EFFECT OF DIFFERENT DOSES OF PINOCEMBRIN ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN RATS

BY

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Abstract

Pinocembrin (PIN), a flavanone found abundantly in honey and propolis, has been reported to have many benefits and medicinal properties. However, its protective effects against carbon tetrachloride (CCl_4) induced hepatotoxicity have not been clarified. The aim of the present study was to investigate the potential hepatoprotective dose of PIN on CCl_4 treated rats. PIN was screened at different doses (10, 20 and 40 mg/kg/day) orally for 7 days, against a single dose of CCl_4 (1 ml/kg, 1:1 mixture with corn oil, i.p.). PIN protected against CCl_4-induced increase in hepatic transaminases, total cholesterol and histopathological changes. The dose of 20 mg/kg PIN was selected for further assessment to address the PIN hepatoprotective mechanisms.

Key words: Pinocembrin, Carbon tetrachloride, liver fibrosis

Introduction:

Liver fibrosis is an integral clinicopathological condition of chronic liver disease predisposing to cirrhosis and hepatocellular carcinoma (HCC) **(Narmada *et al.*, 2013)**. The most common etiologies for liver fibrosis are alcohols, chemicals, and viruses **(Andrade *et al.*, 2005; Cederbaum *et al.*, 2009; Davern *et al.*, 2011)**. Hepatic stellate cells (HSC) undergo activation following liver injury of any cause **(Chu *et al.*, 2013)**, switching from quiescent, vitamin A-storing to activated, vitamin A-losing and α-smooth muscle actin (α-SMA) expressing myofibroblastic phenotypes (MFB) **(Gressner *et al.*, 2007)**. Also, HSC are responsible for most of the excess extracellular matrix (ECM) production, mainly type I collagen, observed in chronic liver fibrosis **(Cong *et al.*, 2013)**.

Carbon tetrachloride (CCl4) is a well-known hepatotoxin that is widely used to induce acute toxic liver injury in a large range of laboratory animals **(Kodai *et al.*, 2007; Campo *et al.*, 2008; Leong *et al.*, 2011)**. A number of studies have shown that CCl4 is metabolized by the P450 enzyme system to yield reactive metabolic products trichloromethyl free radicals, which can initiate the process of lipid peroxidation and ultimately results in the overproduction of reactive oxygen species (ROS) and hepatocyte injuries **(Kodai *et al.*, 2007; Tien *et al.*, 2011)**.

Pinocembrin (5, 7-dihydroxyflavanone, PIN) is a flavanone found abundantly in honey and propolis **(Jaganathan and Mandal, 2009)**. Many studies have established

that PIN possesses multiple activities including neuroprotective, anti-inflammatory, vaso-relaxation, anti-oxidant, anti-microbial, anti-cancer and anti-proliferative effects **(Shi *et al.*, 2011; Lee *et al.*, 2012)**. PIN regulated the production of TNF-α via inhibiting NF-κB, ERK1/2, JNK and p38MAPK in lipopolysaccharide-induced inflammatory responses **(Soromou *et al.*, 2012)**. Propolis also prevented the effects of TGF-β1-induced Smad2 activation pathway in fibrotic lung diseases **(Kao *et al.*, 2013)**.

These findings indicated that PIN might have protective effects on fibrosis but its ability to antagonize liver fibrosis has not been previously examined. This study aims at predicting the ability of different doses of PIN to attenuate acute CCl_4-induced liver fibrosis by measuring liver transaminases, total cholesterol and histopathological examination.

Materials and methods:

Drugs and chemicals

Pinocembrin (purity >99.7%) was purchased from Sichuan Research Center of Traditional Chinese Medicine (Chengdu, China), 2-hydroxypropyl-β-cyclodextrin (HPβCD) from Roquette (France-Europe) and Carbon Tetrachloride (CCl_4) from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Male Wistar rats (180–220 g) were obtained from Nile Co. for Pharmaceutical and Chemical Industries, Egypt. Rats were housed in an air-conditioned atmosphere, at a temperature of 25 8C with alternatively 12 h light and dark cycles. Animals were acclimated for 2 weeks before experimentation. They were kept on a standard diet and water ad libitum. Standard diet pellets (El-Nasr, Abu Zaabal, Egypt) contained not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and a vitamin mixture. The study protocol was approved by the Ethical Committee, Faculty of Pharmacy, Ain Shams University, Egypt.

Experimental design

Screening for the potential hepatoprotective dose of PIN (acute model):

Rats were randomly assigned into five groups (ten animals in each group). Group (I) served as control group and received 1 ml/kg of 20% HPβCD which was used as vehicle for PIN through oral gavage once daily for 7 consecutive days and received corn oil (1ml/kg i.p.) as vehicle for CCl4 (i.p.) on day 5. Group (II) served as CCl4 group and received 1 ml/kg of 20% HPβCD through oral gavage once daily for 7 consecutive day and single dose of CCl4 (1 ml/kg, 1:1 mixture with corn oil, i.p.), to induce liver fibrosis on day 5. Groups (III), (IV) and (V) were PIN pretreated groups, received 10, 20 and 40 mg/kg of PIN dissolved in 20% HPβCD respectively through oral gavage once daily for 7 consecutive days and a single i.p. injection of CCl4 (1ml/kg of 1:1 CCl4: corn oil on day 5), 1 h after PIN treatment.

On day 8, blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by centrifugation at 5000 rpm for 10 min and used for biochemical analysis of hepatic enzymes. Rats were sacrificed and liver tissues were dissected out and washed with ice-cold saline and then were fixed in 10% buffered formaldehyde for histopathological examination.

Assessment of hepatotoxicity indices:

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total cholesterol (TC) were estimated using available commercial kits (Biodiagnostic, Giza, Egypt).

Histopathological examination

For light microscopy, liver specimens were taken from the right lobe and fixed in 10% formalin and processed for paraffin sections of 4 μm thickness. Sections were stained with hematoxylin and eosin according to the method of **(Bancroft *et al.*, 1996)** for routine histopathological examination.

Statistical analysis

Data are presented as mean +SD. Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer as a post hoc test, as appropriate. The 0.05 level of probability was used as the criterion for significance. All statistical analyses and graphs sketching were performed using using GraphPad Prism (ISI1 software, USA) version 5 software.

Results:

Liver transaminases and total cholesterol:

As shown in fig (1), liver function parameters increased with a single dose of CCl_4 including ALT, AST and TC as compared to control rats. These functions have been improved in intoxicated animals pretreated with different doses of PIN (10, 20 and 40 mg/kg), where ALT, AST and TC were significantly lowered similar to the control value at doses of 20 and 40 mg/kg. According to these results, PIN at dose of 20 mg/kg was the most appropriate hepatoportective dose.

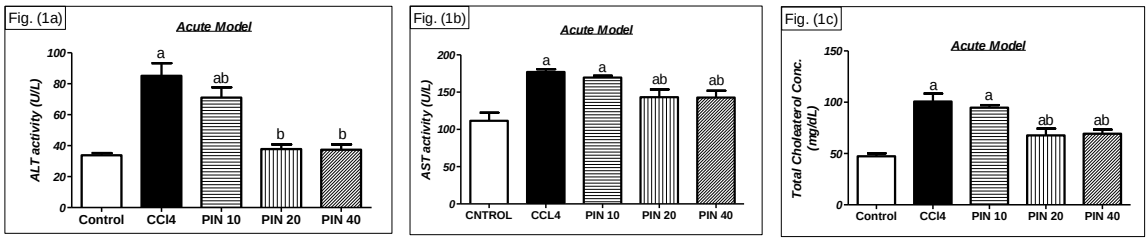


Fig. 1: Data are the mean ± S.D. (n = 8). (a or b) Significantly different from the control or CCl_4 group respectively at $P < 0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test.

Histopathological examination:

Histopathological examination of liver tissue was done to further illustrate CCl_4-induced hepatototoxicity. Control group showed normal architecture of the central veins and surrounding hepatocytes in the hepatic parenchyma and no histopathological alterations were recorded (fig. 2). CCl_4-intoxicated group showed fibrosis (f) in the portal area while the hepatocytes showed vacuolar and ballooning degenerations (d) (fig. 3). Treatment with 10mgkg PIN illustrated fatty change in some of the hepatocytes (arrow) while others showed different other degenerative changes (d) in association with focal inflammatory cells infiltration in between (m) (fig 4). PIN 20mg/kg treatment showed dilatation in the central vein associated with focal inflammatory cells

infiltration (m) in the adjacent degenerated hepatocytes (fig. 5). Finally, treatment with 40mg/kg PIN revealed ballooning degeneration (d) in the hepatocytes (fig. 6). From these results, it is clear that PIN managed to decrease the hepatotoxic effect of a single dose of CCl_4 and the dose 20 mg/kg is the most appropriate dose to be used for further studies. Severity of the reaction is shown in fig. 7 and 8.

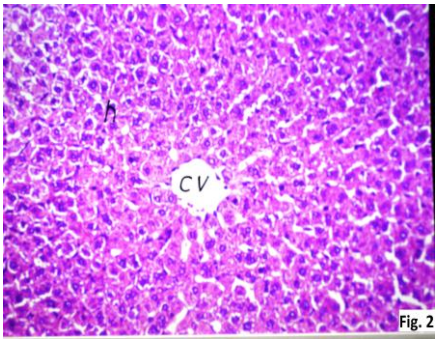


Fig. 2: control group showing normal hepatic architecture.

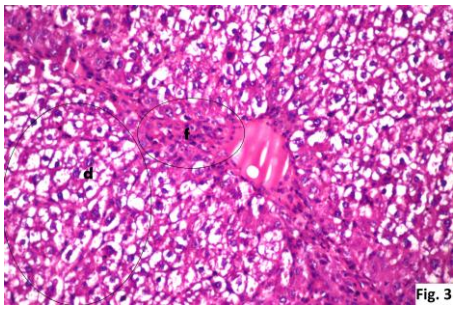


Fig. 3: CCl_4 group showing fibrosis (f) in the portal area while the hepatocytes showed vacuolar and ballooning degenerations (d).

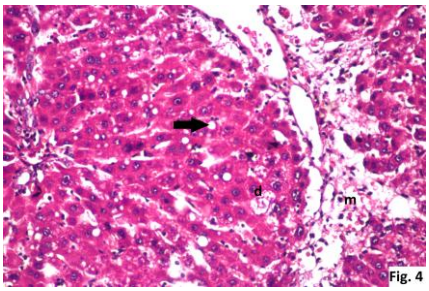


Fig. 4: 10% PIN treated group showing fatty change in some of the hepatocytes (arrow) while others showed different other degenerative changes (d) in association with focal inflammatory cells infiltration in between (m).

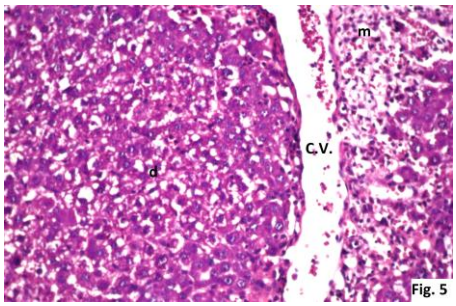


Fig. 5: 20% PIN treated group showing dilatation in the central vein associated with focal inflammatory cells infiltration (m) in the adjacent degenerated hepatocytes

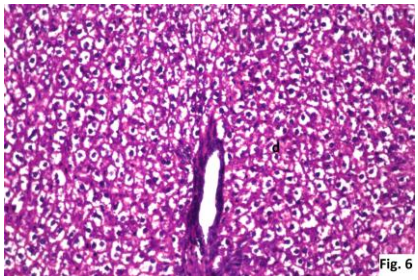


Fig. 6: 40% PIN treated group showing ballooning degeneration (d) in the hepatocytes

Fig. 7: Severity of reaction obtained in histopathological examination of all groups of CCl_4/PIN.

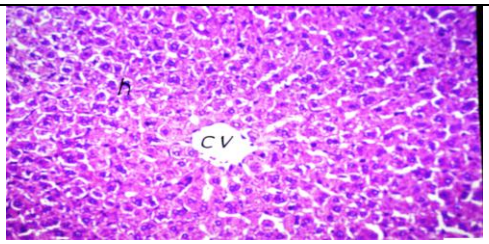
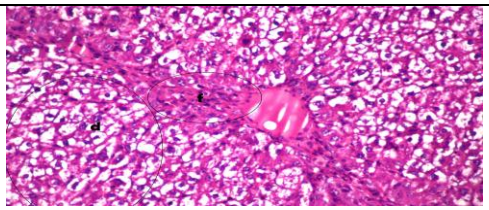
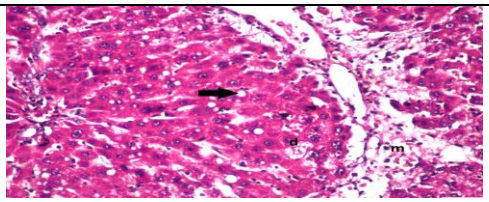
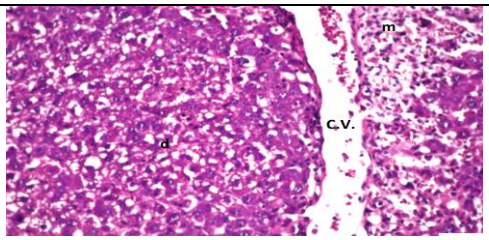
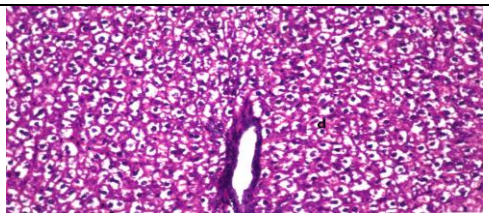
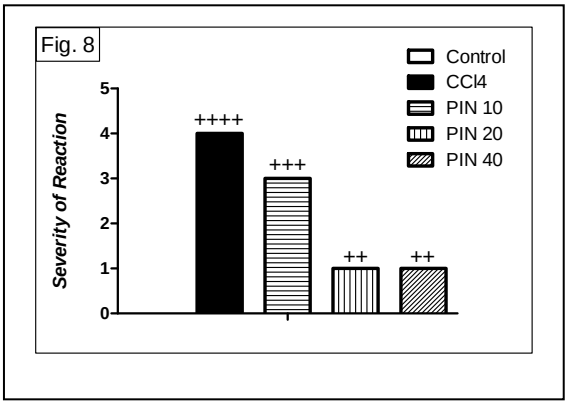
Group		Severity of Reaction
Control		-
CCl4		++++
CCl4+PIN 10		+++
CCl4+PIN 20		++
CCl4+PIN 40		++

Fig. 8: Graphical representation of severity of the reaction in CCl_4/PIN model for determining the effective dose of PIN



Discussion and conclusion:

This study demonstrates the inhibitory effects of different doses of PIN on CCl4- induced hepatotoxicity in rats. CCl4-induced hepatotoxicity was assessed by biochemical analysis of ALT, AST and TC as well as histopathological examination of liver tissue. CCl4-intoxicated group showed a significant elevation in serum ALT, AST and TC levels; these results are in accordance with previous studies **(Mantawy *et al.*, 2012; Ponmari *et al.*, 2014)**. Serum ALT, AST and TC levels have been gradually decreased with the different used doses of PIN reaching the optimal effect with the doses 20 and 40 mg/kg PIN. These data suggested that PIN may have direct hepatoprotective effect against CCl4-induced hepatotoxicity. The above mentioned results were further strengthened by histopathological examination of rats' liver tissue. CCl4 induced extensive fatty change in some of the hepatocytes while others showed different other degenerative changes in association with focal inflammatory cells infiltration in between. All these pathological changes have been previously reported **(Mantawy *et al.*, 2012; Lee *et al.*, 2014)**. The severity of these hepatic changes were gradually ameliorated in intoxicated groups pretreated with different doses of PIN, reaching almost normal hepatic architecture at doses 20 and 40 mg/kg. Accordingly, these data suggest that PIN has a protective role against CCl4-induced hepatotoxicity and the dose 20mg/kg was selected for further investigation in the mechanistic study.

In summary, this study demonstrates for the first time that PIN has potent protective effects against CCl_4-induced hepatotoxicity and that 20mg/kg is the most appropriate hepatoprotective dose. Further future studies are required to elucidate the whole sequential cause-resultant mechanism of PIN, since hepatic fibrosis is a very complicated process.

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تأثير جرعات مختلفة من البينوسيمبرين على التسمم الكبدي الناجم عن رابع كلوريد الكربون في الجرذان

للسادة الدكاترة

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البينوسيمبرين، فلافانون وجد بكثرة في العسل والبروبوليس، سجل العديد من المزايا والخصائص الطبية. ومع ذلك، لم يتم توضيح آثاره الوقائية ضد تسمم الكبد بفعل رابع كلوريد الكربون. وكان الهدف من هذه الدراسة التحقق من جرعة البينوسيمبرين المحتملة لمعالجة التسمم الكبدي الناتج عن رابع كلوريد الكربون على الجرذان. وقد تم حقن البينوسيمبرين بجرعات مختلفة (١٠ و ٢٠ و ٤٠ ملغ / كغ / يوم) عن طريق الفم لمدة ٧ أيام، ضد جرعة واحدة من رابع كلوريد الكربون (١ مل / كجم، ١: ١ خليط مع زيت الذرة في البطن). وقد حمى البينوسيمبرين الكبد من الزيادة في انزيمات الكبد والكوليسترول الناتجة من رابع كلوريد الكربون وقد أكدت هذه النتائج التغيرات التشريحية المرضية. لذلك تم اختيار جرعة من ٢٠ ملغ / كغ لاستخدامها لتقييم آليات عمل البينوسيمبرين في حماية الكبد من التليف.