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Multiple treatment of propolis extract ameliorates carbon tetrachloride induced liver injury in rats

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ABSTRACT

Propolis, a resinous wax-like beehive product has been used as a traditional remedy for various diseases due to a variety of biological activities of this folk medicine. In the present investigation, an attempt has been made to validate hepatoprotective activity of ethanolic extract of propolis (50–400 mg/kg, p.o.) against carbon tetrachloride (CCl_4, 0.5 ml/kg, p.o.) induced acute liver injury in rats. Silymarin, a known hepatoprotective drug was used as a positive control. Administration of CCl_4 altered various diagnostically important biochemical variables. Multiple treatment of propolis significantly prevented the release of transaminases, alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transpeptidase, urea and uric acid in serum; improved the activity of hepatic microsomal drug metabolizing enzymes, i.e., aniline hydroxylase and amidopyrine-*N*-demethylase; significantly inhibited lipid peroxidation and markedly enhanced glutathione in liver and kidney as well as brought altered carbohydrate contents (blood sugar and tissue glycogen), protein contents (serum, microsomal and tissue protein) and lipid contents (serum and tissue triglycerides, serum cholesterol, total and esterified cholesterol in tissue) towards control. Propolis treatment also reversed CCl_4 induced severe alterations in histoarchitecture of liver and kidney in a dose dependent manner. Hepatoprotective activity of propolis at doses of 200 and 400 mg/kg was statistically compared to silymarin and found that propolis exhibited better effectiveness than silymarin in certain parameters, concluded its hepatoprotective potential.

1. Introduction

Evidences developed over the last several years have suggested that various forms of liver injury may be caused by free radical formation and subsequent oxidative stress. It is believed that reactive oxygen species (ROS), such as hydroxyl radical, superoxide radical anion and nitric oxide may injure cell membranes through lipid peroxidation and modify or damage biomolecules, i.e., proteins, lipids, carbohydrates and DNA *in vitro* and *in vivo* (Halliwell, 1996; Graziewicz et al., 2002). Significant cellular damage occurs when the amount of produced free radicals exceeds the capacity of endogenous cellular antioxidant defense system. Many natural products contain active chemicals that are metabolized by phase I and phase II pathways of the cytochrome P450 (CYP450) enzyme system and also serve as substrates for certain transporters. Due to their interaction with CYP450 enzymes and transporters, there is a potential to modulate the activity of these drug metabolizing enzymes and transporters by administration of natural components (Venkataramanan et al., 2006). About 600 commercial preparations with claimed liver protecting activity are available all over the world and more than 100 medicinal plants belonging to 40 families are used for poly herbal formulations in India (Ahmed et al., 2002). In fact, the herbs/plants are the oldest friend of mankind because they not only provide food and shelter but also serve the humanity to cure different ailments (Gilani and Rahman, 2005). According to a WHO report, about three-quarter of the world population relies upon the traditional medicines for health care.

Propolis, a resinous wax-like beehive product is prepared by honeybees from plant materials and also known as bee glue (Moreno et al., 2000). It has been used as a traditional remedy for various diseases in folk medicine, as a constituent of bio-cosmetics and as health food (Kujumgiev et al., 1999). It is believed that it can cure heart disease, diabetes and even cancer (Hirota et al., 2000; Na et al., 2000). Several biological properties of propolis including tumor cell arrest, antibiotic, anti-inflammatory and antioxidant have also been reported (Banskota et al., 2000; Moreno et al., 2000; Cai et al., 2004). It contains esters of phenolic acids and flavonoids especially cafeates and ferulates, which have been identified as antibacterial, antiviral and antifungal agents (Marcucci, 1995; Vennat et al., 1995). Caffeic acid phenethyl ester (CAPE) is one of the main components of ethanolic extract of propolis (Kartal et al., 2003), which has been reported to prevent diabetes-induced decrease of IGF-I mRNA and IGF-II mRNA in liver (Park and Min, 2006), doxorubicin induced cardiotoxicity (Fadillioglu et al., 2004) and focal cerebral ischemia injury in rats (Tsai et al.,

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2006). Aqueous extract of propolis has prophylactic hepatoprotective effect against CCl_4 induced injury (El-Khatib et al., 2002). We have previously reported the treatment dependent (prophylactic/curative; Shukla et al., 2004), dose dependent (Shukla et al., 2005) and duration dependent (Bhadauria et al., 2007) hepatoprotective effects of propolis against acute single administration of CCl_4 induced liver injury using female rats those are more susceptible to CCl_4 toxicity than males (Mehendale and Thakore, 1997). We have extended our study through this work to confirm the hepatoprotective potential of propolis using various specific biochemical markers such as LDH, γ-GT, urea, triglyceride, cholesterol, protein in serum and blood sugar; hepatic CYP2E1 and CYP1A2 enzymes, lipid peroxidation (LPO) and protein in microsomal fraction as well as hepatorenal glycogen, protein, triglycerides and total and esterified cholesterol contents along with histoarchitecture of liver.

2. Materials and methods

2.1. Maintenance of animals

Female albino rats of *Sprague-Dawley* strain (3 animals/cage; 130 ± 10 g body wt) were obtained randomly from the departmental animal facility where they were housed under standard husbandry conditions (25 ± 2 °C temp, 60–70% relative humidity and 12 h photoperiod) and had access to standard rat feed (Pranav Agro Industries, New Delhi, India) and water *ad libitum*. Experiments were conducted in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India and experimental protocols were approved by the Institutional Ethical Committee (CPCSEA/501/01/A) of Jiwaji University, Gwalior, India.

2.2. Chemicals

CCl_4, silymarin and other chemicals were procured from Ranbaxy, New Delhi, India; Sigma–Aldridge Company and Himedia Laboratories Ltd. Mumbai, India. Different kits for estimation of biochemical parameters were purchased from E-Merck. Crude propolis was gifted by Prof. O. P. Agrawal, Senior Entomologist, School of Studies in Zoology, Jiwaji University, Gwalior, India who collected it from the hive of *Apis mellifera*.

2.3. Preparation and administration of doses

CCl_4 was dissolved in liquid paraffin and administered orally (0.5 ml/5 ml/kg) according to Anand et al. (1994). A series of extraction was performed to yield ethanolic extract of propolis (62.8%, w/w) and kept at 4 °C for further use. Aqueous suspension of propolis (50, 100, 200 and 400 mg/kg, p.o.) and silymarin (50 mg/kg, p.o.) were prepared in gum acacia (Shukla et al., 2004) and silymarin was given as positive control. Equal amount of liquid paraffin (LP) or 1% gum acacia suspension (GAS, 5 ml/kg) was given as vehicles to control animals.

2.4. Experimental protocol

Animals were divided into eight groups of six each. Group 1 served as normal control and was treated with vehicles only. Groups 2–8 were administered CCl_4 and group 2 and 3 were treated as experimental controls. Groups 4–8 received multiple treatments with propolis and silymarin respectively after CCl_4 administration (see Scheme 1). Animals of all the groups were euthanized after 24 h of the last treatment and the estimations of various biochemical endpoints were carried out by frequently used older methods that the authors believe are trustworthy and sensitive.

2.5. Assessment of liver and kidney function tests

Blood samples were drawn by puncturing retro-orbital venous sinus, centrifuged and serum was isolated for estimation of transaminases (AST & ALT; Reitman and Frankel, 1957), alkaline phosphatase (SALP; Halk et al., 1954) and lactate dehydrogenase (LDH; Wroblewski and La Due, 1955). γ-Glutamyl transpeptidase (γ-GT), urea and uric acid were estimated using kit (E-Merck) according to the manufacturer's instructions and absorbance was read on a Merck auto-analyzer (Micro lab 200).

2.6. Study of oxidative stress

Liver and kidney of each rat were promptly removed out to determine LPO. The amount of malondialdehyde (MDA) formed was quantitated by reaction with thiobarbituric acid (TBA) and used as an index of LPO (Sharma and Krishna Murti, 1968). About 1 ml of homogenate, prepared in KCl (0.15 M) or microsomes were incubated at 37 °C for 30 min and proteins were precipitated by adding 1 ml chilled TCA (10%) then centrifuged at 450g for 15 min. Supernatant and TBA solution (0.67%) of 1 ml each were kept in boiling water bath for 10 min and after cooling, optical density was noticed at λ535 nm.

Reduced glutathione (GSH) was estimated in both organs using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Brehe and Burch, 1976). Hepatic and renal homogenates were prepared in 0.25 M sucrose solution. About 0.1 ml of homogenate and 0.9 ml of distilled water was added with 1.0 ml sulfosalicylic acid (10%) followed by centrifugation at 1000g for 10 min. Blank and standards were prepared by taking 0.5 ml of distilled water and 0.5 ml of GSH standard respectively. About 0.5 ml of supernatant was added with 4.5 ml of tris buffer (pH 8.23). Color was developed by adding 0.5 ml of DTNB solution and optical density was recorded at λ412 nm.

2.7. Microsomal drug metabolizing enzymes (MDMEs)

Fresh liver tissues were used for preparation of microsomes by the calcium precipitation method (Schenkman and Cinti, 1978). Aniline hydroxylase (AH) activity was assayed by measuring intensity of blue colored conjugate of phenol and *p*-amino phenol (PAP) and expressed as *n* moles PAP/min/g liver (Kato and Gillette, 1965). Briefly, 500 μl tris acetate buffer (0.2 M), 100 μl NADPH (1.2 mM), 100 μl $MgCl_2$ (25 mM) and 100 μl aniline (80 mM) were added with 200 μl microsomes and incubated at 37 °C for 20 min then 200 μl chilled TCA (30%) was added and centrifuged at 1050*g* for 10 min. About 750 μl of supernatant, *p*-aminophenol (20 μM) and TCA (5%) were taken for test, standard and blank respectively. Added 100 μl Na_2CO_3 (30%) and 500 μl phenol (2%) in all the tubes and kept at room temperature for 30 min then optical density was noted at λ630 nm against blank.

S. No.	Treatments	Day 1-3	Day 4-6	Day 7
Group 1	Control	LP (5 ml/kg, p.o.)	GAS (5 ml/kg, p.o.)	Euthanized
Group 2	Exp. Control 1 (3 days)	GAS (5 ml/kg, p.o.)	CCl_4 (0.5 ml/kg, p.o.)	Euthanized
Group 3	Exp. Control 2 (6 days)	CCl_4 (0.5 ml/kg, p.o.)	GAS (5 ml/kg, p.o.)	Euthanized
Group 4	-	CCl_4 (0.5 ml/kg, p.o.)	Propolis (50 mg/kg, p.o.)	Euthanized
Group 5	-	CCl_4 (0.5 ml/kg, p.o.)	Propolis (100 mg/kg, p.o.)	Euthanized
Group 6	-	CCl_4 (0.5 ml/kg, p.o.)	Propolis (200 mg/kg, p.o.)	Euthanized
Group 7	-	CCl_4 (0.5 ml/kg, p.o.)	Propolis (400 mg/kg, p.o.)	Euthanized
Group 8	Positive control	CCl_4 (0.5 ml/kg, p.o.)	Silymarin (50 mg/kg, p.o.)	Euthanized

Scheme 1.