Protective Effects of Propolis and Probiotic Lactobacillus acidophilus against Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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Article Info

Article History:
Received: 10 February 2019
Revised: 4 April 2019
Accepted: 26 April 2019
ePublished: 30 September 2019

Keywords:
- Carbon tetrachloride
- Propolis
- Lactobacillus acidophilus
- Oxidative stress
- Hepatotoxicity

Abstract

Background: Propolis (PRS) and probiotic bacteria Lactobacillus are natural products used as dietary supplement for their therapeutic benefits. This study was performed to examine the possible hepatoprotective effect of PRS and probiotics (PRCs) against carbon tetrachloride-induced liver injury.

Methods: Experimentally, intoxicated rats received 0.5 ml/kg CCl4 (i.p.) daily for six days, pretreated rats received per os PRS 100 mg/kg or PRCs 10⁷ CFU for six days followed by a single dose of 0.5 ml/kg CCl4. Control groups received either PRS, PRCs or olive oil for six days. Then, serum biochemistry (total protein, cholesterol, triglycerides and albumin) and oxidative stress parameters were measured.

Results: We showed that CCl4 treatment was associated with an increase of the serum aspartate amino transferase (AST), alanine aminotransferase (ALT), cholesterol and triglycerides levels. In parallel, serum total protein, albumin and blood sugar levels were significantly decreased. Regarding the oxidative stress parameters, catalase and glutathione S-transferase (GST) levels were lower, conversely to the lipid peroxidation (MDA).

Conclusion: Our results strongly support that administration of PRS and PRCs may significantly protect liver against CCl4-induced toxicity by enhancing antioxidative stress pathway and preventing lipid peroxidation.

Introduction

Liver oxidative stress results from an inappropriate balance between reactive oxygen species (ROS) production and clearance,1 which can seriously damage normal hepatic functions, generating various chronic diseases such as steatosis, chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma.2 Furthermore, increasing evidences point out that free radicals play a crucial role in the various steps that initiate and trigger the progression of liver diseases.3 Carbon tetrachloride is an environmental pollutant that has many adverse effects on liver, kidney, heart as well as blood by generating free radicals and increasing lipid peroxidation. This latest is one of the main mechanisms explaining CCl4-induced liver toxicity.4 In the liver, cytochrome P450 enzymes metabolize CCl4 into trichloromethyl radical, which rapidly reacts with oxygen to form the highly reactive derivative trichloromethyl peroxy radical. Both radicals initiate lipid peroxidation of plasma membrane phospholipids to form lipid peroxidation product causing liver injury.5 Propolis (PRS) is a natural resinous material, with complex mixed compounds, prepared by honeybees from different plant species to use it as beehive sealant. Several compounds have been identified from PRS, including flavonoids, terpenoids, β-steroids, phenolic acids, amino acids and derivatives of sesquiterpenes, sugars and sugar alcohols.6 These constituents, in particular phenolic acids, terpenoids and flavonoids, give PRS the ability to perform numerous functions. Evidence has been collected that PRS shows hepatoprotective,7 antioxidant, antimicrobial,8 anticancer and immunomodulatory properties.9 Thus, ethanolic PRS extract has an important capacity in scavenging free radicals and could thus be used as a source of natural antioxidant.10 Probiotics (PRCs) are live microorganisms that can improve the intestinal flora and promote human health when administered in adequate amounts.11 PRCs are reported to serve as supplement to the host microflora and provide protection against many diseases that affect the gastrointestinal tract. PRCs also stimulate, modulate and regulate the host's immune and inflammatory responses,12 as well as most chronic liver diseases such as alcoholic and nonalcoholic liver diseases, viral hepatitis and liver...
 Cirrhosis. Besides, PRCs have been shown to have potent antioxidative activity and are able to reduce the risk of accumulation of ROS during the ingestion of food. Ethnobotanical studies have pointed out for many years the growing interest in the use of bioactive substances such as PRS and PRCs in folk medicine as well as nutritional supplements because of their beneficial effects on the body. As the biological activity of propolis is closely related to its botanical origin and geographical location, to the best of our knowledge, this study is the first one that shows the antioxidiant effect of Algerian propolis on the hepatotoxicity induced by CCl₄. Moreover, little information is known about the antioxidiant activity of the L. acidophilus strain against CCl₄-induced liver toxicity. Based on these evidences, the aim of the present study was to investigate the hepatoprotective effect of both ethanol extract of propolis and probiotic Lactobacillus on CCl₄-induced liver injury.

Material and Methods

Animal handling

Male Wistar rats weighing 140 ± 15 g were supplied from institute of veterinary sciences El Khroub, Constantine (Algeria). They were kept in the animal house under standard environmental conditions (22 ± 2 °C with 12–12 h dark–light cycle and 50 ± 10% humidity). The rats were fed on standard rodent pellet diet and drinking water ad libitum. The rats were acclimated for 1 week prior to the beginning of the experiments. All experiment steps were conducted in accordance with the institutional ethics committee regulations and guidelines on animal welfare of the University of Jijel (Algeria).

Preparation of propolis extract

Propolis extract was supplied by Laboratory of Molecular Toxicology, University of Jijel (Algeria). PRS samples were collected from bee keeping areas (Latitude N 36.755506, Longitude E 5.807074), located at Chaddia (Kaous), 10 km in the South-Eastern region of Jijel (Algeria). The extraction was performed as described previously. In brief, 100 grams of raw PRS was added to 900 ml of 95 % ethanol solution and incubated for 15 days with shaking. After filtration, the solution was evaporated using rotary evaporator and freeze dryer. The resulting dry extract was let in 70% ethanol for steeping overnight. After evaporation, the ethanolic extract of propolis (EEP) was obtained. To prepare the solution, PRS extract dissolved in a mixture of water and ethanol (1:1 v/v) and administered 100mg/kg body weight of rats for six consecutive days.

Preparation of probiotic strain

Lactobacillus acidophilus (L. acidophilus) strain was grown in MRS broth (Biokar, Pantin France) for 18 h at 37 °C, then centrifuged at 3000 rpm for 15 min, washed in normal saline solution and re-suspended in saline water to obtain a final concentration of 10⁹ colony-forming units (CFU) per mL. The bacterial suspension was administered by gavage to each rat in respective groups.

CCL₄-induced hepatotoxicity in rats

Thirty rats were randomly and equally divided to six groups: rats of group 1 received vehicle only (olive oil) for six days and served as control. Rats of groups 2 and 3 received 100mg/kg propolis dissolved in a mixture of water and ethanol (1:1 v/v) and 1 ml of probiotics at a concentration of 10⁹ CFU/mL, respectively, for six consecutive days. Rats of groups 4 and 5 pretreated with the same dose of PRS and PRCs respectively, and intoxicated two hours after the last injection by a single intraperitoneal dose of 0.5 ml/kg CCl₄ in olive oil (1:1 v/v). Rats of group 6 received 0.5 ml/kg CCl₄ in olive oil (1:1 v/v) intraperitoneally for six consecutive days. After 24 hours of the last injection, blood was collected from retro-orbital venous plexus and centrifugated at 3300 rpm for 10 min, serum was collected and frozen at -20°C for the biochemical analysis. Rats were anaesthetized with chloroform, then decapitated and the liver tissues were dissected, washed immediately with 0.9% ice-cold saline solution. One g of liver was added to 9 ml of phosphate buffer solution (0.1 M pH 7.4) containing KCl (1.15 M) and homogenized in a small Potter-Elvehjem homogenizer. The homogenate was centrifuged twice at 4000 rpm for 10 min at 4°C and then at 10000 rpm for 30 minutes at 4°C. The supernatants thus obtained were used for measuring the enzyme activities.

Determination of serum biochemistry

Aspartate aminotransferase (AST) and alanine transaminase (ALT) were measured by reaction with 2,4 dinitrophenylhydrazine (DNPH) in alkaline solution as described by the method of Reitman and Frankel. Serum total protein was estimated using Bradford reagent as standard. Blood sugar levels were measured by the method of Dubois et al. Cholesterol, triglycerides and albumin levels were determined using SPINREACT Kit according to the manufacturer’s instructions.

Determination of liver oxidative stress parameters

Hepatic tissue catalase (CAT) activity was assayed according to the method described by Claiborne. Briefly, the assay mixture consists of 50 µl of cytosolic fraction and 2.95 ml of 19mM H₂O₂ (prepared in 0.1M phosphate buffer, pH 7.4) in a final volume of 3 ml per measurement. Changes in absorbance were recorded at 240 nm. CAT activity was calculated as nmol H₂O₂ consumed/min/mg protein. Protein concentrations were determined by the method of Bradford. Gluthatine-S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (prepared in 95° ethanol) as described by Habig et al. After incubation, changes in absorbance were determined at 340 nm and the activity of GST was expressed as nmol of thioether formed/gram protein/minute. Lipid peroxidation was assayed using the method described by Botsoglou et al. by measuring the formation of thiobarbituric acid reactive substances (TBARS). The product was evaluated by spectrophotometer at 530 nm and the results were expressed as nmol/g tissue.
**Statistical analysis**

All data are presented as mean ± standard error of the mean. The comparison between groups was performed using one-way ANOVA followed by Tukey’s multiple comparison test. P < 0.05 was considered statistically significant.

**Results**

**Effects of propolis and probiotics on CCl4-induced changes on liver function**

AST, ALT and albumin and total serum proteins were estimated in serum samples as biomarkers of liver function. Exposure to CCl4 (0.5ml/kg for six days) resulted in liver injury as indicated by a significant increase in serum AST (p < 0.01) and ALT (p < 0.01), compared to the control. However, rats pretreated with PRS showed significantly lower levels of serum AST (p < 0.01) and ALT (p < 0.05) than rats in CCl4 group. These data indicate that PRS clearly presented significant protective effects against CCl4-induced liver injury (Figure 1). Interestingly, similar effects were observed with PRCs, as shown by AST and ALT levels (p < 0.05).

**Effects of propolis and probiotics on CCl4-induced changes of other blood biochemical endpoints**

Chronic administration of CCl4 increased cholesterol, triglycerides and blood sugar levels (p < 0.01), whereas significant decrease was observed in both serum total proteins (P < 0.001) and albumin levels (p < 0.01). PRS and PRCs pretreatment significantly restored the levels of cholesterol, triglycerides and blood sugar as well as albumin and serum total protein levels towards control values (Figure 2). Compared with control group, PRS seemed to be more efficient than PRCs on the liver parameters, suggesting a more efficient antioxidant activity.

**Effects of propolis and probiotics on CCl4-induced changes in liver oxidative stress parameters**

To investigate the ability of PRS and PRCs to enhance the antioxidant status of liver, we assessed CAT and glutathione-S-transferase in liver tissue homogenate. As expected, treatment with CCl4 induced a high production of ROS marked by a significant decrease in CAT (p < 0.001) and GST (p < 0.01) activities compared to the control group. Conversely, rats pretreated with PRS and PRCs displayed significant increase in hepatic CAT and GST activities (p < 0.001) compared to intoxicated rats. To evaluate the liver oxidative injury, lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA), the final product of polyunsaturated fatty acids peroxidation. As shown in Figure 3, MDA production (p < 0.001) was higher in the livers of rats exposed to CCl4, while pretreatment with PRS and PRCs significantly prevented this increase (p < 0.001).

**Discussion**

Carbon tetrachloride is metabolized in liver by cytochrome P450s to yield highly reactive free intermediate radicals. These products can bind to different biological macromolecule, leading to the release of transaminases from hepatocytes cytoplasm to blood circulation. The significant increase in the activity of liver markers such as serum AST and ALT is a marker of the liver injury in the experimental rats. This elevation of serum ALT and AST has been associated with peroxidation damage of liver cells. Interestingly, rats pretreated with PRS or L. acidophilus showed a significant reduction of AST and ALT levels compared to the group receiving CCl4, which suggested a putative hepatoprotective effect. Previous studies have shown similar results on hepatoprotective of propolis, but only a few studies have attempted to determine the protective role of probiotics on CCl4-induced liver toxicity. As shown in Figure 2, CCl4 caused an increase of serum cholesterol and triglycerides levels (p < 0.01), which was reversed by a pretreatment with PRS or L. acidophilus. This is in agreement with earlier study pointing out that PRS modulates cholesterol levels and protects the cardiovascular system through an anti-inflammatory action.
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Figure 2. Therapeutic potential of propolis (PRS) and probiotics (PRC) on CCl₄-induced alterations in serological parameters. (A) Cholesterol. (B) Triglycerides. (C) Serum total proteins. (D) Serum albumin. (E) Blood sugar. Data are expressed as mean ± SD (n=5). *P < 0.05 versus the control group; **P < 0.01 versus the control group; ***P < 0.01 versus the CCl₄ group; ###P < 0.001 versus the CCl₄ group.

Others studies have also shown that probiotic supplementation can significantly reduce serum lipid levels, reducing hence the risk of developing coronary artery disease and heart attack.27,28 One important point regards the fact that pretreatment with PRS or L. acidophilus reversed the effects of CCl₄ on the reduction of serum total proteins, and albumin particularly. Moreover, administration of PRS and L. acidophilus prevented rats against CCl₄-induced hyperglycemia. Even though the presence of bioactive components preventing beta-cell apoptosis and enhancing insulin secretion has been hypothesized,29 the precise molecular mechanism has not been deciphered so far. The stimulation of GLUT4 translocation in skeletal muscle, which enhanced glucose uptake,30 the down regulation of glucose-6-phosphatase,31 as well as the modulation of the anti-inflammatory and immunomodulatory activities through the NF-κB pathway32 have been proposed however. Altogether, the fact that propolis and L. acidophilus can improve hyperglycemia and hyperlipidemia is a promising field to investigate for natural product therapy.

The GSTs are major phase II detoxification enzymes that conjugates xenobiotics or their metabolites with glutathione to endogenous water-soluble substrates.33
Protective Effect of Propolis and Probiotics against Hepatotoxicity

Antioxidants defense system also are supported by others antioxidant enzymes like catalase that limits the formation of ROS. Previous studies have shown that free radicals attack antioxidant defense system, leading to the impairment of antioxidant components such as CAT, GST, GPx and GSH.34 In the present study, we have observed that CCl₄ treatment resulted in a significant deficiency of the enzymatic antioxidant defense system in the liver (GST and catalase). Conversely, pretreatment with PRS and L. acidophilus restored GST and catalase activities so that the values have returned to control levels. The hepatoprotective effects of PRS and PRCs on CCl₄-induced liver injury have been linked to their antioxidative properties. The anti-oxidative activity of propolis has previously been reported to be associated to its chemical composition.35 MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. In the current study, the levels of formed MDA were statistically significantly increased after CCl₄-administration compared to the control group. We have hypothesized that the pretreatment with PRS and L. acidophilus completely prevented liver cell membrane against lipid peroxidation, which can directly be attributed to the anti-oxidative activity of both PRS and L. acidophilus (Figure 4). Najafi et al. showed that PRS can act as free radical scavenger and inhibitor of oxygen free radical production.36 Indeed, it has been well established that probiotic bacteria can improve the antioxidant system, reduce injury caused by oxidation and decrease free radical generation.37

Over all, the metabolism of CCl₄ by cytochrome P450 generates trichloromethyl radical (CCl₃⁺) that quickly reacts with oxygen to yield the trichloromethylperoxy radical (CCl₃O₂⁺). These free radicals can bind covalently to cellular macromolecules such as polyunsaturated fatty acids in cell membranes causing lipid peroxidation (Figure 4). This affects the permeability of membranes leading to the loss of membrane integrity, the leakage of microsomal enzymes and subsequently hepatic failure and liver cell damage.38 The lipid peroxidation also generates reactive aldehydes that bind to proteins and DNA, affecting their function leading to protein degradation that contributes to the hepatotoxicity,39 and RNA hypomethylation that leads to protein synthesis disruption and carcinogenicity, respectively.40 CCl₄ metabolites could also inhibit lipoprotein secretion leading to liver steatosis. The hepatotoxicity of CCl₄ is mediated by several signaling pathway such TNFα and NO that lead to inflammation and apoptosis, while TGFα and β induce fibrosis.38 The antioxidant and anti-inflammatory effects of PRS are due to its high content in polyphenols, flavonoids and phenolic acids contents that act as reducing agents, lipid peroxidation inhibitors and free radical scavengers.41

Figure 3. Effects of propolis and probiotics on CCl₄-induced changes on parameters of oxidative stress in the liver of different experimental groups. (A) Liver catalase activity. (B) Liver glutathione-S-transferase (GST). (C) Lipid peroxidation (MDA). Data are expressed as mean ± SD (n = 5). **P < 0.01 versus the control group; ***P < 0.001 versus the control group; ###P < 0.001 versus the CCl₄ group.
Furthermore, PRS is able to induce antioxidant enzymes as well as phase II detoxifying enzymes such as glutathione S-transferase through the activation of the transcription factor Nrf2 (responsible for regulating antioxidant genes). The PRCs have also shown to reduce inflammation through the down regulation of proinflammatory genes like IL-1, IL-6, IFNγ and TNFα.

Even more, PRCs can enhance total antioxidant status by scavenging free radicals, increasing glutathione and reducing proinflammatory cytokines.

Conclusion

Our results indicated that CCl₄ administration in rats disrupted plasma glycemic and lipidemic profile, and increased oxidative stress in liver. Conversely, propolis and L. acidophilus intake antagonized these adverse alterations without any apparent negative effects. Altogether, this study showed that propolis and L. acidophilus can further be used as a source of drug or dietary supplement development in the prevention and/or treatment of liver damages induced by chemicals.

Conflict of interests

The authors claim that there is no conflict of interest.


