT activities were observed in the group treated with 100 and 300 mg/mL, respectively. Moreover, the treatment with *P. brevitarsis* larvae at 100 and 300 mg/mL significantly reduced PT and aPTT values, and increased the fibrinogen level, when compared to the CCl_4-treatment alone ($P < 0.05$). In intoxicated rats, the doses of *P. brevitarsis* larvae greater than 300 mg/kg did not enhance its protective action (1,000 mg/mL group) or was not as effective (3,000 mg/mL group). The results indicate that *P. brevitarsis* larvae revealed a protective effect against CCl_4 acute hepatotoxicity including coagulation disorders and the optimum dose was 300 mg/kg.

Key words: Carbon tetrachloride, Coagulation disturbance, Hepatoprotective, *Protaetia brevitarsis seulensis* (Kolbe), Pycnogel

Introduction

Liver diseases are a serious health problem, because the liver is important for the regulation of physiological functions, including detoxification and deposition of xenobiotics (Ilyas *et al*. 2016; Wolf 1999). Therefore, when the liver has been damaged by hepatotoxic reagents, such as viruses, alcohol, and other chemical reagents, the whole-body metabolism is seriously disturbed. The liver may be adversely affected by steroids, vaccines, and antiviral drugs used to treat liver diseases, especially when administered long term. Therefore, chemical therapeutics should be substituted by herbal and/or insect extracts with improved effectiveness and safety profiles.

Carbon tetrachloride (CCl_4) has been commonly used to research the mechanism of hepatic injury in animal models because the chemical hepatic injuries induced by CCl_4 in rat and mouse models were found to be similar to those in humans (Jhonston & Kroening 1998). Previous studies reported that CCl_4 administration induced liver-related diseases, such as degeneration of the blood coagulative and fibrinolytic functions in experimental animals

(Vazquez *et al*. 1990). Since most coagulation and fibrinolysis-related process are synthesized in the liver, abnormal coagulation process can be affected by acute or chronic liver injury/disease (Arıcı & Çetin 2011).

The larvae of *Protaetia brevitarsis seulensis* (Kolbe), belonging to family Cetoniidae, are white-spotted flower chafers, which are widely distributed in East Asian countries and in Europe (Kwon 2009). *P. brevitarsis seulensis* has been reported to possess physiological functions such as anti-oxidant, anti-microbial, and anti-cancer activities, and to exhibit protective effects against liver-related disorders (Hah *et al*. 2005; Park *et al*. 1994; Yoo *et al*. 2007). Furthermore, a study by Kang *et al*. (2012) has demonstrated that diet containing extracts of *P. brevitarsis* larvae-fed fermented *Aloe vera* inhibits lipid peroxidation and increased glutathione contents in liver of CCl_4-induced intoxicated rat. Although *P. brevitarsis* larvae have not been fully elucidated as protective mechanisms, their remarkable biological profiles in various diseases have attracted much attention, and they have been safely used for long periods of time in many countries. However, there is no systematic characterization of the effective dosages of *P. brevitarsis* larvae for protection against CCl_4-induced liver injuries including coagulation disturbance. Therefore, the purpose of this study was to determine the optimum dose by characterizing the limits of *P. brevitarsis* for protection in CCl_4-induced hepatic injury rat and to provide basic research data as functional ingredient.

Pycnogenol (PYC), plant extract from the bark of the French maritime pine *Pinus pinaster,* containing 65–75% of catechin and epicatechin, is commonly consumed as a dietary food supplement. Therefore, PYC has biological benefits in many chronic diseases such as diabetes, obesity, dyslipidemia, and oxidative stress-related damage (Atta *et al*. 2020; Gulati, 2015; Parveen *et al*. 2013). Furthermore, many studies demonstrated the protective effects of PYC against the deterioration of hepatic functions due to free radicals in toxic- and chemical-induced hepatotoxicity (Yang *et al*. 2008). Also, PYC was compared as a reference in this study.

Materials and methods

Materials

The *Protaetia brevitarsis* larvae powder used in our experiments was obtained from Hangum Biotech, Inc., Jecheon-si, Korea. Pycnogenol (US patent #6,372,266), which is extracted from the bark of the French marine pine, was donated by Now Foods Ltd (Bloomingdale, IL, USA). All chemicals used in the experiment were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA), and were of analytical grade.

Animals

Sprague–Dawley rats (160–200 g) aged 6 weeks were obtained from Samtako Bio Korea (Seoul, Korea). The rats were housed in polycarbonate cages in a controlled room at 22 ± 2°C and 55–60% relative humidity where a 12-h light/12-h dark cycle was provided. The animals had *ad libitum* access to commercial pellet chow and sterilized drinking water. All procedures complied with the Institutional Animal Care (Approval number: CBIACUC-19262ET1), according to the Animal Protection Act [Enforcement Date 27 Aug. 2019] [Act No.16544, 27 Aug. 2019, Partial Amendment].

Experimental design

In the experiment, 64 rats were evenly divided into eight groups. Group 1 served as normal control and was administered distilled water intraperitoneally for 5 days. Group 2 (reference group) was administered *P. brevitarsis* larvae (3,000 mg/kg, dissolved in physiological saline) intraperitoneally once daily for 5 days at dose level of 10 mL/kg. To induce live injury, the rats in Groups 3–8 were administered CCl_4 (4.0 mg/kg, 25% CCl_4 in olive oil) intraperitoneally twice a week. Group 3 (CCl_4 group) was administered CCl_4 only. Group 4 was administered PYC (500 mg/kg, dissolved in physiological saline) intraperitoneally daily for 5 days at dose level of 10 mL/kg. Groups 5–8 were administered *P. brevitarsis* larvae at 100, 300, 1,000, and 3,000 mg/kg intraperitoneally, respectively, and dose level of 10 mL/kg for 5 days. All treated rats were anesthetized by isoflurane for blood sample collection 24 h after CCl_4 administration. Blood samples were gathered in sodium citrate tubes under anesthetic and subjected to centrifugation for 20 min at 3,000 rpm, and the serum was separated and stored at −70°C for biochemical analysis. Liver tissue samples were collected for histopathological examination and biochemical analysis.

Liver biomarker assay

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a commercial automatic biochemical analyzer (Hitachi 2030, Hitachi, Tokyo, Japan), according to the manufacturer's instructions.

Coagulation assays

Prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen levels were measured by automatic coagulation analyzers (CA660, Sysmex Co., Kobe, Japan).

Histopathological assays

Liver tissues were dissected, fixed in 10% buffered formalin, and then embedded in paraffin. Afterwards 4 μm thick sections were prepared, stained with Hematoxylin and eosin (H&E), and then observed by light microscopy. The histological scoring of hepatic damage (necrosis, steatosis, and hemorrhage) was evaluated and graded (Grade 0, none; 1, minimal; 2, mild; 3, moderate; 4, severe).

Statistical analysis

The data are expressed as the mean ± standard deviation. Means were compared between groups using one-way analysis of variance (ANOVA) by Duncan's multiple range at $P < 0.05$. SPSS software version 21 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Effect of *P. brevitarsis* larvae on serum biochemical parameters

After the administration of the CCl_4, serum AST and ALT activities increased drastically, indicating that severe hepatotoxicity was caused by the CCl_4 ($P < 0.05$). In contrast, no treatment-related effect was observed on the absolute or relative weight of the liver and necropsy findings in the CCl_4-treated rats (data not shown). The protective effects of treatment with *P. brevitarsis* larvae against abnormal liver function biomarkers induced by CCl_4 are presented in Figure 1. The CCl_4 group showed much higher activities of serum AST and ALT compared to the control group, respectively. The serum AST activities significantly decreased in 100, 300, and 1,000 mg/kg groups ($P < 0.05$), but at the dose of 3,000 mg/kg, AST level started to elevate. Meanwhile, the level of ALT activities was decreased from 100 mg/kg group to 300 mg/kg group, at which the lowest ALT level was detected. In addition, at the subsequent dose (1,000 mg/kg), ALT level began to increase, and restored to CCl_4 level at 3,000 mg/kg group. In particular, the ameliorative liver function value in the group treated with *P. brevitarsis seulensis* at lower dose of 100 or 300 mg/kg was superior to that of the PYC-treated group (Fig. 1).

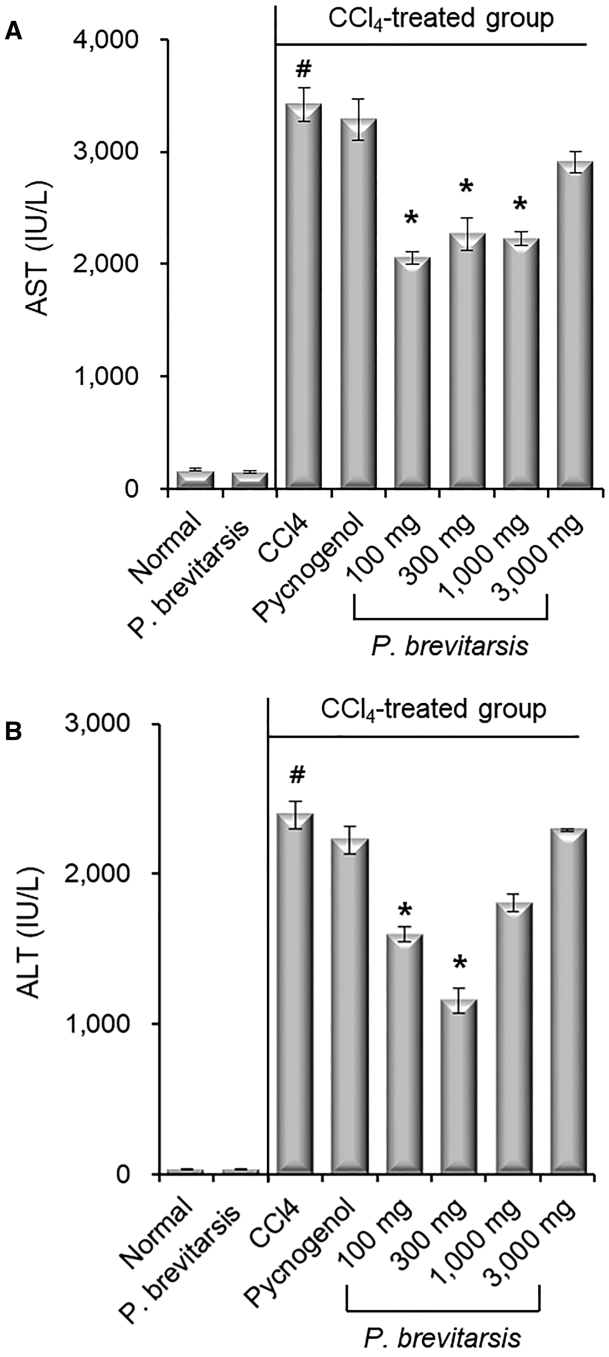

FIGURE 1 Effects of *Protaetia brevitarsis* larvae on serum liver function parameters for (A) aspartate aminotransferase (AST) and (B) alanine aminotransferase (ALT) in CCl_4-intoxicated rats.# $P \leq 0.05$ vs. normal control, * $P \leq 0.05$ vs. CCl_4 control.

Effect of *P. brevitarsis* larvae on coagulation markers

Blood-coagulation factors, such as PT, aPTT, and fibrinogen levels, were measured. As shown in Figure 2, compared to the normal group, treatment with *P. brevitarsis* larvae alone neither significantly affected PT and aPTT nor decreased the fibrinogen levels. PT and aPTT were prolonged significantly, and the plasma fibrinogen level decreased significantly in the CCl_4 group compared to the control group in serum ($P < 0.05$). When compared to the CCl_4-only treated group, treatment with 100 or 300 mg/kg *P. brevitarsis* larvae prior to the administration of CCl_4 led

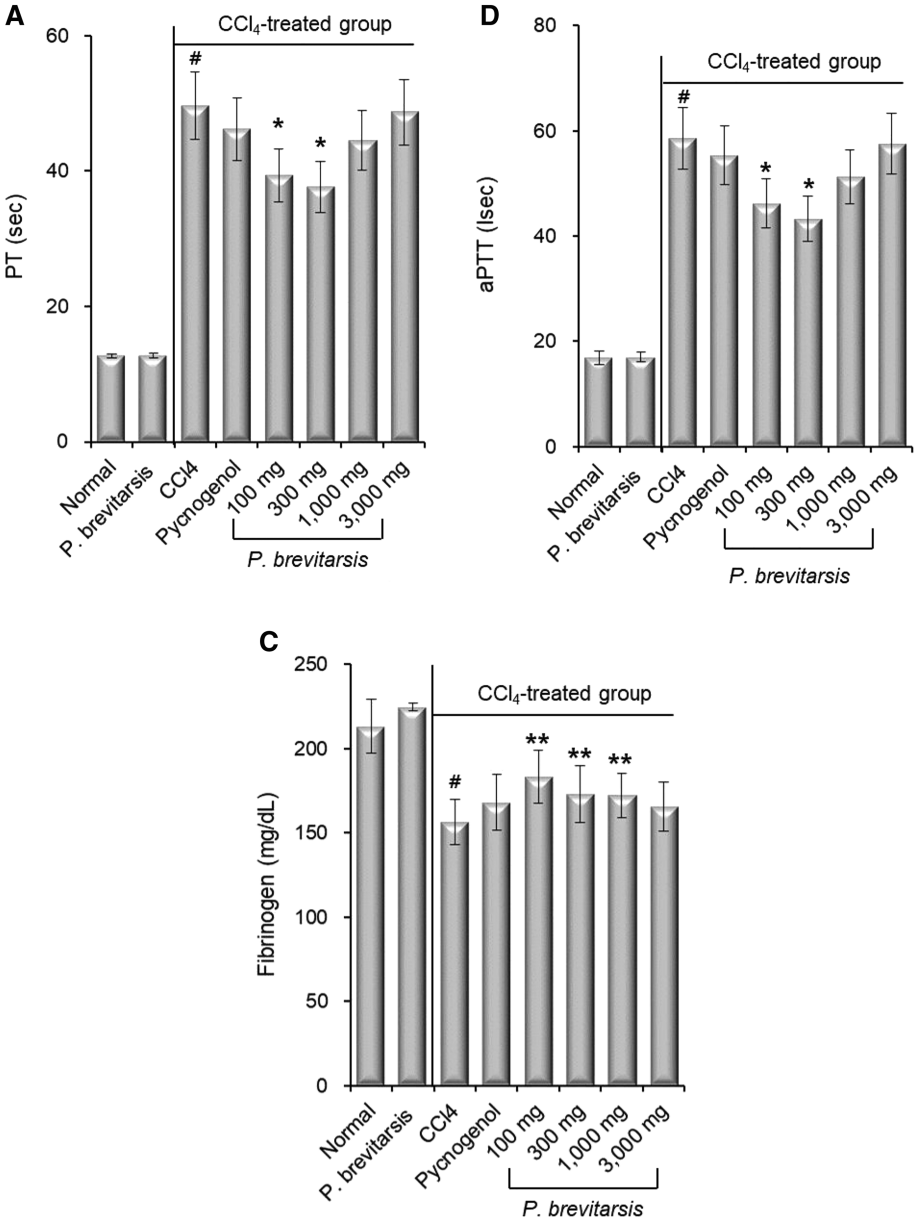

FIGURE 2 Effects of of *Protaetia brevitarsis* larvae on the coagulation parameters for (A) prothrombin time (PT), (B) activated partial thromboplastin time (aPTT), and (c) fibrinogen level parameters in CCl_4-intoxicated rats. $^{\#}P < 0.05$ compared with the control group. *Significantly lower than the CCl_4 group ($P < 0.05$) ** Significantly higher than the CCl_4 group ($P < 0.05$).

to a significant decrease ($P < 0.05$). However, at the dose of 1,000 mg/mL, the both level of PT and aPTT increased again near to CCl4 level. In fibrinogen level (Fig. 2C), the all *P. brevitarsis* larvae treated group showed higher level than CCl_4 group, and highest level was detected in the group treated with 100 mg/kg.

Histopathology

The protective effect identified through biochemical assays was confirmed by histopathological observations and parameters of the H&E stained liver tissues (Fig. 3 and 4). The liver tissues from control rats showed a classical structure, with normal central veins and hepatic lobules (Fig. 3A). Similarly, the liver tissues from the rats treated with *P. brevitarsis* larvae (Fig. 3B) alone were also shown to be normal. In contrast, the CCl_4-treated group had drastic changes in the liver, characterized by broad infiltration of inflammatory cells, fatty acid accumulation, and focal necrosis, as well as congestion of the central vein (Fig. 3C). However, treatment with *P. brevitarsis* larvae attenuated the histological characteristics of liver injury, as confirmed by less fatty changes and by decreased fibrosis and apoptosis (Fig. 2E, 2F and 2G), except for the group treated at a dose of 3,000 mg/kg (Fig. 2H). In addition, the improvement of liver histology by the treatment with *P. brevitarsis* larvae at 100

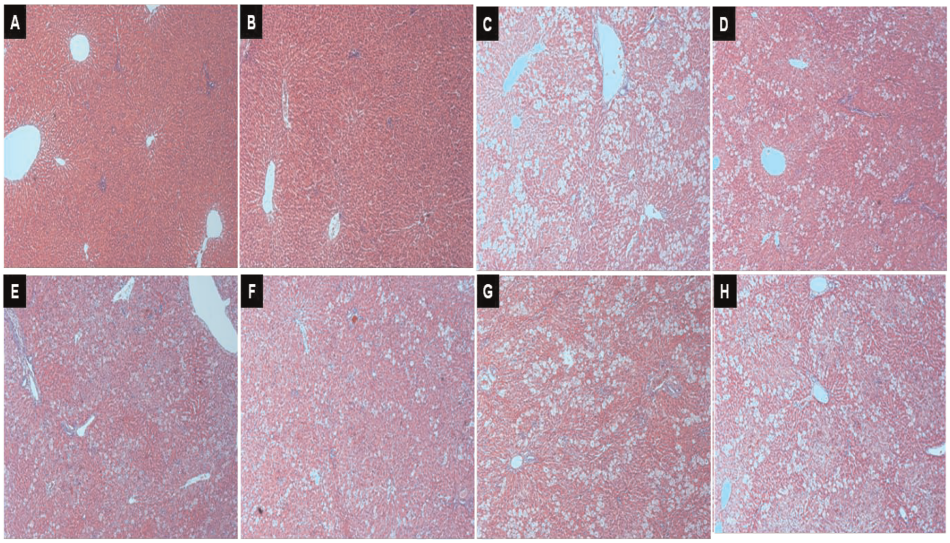

FIGURE 3 Histological images in the control group (A), groups with *Protaetia brevitarsis* powder 3,000 mg/kg (B), CCl_4-intoxicated groups (4 mL of 25% CCl_4 in olive oil) (C), CCl_4-intoxicated groups treated with pycnogenol 500 mg/kg (D), CCl_4-intoxicated groups treated with *Protaetia brevitarsis* powder 100 mg/kg (E), 300 mg/kg (F), 1,000 mg/kg (G), or 3,000 mg/kg (H). All sections were stained with haematoxylin and eosin (400 × for all panels).

or 300 mg/kg was significant, and this was comparable to that treated by PYC (Fig. 2D).

Discussion

Edible insects have been paid considerable attention in the food or pharmaceutical field as a new biological resource. Among the edible insects, *P. brevitarsis* larvae were considered useful in the prevention of diverse liver injuries caused by oxidative stress. In the present study, the ability of *P. brevitarsis* larvae to protect against CCl_4-induced hepatotoxicity and coagulation disturbance was investigated in comparison with that of PYC.

The liver is the most vital organ for the detoxification of endogenous and exogenous metabolites in the human body since it is the target organ of all toxic chemicals. CCl_4 is a hepatotoxin that is the most commonly used to cause acute liver injuries, as has been demonstrated by much research literature on experimental animals (Jhonson and Kroening, 1998). In addition, the liver plays an important role for regulating hemostatic systems such as the clotting and fibrolytic process, and platelet aggregation. Hence, acute or chronic liver injury/diseases are fully concerned with coagulation disorders such as reduced synthesis of coagulation and fibrolysis process, hyperfibrinolysis, deficit of platelets, and accelerated intravascular coagulation (Amitrano *et al*. 2002).

As expected, the administration of a single oral dose of CCl_4 at 4 mg/kg led to a dramatic elevation in the serum AST and ALT activities and an increased prevalence and severity of histopathological hepatic lesions in rats indicating that the single oral dose of CCl_4 induced significant hepatotoxicity. However, the protective effect of treatment with *P. brevitarsis* larvae against CCl_4-induced hepatotoxicity including coagulation disturbance in rats was significant. Serum aminotransferase activities (e.g. AST and ALT) have been well known to be indicators of liver injury, which has led to alteration of their transport function and membrane permeability, resulting in leakage of enzymes from the cells (Yang *et al*. 2008). Therefore, the severe damage to hepatic tissue membranes induced by CCl_4 can be identified with the marked release of AST and ALT into the circulation. In the present study, the serum AST and ALT activities of CCl_4 (at 4 mL/kg) group were drastically increased, indicating that the administration of CCl_4 induced an acute hepatotoxicity (Fig. 1). These effects were confirmed by histopathological evidence such as extensive hepatocellular degeneration and necrosis, fatty changes, inflammatory cell infiltration, congestion, and sinusoidal dilatation (Fig. 3 and 4). The results are consistent with those of previous studies (Lee & Jeong 2002; Recknagel *et al*. 1989; Wong *et al*. 2003). Treatment with *P. brevitarsis* larvae significantly alleviates the elevated serum AST and ALT activities induced by CCl_4 in a dose-dependent manner ($P < 0.05$), indicating the protective effects of *P. brevitarsis* larvae against the acute intoxication of CCl_4.

It is well known that the aPTT is used to measure to the intrinsic pathway coagulation, while PT is associated with the extrinsic pathway coagulation in plasma (Dang *et al*. 2015). In the present study, aPTT and PT values were significantly prolonged in the CCl_4-treated group when compared to normal control groups ($P < 0.05$), which can be probably ascribed to the depression of intrinsic or extrinsic coagulation factors (Okazaki *et al*. 1986; Vazquez *et al*. 1990). Treatment with *P. brevitarsis* larvae at 100 and 300 mg resulted in a significant decrease in PT and aPTT compared with the CCl_4 group ($P < 0.05$). This result suggests that *P. brevitarsis* larvae

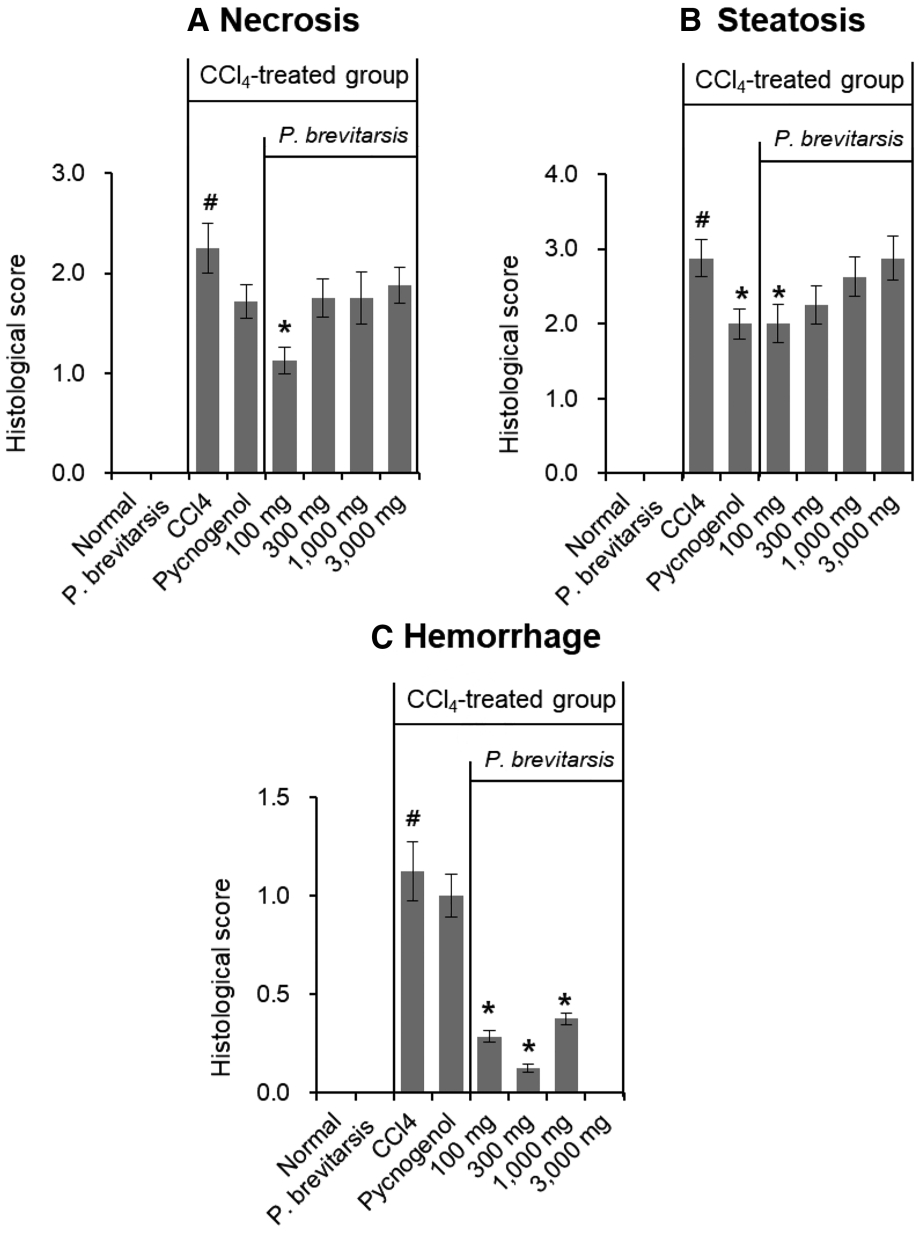

FIGURE 4 Effects of of *Protaetia brevitarsis* larvae treatment on histological score for (A) necrosis, (B) steatosis, and (C) hemorrhage in CCl_4-intoxicated rats.

contributed to the regulation of the coagulation process in rats with CCl_4–induced liver injury. The fibrinogen level in the CCl_4 group was significantly lower when compared to the normal control group in this study. Fibrinogen is synthesized in the hepatic parenchymal cells of liver (Thapa & Walia, 2007). The observed decrease in the fibrinogen level may be attributed to the disordered synthetic function of the parenchymal cells caused by CCl_4. Treatment with *P. brevitarsis* larvae prior to the injection of CCl_4 significantly increased the fibrinogen level compared to the CCl_4 group ($P < 0.05$). Hence *P. brevitarsis* larvae might be responsible for stimulating the synthesis of fibrinogen in the liver (Arıcı & Çetin 2011). The dose-related decrease in the prevalence and severity of histopathological hepatic lesions identified through histopathological examination also confirmed the phenomenon. Interestingly, the treatment with *P. brevitarsis* larvae alleviated histological changes induced by CCl_4, and the effect was comparable to that of pycnogenol.

Conclusions

The present study demonstrated the optimal dose for obtaining effective protection with *P. brevitarsis* larvae against

CCl_4-intoxicated rats. A loss of protective effects was found when the dose of *P. brevitarsis* larvae increased to more than 300 mg/kg. Increasing the dose of *P. brevitarsis* larvae to 1,000 or 3,000 mg/kg did not enhance the degree of protection of CCl_4-induced injury. In particular, *P. brevitarsis* larvae at 3,000 mg/kg notably lost its effectiveness for providing protection. Therefore, maximal concentration of *P. brevitarsis* larvae exerting effective hepatoprotection would be expected to be in the range of 100–300 mg/kg dose and these findings are useful for use of *P. brevitarsis* as a therapeutic agent in liver injury-related patients.

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Compliance with ethical statement

This study protocol was reviewed and approved by the Institutional Animal Care (Approval number: CBIACUC-19262ET1) based on Animal Protection Act [Enforcement Date 27. Aug, 2019] [Act No.16544, 27. Aug, 2019, Partial Amendment].

Conflict of interest statement

All authors declare no conflict of interest.

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