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RESEARCH PAPER

The protective effect of *Protaetia brevitarsis seulensis* against CCl_4-induced hepatotoxicity in rats

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Abstract

The present study was performed to investigate the protective effect of *Protaetia brevitarsis seulensis* (Kolbe) against carbon tetrachloride (CCl_4)-induced liver damage and coagulation disturbances in rats, in comparison to silymarin. Rats were orally pre-treated with *P. brevitarsis* larvae and silymarin at a dose of 300 mg/kg once daily for 5 days, and then a single dose of CCl_4 (4 mL/kg i.p.). *P. brevitarsis* larvae showed significant hepatoprotection by reducing the liver enzymes (AST, ALT and ALP) activity levels that had been raised by CCl_4 administration. In addition, *P. brevitarsis* larvae significantly restored prothrombin time (PT) and activated partial thromboplastin time (aPTT) values to nearly silymarin level, which were abnormalized after CCl_4 administration. The histopathological results of liver tissues treated with *P. brevitarsis* larvae supported the serum biochemical results, indicating improved liver functions. The current study showed that *P. brevitarsis* larvae have potential against CCl_4-induced liver damage and coagulation disorders.

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

Introduction

The liver is the most significant organ in the human body for the detoxification of endogenous and exogenous metabolites, and is the target organ of all toxic chemicals. Additionally, the liver plays an essential role in regulation of the clotting process, and acute and chronic liver diseases are almost invariably associated with coagulation disorders. Because haemostatic systems are associated with the coagulation, fibrolytic activity, and platelet aggregation, many factors are responsible for coagulation disorders as follows: decreased synthesis of coagulation and fibrolysis factors, hyperfibrinolysis, deficiency of platelet, and accelerated intravascular coagulation (Amitrano *et al*. 2002).

CCl_4 is one of the most commonly used hepatotoxins to cause acute liver injury that has been demonstrated by many research literatures in experimental animals (Jhonston & Kroening 1998). Previous studies have suggested that CCl_4 administration in experimental animal studies induced liver-related diseases such as degeneration of the blood coagulative and fibrinolytic functions (Vazquez *et al*. 1990). Because most coagulation and fibrinolysis-related factors are synthesized by the liver, acute or chronic hepatic damage would be related to abnormal coagulation process (Arıcı & Çetin 2011).

The larvae of *Protaetia brevitarsis seulensis* (Kolbe) (Family: Cetoniidae) is a white-spotted flower chafer and is widely founded in East Asian countries such as Korea, China, Japan, and Taiwan, and in Europe (Kwon 2009). *P. brevitarsis seulensis* has been reported to possess anti-oxidant (Hah *et al*. 2005), anti-microbial (Park *et al*. 1994), and anti-cancer (Yoo *et al*. 2007) activities. Recently, it is reported that indole alkaloids isolated from *P. brevitarsis seulensis* inhibited the blood coagulation pathways by inhibited Factor Xa activity and thrombin production as well as the platelet aggregation *in vitro* and *in vivo* (Lee *et al*. 2017). However, the protective effect of *P. brevitarsis* larvae against CCl_4-induced hepatotoxicity has not been reported. However, there are no reports on the protective effect of *P. brevitarsis* larvae against CCl_4-induced liver injury including coagulation disturbance. Therefore, the current

study examined the protective effect of *P. brevitarsis* larvae against the CCl_4-induced hepatic injury in SD-rat. In addition, silymarin, a mixture of flavonoligans with clinically proven hepatoprotective effect, was compared as a reference (Stickel & Schuppan 2007). To our knowledge, the protective effects of *P. brevitarsis* larvae, compared to silymarin, on CCl_4-induced liver damage in rats have not previously been reported.

Materials and methods

Materials

The *Protaetia brevitarsis* larvae powder used in the experiments was obtained from Hangum Biotech, Inc., Korea. All chemicals used in the research were of analytical grade, and were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA).

Animals

The protective effects of the *P. brevitarsis* larvae against CCl_4-induced hepatotoxicity in Sprague–Dawley rats (270–300) g were then measured. They were obtained from Samtako Bio Korea (Seoul, Korea). Rats were housed in polycarbonate cages in controlled room (22 ± 2°C; 55–60% relative humidity) under 12 h-light and 12 h-dark cycle. Animals had *ad libitum* access to standard pellet chow and drinking water. All procedures complied with the Institutional Animal Care (Approval number: CBIACUC-19262ET1), according to Animal Protection Act [Enforcement Date 27. Aug, 2019] [Act No.16544, 27. Aug, 2019, Partial Amendment].

Experimental design

Forty-eight rats were divided into six groups of eight rats each. Group 1 served as the normal control group, and received only the vehicles for 5 days orally. Groups 2 and 3 were orally given with *P. brevitarsis* larvae and silymarin at 300 mg/kg for 5 days, respectively. Group 4 served as CCl_4 control, and received distilled water for 5 days orally, and on the fifth day CCl_4 (25% in olive oil; 4 mg g/kg, i.p.). Group 5 was orally pre-treated with *P. brevitarsis* larvae (300 mg/kg) for 5 days, before treatment with CCl_4 (4 mg/kg, i.p.). Group 6 was orally pre-treated orally with silymarin (300 mg/kg) for 5 days, before treatment with CCl_4 (4 mg/kg, i.p.). The effective dose of *P. brevitarsis* larvae was based on preliminary experiment. At the end of the experiment, blood samples of fasted rats were collected in sodium citrate tube, allowed to coagulate for 30 min at room temperature, and then centrifuged at 3,000 rpm at 20 min. The clear supernatants were quickly removed and serum was stored at −70°C for biochemical analysis. While a portion of liver tissue were dissected and fixed in 10% neutral buffered formalin for 24 h collected for histopathological examination. The remaining livers were frozen quickly in liquid nitrogen and stored at −70°C for biochemical analysis.

Liver biomarker assay

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were assayed measured using automatic biochemical analyzer (Hitachi 2030, Hitachi Ltd, Japan).

Coagulation assays

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

Histopathological assays

Liver tissues were surgically excised, and liver slices were cut and fixed in 10% buffered formalin, and embedded in paraffin. Tissue sections of 4 μm thick were prepared, stained with Hematoxylin and eosin (H&E), and then examined with light microscopy.

Statistical analysis

The data are expressed as the mean ± standard deviation. The analysis of variance (ANOVA) was used to compare means among groups by Duncan's multiple range at $P < 0.05$. Statistical analyses were carried out using SPSS software version 21 (SPSS Inc., Chicago, IL, USA).

Results

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

The hepatoprotective effects of *P. brevitarsis* larvae on serum biochemical parameters in CCl_4-itoxicated rates are presented in Table 1. As shown in Table 1, serum AST, ALT, and ALP enzymes in the normal control group were 115.8 ± 8.5, 48.5 ± 1.8, and 1,066.6 ± 35.1 U/L, respectively. CCl_4 administration markedly raised serum level of AST, ALT, and ALP when compared to normal control, *P. brevitarsis* larvae and silymarin groups, suggesting CCl_4-induced hepatotoxicity. These increases were attenuated by pre-treatment of *P. brevitarsis seulensis* in ALT, AST, and ALP ($P < 0.05$), compared with the CCl_4 group. In particular, the ameliorative liver function in group with *P. brevitarsis*

Table 1 Effects of *Protaetia brevitarsis* larvae on serum biochemical parameters in CCl_4-intoxicated rats

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Normal control	115.8 ± 8.5	48.5 ± 1.8	1,066.6 ± 35.1
P. brevitarsis larvae	119.3 ± 8.6	46.3 ± 2.0	1,128.6 ± 48.8
Silymarin	120.6 ± 6.4	37.1 ± 2.3	879.3 ± 44.0
CCl_4	541.5 ± 38.9$^{\#}$	291.0 ± 18.8$^{\#}$	2,013.9 ± 38.9$^{\#}$
CCl_4 + *P. brevitarsis* larvae	362.7 ± 20.9*	149.0 ± 9.9*	1,673.6 ± 13.6*
CCl_4 + Silymarin	307.5 ± 10.9*	129.4 ± 10.8*	1,519.0 ± 27.1*

PBS, *Protaetia brevitarsis* larvae, Data are expressed as mean ± SD (n = 8).
One-way ANOVA Tukey *post hoc*:
$^{\#}P \leq 0.05$ vs. control group,
$^{*}P \leq 0.05$ vs. CCl_4.

seulensis was equivalent to that of the silymarin-treated group (Table 1).

Effect of *P. brevitarsis* larvae on PT, aPTT, and fibrinogen level

As presented in Figure 1, *P. brevitarsis* larvae and silymarin groups does not cause any significant alteration in the coagulation variables, as compared to the normal control. However, aPTT and PT values were significantly elongated in CCl_4-treated group. Pre-treatment with *P. brevitarsis* larvae and silymarin prior to the administration of CCl_4 resulted in significant decrease in PT and aPTT as compared to CCl_4 group ($P < 0.05$).

Fibrinogen level in CCl_4 group was significantly decreases as compared with normal control group ($P < 0.05$), as shown in Figure 1. Meanwhile, pre-treatment with *P. brevitarsis* larvae and silymarin prior to the administration of CCl_4 significantly increased fibrinogen level, compared to the CCl_4 group ($P < 0.05$).

Histopathology

The protective effect from biochemical assays was confirmed by histopathological observations of the H&E stained liver tissues (Fig. 2). The liver tissue from normal control rats showed a classical structure with normal central vein and

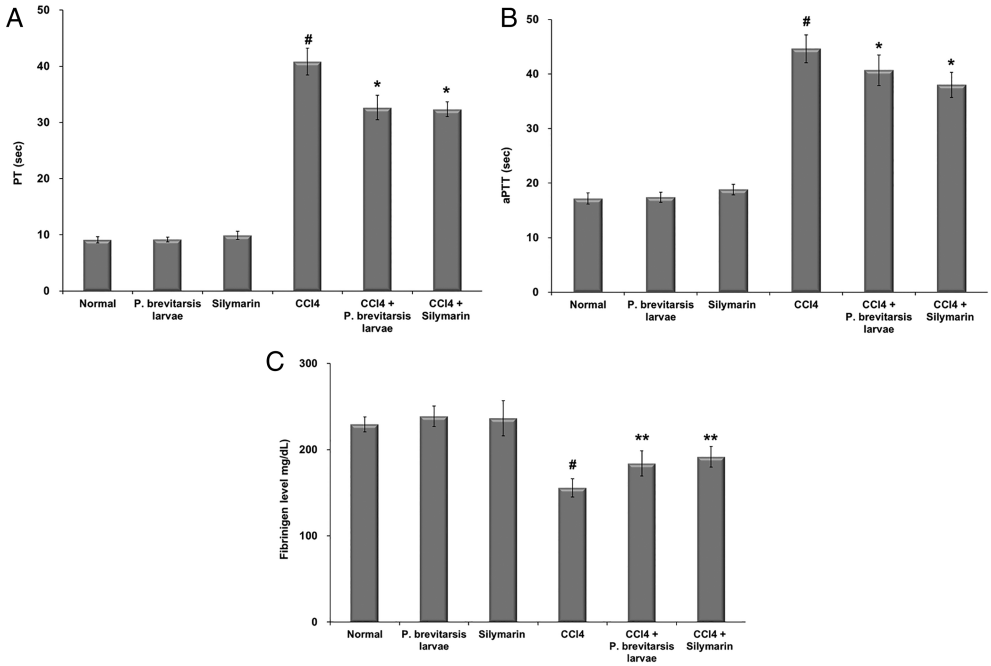

Figure 1 Effects of *Protaetia brevitarsis* larvae on (a) PT, (b) aPTT, and (c) fibrinogen concentrations in CCl_4-intoxicated rats.PT, Prothrombin time; aPTT, activated partial tromboplastin time; CCl_4, carbon tetrachloride.$^{\#}P < 0.05$ compared with the control group.* Significantly lower than the CCl_4 group ($P < 0.05$).** Significantly higher than the CCl_4 group ($P < 0.05$).

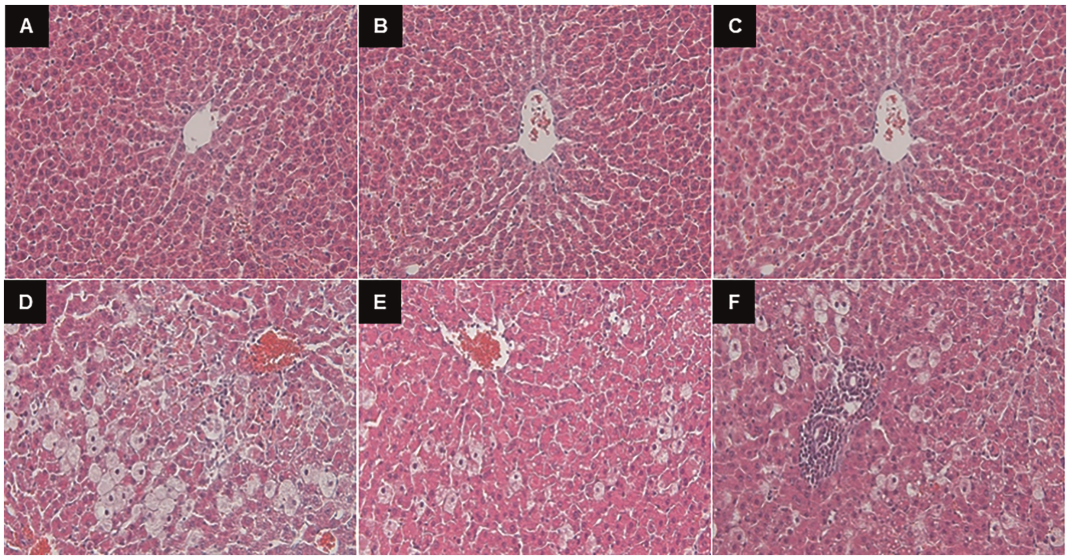

Figure 2 Effects of *Protaetia brevitarsis* larvae and silymarin on the histological changes induced by CCl_4 administration in rats. (A) Control group, (B) rats treated with *Protaetia brevitarsis* larvae (300 mg/kg), (C) rats treated with silymarin (300 mg/kg), (D) rats treated with CCl_4 (4 mL of 25% CCl_4 in olive oil), (E) rats treated with *Protaetia brevitarsis* larvae (300 mg/kg) and CCl_4, and (F) rats treated with silymarin (300 mg/kg) and CCl_4. All sections were stained with haematoxylin and eosin (400 × for all panels).

hepatic lobule (Fig. 2A). Similarly, liver tissues from rats treated with *P. brevitarsis* larvae or silymarin (Fig. 2 B and Fig. 2 C) alone also revealed normal liver tissue. On the contrary, CCl_4-treated rat caused severe liver changes characterized by broad infiltration of inflammatory cells, fatty accumulation, and focal necrosis, as well as congestion of central vein (Fig. 2D). However, pre-treatment with *P. brevitarsis* larvae ameliorated the histological features of liver injury, as confirmed by less fatty alteration, less and decreased fibrosis and apoptosis (Fig. 2E). Furthermore, the *P. brevitarsis* larvae treatment alleviated histological changes induced by CCl_4 treatment, which was comparable to that of silymarin (Fig. 2F).

Discussion

As a new biological resource in the food or pharmaceutical industries, the considerable attention of edible insects has increased. The current study focused on the protective effect of *P. brevitarsis* larvae against the CCl_4-induced hepatic injury including coagulation disturbance in SD-rat, compared to silymarin. The liver is the most significant organ in the human body for the detoxification of endogenous and exogenous metabolites, and is the target organ of all toxic chemicals. CCl_4 is one of the most common hepatotoxins to cause acute liver injury that has been demonstrated by numerous research literature in experimental animals (Jhonston & Kroening 1998). Additionally, the liver plays an essential role in regulation of the clotting process, and acute and chronic liver diseases are almost invariably associated with coagulation disorders. Because haemostatic systems are associated with the coagulation, fibrolytic activity, and platelet aggregation, many factors are responsible for coagulation disorders as follows: decreased synthesis of coagulation and fibrolysis factors, hyperfibrinolysis, deficiency of platelet, and accelerated intravascular coagulation (Amitrano *et al*. 2002).

Serum AST, ALT, and ALP are sensitive indicators directly implicated in liver damage in clinical findings, and are present in high concentrations in the liver under normal conditions. When there is hepatocellular damage, these enzymes will be secreted into the bloodstream and then, their levels elevate (Drotman & Lawhan 1978). This increase in the serum AST, ALT, and ALP enzyme levels in CCl_4-treated animals indicates hepatic cell damage (Wolf 1999). In the present study, the levels of all serum enzymes are considerably increased in the CCl_4-treated group as compared normal control groups that are characteristic features of hepatic injury induced by toxins (Shen *et al*. 2015). Pre-treatment with *P. brevitarsis* larvae attenuated the increase in AST, ALT, and ALP. These restorations could be ascribed to prevention of leakage of intracellular enzymes by stabilized liver cell membrane, which reflects protective effects against the hepatic damage caused by CCl_4 (Giannini *et al.* 2005).

The aPTT is related to the intrinsic pathway coagulation activity and PT is used to measure the extrinsic pathway

coagulation activity of coagulation in plasma (Dang *et al*. 2015). Neither *P. brevitarsis* larvae nor silymarin is known to influence PT or aPTT values. To our knowledge, the protective effects of *P. brevitarsis* larvae, compared to silymarin, on CCl_4-induced blood coagulation disturbances in rats have not previously been reported. In the present study, aPTT and PT values were significantly elongated in the CCl_4-treated group when compared normal control groups, as demonstrated by other studies that could be attributed to the depressions of extrinsic/intrinsic coagulation factors (Okazaki *et al*. 1986; Vazquez *et al*. 1990). Pre-treatment with *P. brevitarsis* larvae and silymarin prior to the administration of CCl_4 resulted in significant decrease in PT and aPTT as compared to CCl_4 group ($P < 0.05$). This result suggests that *P. brevitarsis* larvae and silymarin contributed to the regulation of the coagulation system in rats with CCl_4-induced liver damage.

Fibrinogen level in the CCl_4 group was significantly decreased as compared with normal control group, as in our study. Fibrinogen is synthesized in the liver. In particular, the hepatic parenchymal cells in liver are responsible for synthesis of fibrinogen (Thapa & Walia 2007). The observed decrease in fibrinogen level may be attributed to the dysfunction in the synthesizing activity of the parenchymal cells because of liver dysfunction brought on by CCl_4. Our result indicated that pre-treatment with *P. brevitarsis* larvae and silymarin prior to the injection of CCl_4 significantly increased fibrinogen level, compared to the CCl_4 group ($P < 0.05$). So, it is considered that *P. brevitarsis* larvae and silymarin might be responsible for stimulating the synthesis of fibrinogen in the liver (Arıcı & Çetin 2011).

Histopathological results are in accordance with these biochemical data; necrosis, steatosis, hemorrhage and vacuolization in the liver tissue CCl_4-treated group were observed, as compared normal control groups. Interestingly, the *P. brevitarsis* larvae treatment alleviated histological changes induced by CCl_4, treatment and was comparable to that of silymarin.

Conclusions

The present study demonstrates that *P. brevitarsis* larvae were able to restore the pathological disorder of CCl_4 intoxication. This was revealed by less histological changes in liver in addition to the recovery of hepatic biomarkers (AST, ALT, and ALP), and a maintenance of coagulation homeostatis in a way comparable to that of pycnogenol. Therefore, *P. brevitarsis* larvae possess substantial hepatoprotective effects against CCl_4-induced liver damage in rat. However, further studies will be needed to investigate the active component as well as mechanisms responsible for the implicated protective effect of *P. brevitarsis* larvae.

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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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