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Reversal of acetaminophen induced subchronic hepatorenal injury by propolis extract in rats

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ABSTRACT

The ethanolic extract of propolis (200 mg/kg, p.o.) was evaluated against acetaminophen (APAP; 20 mg/kg, p.o.) induced subchronic hepatorenal injury in rats. Administration of APAP significantly increased the release of serum transaminases, alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transpeptidase, bilirubin and serum proteins, whereas concomitantly decreased hemoglobin, blood sugar and albumin. Hepatorenal reduced glutathione and activities of superoxide dismutase and catalase, hepatic CYPs i.e., aniline hydroxylase and amidopyrine-*N*-demethylase were significantly decreased after APAP intoxication. Lipid peroxidation showed significant elevation in both organs significantly after APAP assault. Total proteins, glycogen contents and the activities of certain metabolic enzymes i.e., adenosine triphosphatase, alkaline phosphatase and acid phosphatase were altered after APAP administration. Propolis extract exhibited curative effects by reversing APAP induced alterations in blood biochemical variables, CYP enzymes and markers of oxidative stress. Histopathological analysis of liver and kidney was consistent with the biochemical findings and led us to conclude the curative potential of propolis against APAP induced hepatorenal injury.

1. Introduction

The acetaminophen (APAP) is a widely used antipyretic and analgesic drug, which at overdose, causes extensive hepatic (Sener et al., 2006) and renal damage (Isik et al., 2006). The APAP is metabolized primarily by conjugation with glucuronic acid and sulphate in the liver. Less than 5% of the taken dose is metabolized by hepatic cytochrome P450s (CYP) to *N*-acetyl-*p*-benzoquinone imine (NAPQI), a chemically reactive metabolite. Alterations in the enzymatic activity of CYP even after minimal injury are used as a sensitive indicator of APAP induced liver damage (Sultatos et al., 1978). The toxic dose of APAP reduces the activity of CYP including aminopyrine-*N*-demethylase (AND) (Speck and Lauterburg, 1991) and aniline hydroxylase (AH) (Strubelt et al., 1979). The NAPQI either binds irreversibly to cellular macromolecules resulting to cytotoxicity (Jollow et al., 1973) or can react with glutathione (GSH) and is rendered non-toxic (Mitchell et al., 1973) that leads GSH depletion by as much as 90%. Because GSH is essential for the protection of thiol and other nucleophilic groups in proteins from the toxic metabolite of APAP, the concentration of intracellular GSH, therefore, is the key determinant of the extent of APAP induced hepatic injury (Ross, 1988). The binding of NAPQI with cellular proteins leads to necrosis in liver (Dahlin et al., 1984), which subsequently alters the liver function tests (LFTs). Reactive oxygen species (ROS) and nitrogen intermediates, produced by hepatic parenchymal and non-paranchymal cells are believed to be important factors contributing to APAP induced injury (Michael et al., 1999). In addition, the antioxidative enzymes such as, superoxide dismutase (SOD) and catalase (CAT) also play an important role in the modulation of APAP induced oxidative damage.

Propolis is a resinous substance to that honey bees collect from different plant exudates and use it to fill the gaps and to seal the parts of the hive (Marcucci et al., 2001). Chemical properties of propolis are not only beneficial to bees but have general pharmacological value as a natural mixture (Garedewa et al., 2004). Several empirical and clinical findings point to the fact that propolis may be more effective against pathogenic microorganism than conventional medications (Higashi and de Castro, 1994). Flavonoids and phenolics are the major complementary compounds of propolis (Ivanovska et al., 1995) that has beneficial effects as natural antioxidants (Basnet et al., 1997) and prevent oxidative damage of DNA caused by reactive oxygen species. The antioxidant effects may be a result of a combination of radical scavenging and an interaction with enzyme functions (Benkovic et al., 2007). Some components of the propolis are absorbed and circulate in the blood and behave as hydrophilic antioxidant and save vitamin C (Sun et al., 2000). Furthermore, the propolis extract has been reported to have a broad

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spectrum of biological activities, including anti-hyperalgesic (de Campos et al., 1998), antiparasitic (Dantas et al., 2006), antiproliferative (Russoa et al., 2004), immuno-enhancement (Orsolic et al., 2002), immunomodulatory (Orsolic and Basic, 2003), neuroprotective (Shimazawa et al., 2005) and radiation-induced damage (El-Ghazaly and Khayyal, 1995). Synergism between propolis and antibiotic (Krol et al., 1993), antimicrobial agents (Stepanovic et al., 2003) and with chelators against metal intoxication (Nirala et al., 2008) has also been observed.

Recently, our group has reported that ethanolic extract of propolis reverses carbon tetrachloride induced acute (Bhadauria et al., 2007a,b, 2008) and subchronic hepatic injury (Bhadauria et al., 2006) as well as APAP induced acute toxicity (Nirala and Bhadauria, 2008). Based on these findings, we continued to investigate the curative effect of propolis against APAP induced subchronic toxicity considering the measurement of CYP enzymes, GSH, thiobarbituric reactive substances (TBARS), LFTs, antioxidant enzymes (SOD and CAT) as well as histopathological analysis.

2. Materials and methods

2.1. Animals and chemical

Female Sprague–Dawley rats (8–10 weeks old having 130 ± 10 g body wt) were obtained 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) with standard rat feed (Pranav Agro Industries, 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), India and experimental protocols were approved by the institutional animal ethics committee (CPCSEA/501/01/A). The APAP, silymarin and other chemicals were procured from Sigma–Aldrich, Ranbaxy and Himedia Laboratories Ltd., India. Crude propolis was obtained as generous gift from Prof. O. P. Agrawal, Senior Entomologist, School of Studies in Zoology, Jiwaji University, who collected it from the hives of *Apis mellifera*.

2.2. Experimental design

The APAP (20 mg/kg) was suspended in warm distilled water and administered orally according to Khedun et al. (1993). A series of extraction was performed to yield ethanolic extract of propolis (62.8%, w/w) as described previously and kept at 4 °C for further use (Shukla et al., 2004). Aqueous suspension of propolis (200 mg/kg, p.o.) and silymarin (50 mg/kg, p.o.) were prepared in 1% gum acacia and silymarin was given as positive control (Bhadauria et al., 2007a,b). Equal amount of double distilled water or 1% gum acacia suspension were given as vehicle to control animals. Animals were divided into five groups consisting of six animals each and groups 2–5 received APAP for 21 days and treated as follows:

- Group 1: control, received vehicle only.
- Group 2: experimental control-1, received APAP only for 21 days.
- Group 3: experimental control-2, received APAP (21 days) followed by gum acacia suspension (5 days).
- Group 4: received APAP (21 days) followed by propolis (5 days).
- Group 5: positive control, received APAP (21 days) followed by silymarin (5 days).

Animals of all the groups were euthanized after 48 h of final administration; blood was collected, serum was isolated to assess various biochemical variables. Tissue samples from the liver and kidney were immediately processed for the biochemical analysis and histological preparations.

2.3. Isolation of serum and homogenate preparation

After keeping the blood for 1 h at room temperature, serum was isolated by centrifugation at 1000 × *g* for 15 min and stored at −20 °C until analyzed. Tissue samples of liver and kidney were homogenized with ice-cold 150 mM KCl for the determination of TBARS and CAT activity. For the estimation of GSH and SOD activity tissues were homogenized respectively in 1% sucrose and normal saline solution. The homogenates (10%, w/v) of liver and kidney were prepared in chilled hypotonic solution for total proteins, total cholesterol, adenosine triphosphatase (ATPase), acid and alkaline phosphatase (ACPase and ALPase).

2.4. Blood biochemical analysis

Blood was immediately used for the estimation of hemoglobin (Swarup et al., 1992) and blood sugar (Asatoor and King, 1954). Serum was used for the estimation of transaminases (AST and ALT) (Reitman and Frankel, 1957), alkaline phosphatase (SALP) (Halk et al., 1954), lactate dehydrogenase (LDH) (Wroblewski and La Due, 1955) and serum protein contents (Lowry et al., 1951). γ-Glutamyl transpeptidase (γ-GT), serum bilirubin and serum albumin were estimated using E-Merck's kit according to the manufacturer's instructions.

2.5. Preparation of microsome and assessment of hepatic CYP activity

Liver tissues were homogenized with Tris–HCl buffer (10 mM, pH 7.4) under cold conditions for the preparation of microsomes. Microsomes were obtained by calcium precipitation method (Schenkman and Cinti, 1978) for the estimation of CYP enzymes. The activity of AH was assayed by measuring the intensity of blue colored conjugate of phenol and *p*-amino phenol (PAP) at λ 630 nm (Kato and Gillette, 1965) and expressed as *n* moles PAP/(min g) liver. The AND activity was determined by measuring formaldehyde (HCHO) formed at λ 415 nm and expressed as *n* moles HCHO/(min g) liver (Cochin and Axelrod, 1959).

2.6. Glutathione and TBARS assays in liver and kidney

The GSH measurement was performed using dithionitrobenzoic (DTNB) acid (Brehe and Burch, 1976) and optical density was recorded immediately at λ 412 nm. The GSH level was calculated using an extinction coefficient of 13600 M^{-1} cm^{-1} and expressed as μmol GSH/g tissue. The TBARS was assayed for LPO (Sharma and Krishna Murti, 1968) and the LPO was expressed in terms of nmol MDA/g tissue using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.7. Assessment of CAT and SOD in liver and kidney

The CAT activity was determined as per method of Aebi (1984). Decomposition of H_2O_2 was monitored by a decrease in the absorbance at λ 240 nm. H_2O_2 concentration was calculated using extinction coefficient of 0.0394 mM^{-1} cm^{-1} and CAT activity was expressed as *n* moles H_2O_2/(min mg) protein. The SOD was determined by estimating inhibition of auto oxidation of epinephrine (Mishra and Fridovich, 1972). Specific activity was the % inhibition of auto oxidation of epinephrine by the enzyme per min and expressed as unit/mg protein.

2.8. Tissue biochemical analysis

Fresh tissues of liver and kidney were immediately processed for the estimation of glycogen by anthrone reagent method (Seifter et al., 1950). Total proteins were measured using BSA as standard by Lowry et al. (1951). Method of Zlatkis et al. (1953) was followed for the estimation of total cholesterol in liver and kidney. Estimation of metabolic enzymatic activities included acid and alkaline phosphatase (ACPase and ALPase) (Halk et al., 1954) and adenosine triphosphatase (ATPase) (Seth and Tangari, 1966).

2.9. Histopathological analysis

For light microscopic observations, samples from the liver and kidney were fixed in Bouin's fixative and processed routinely for embedding in paraffin. Tissue sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and examined under photomicroscope.

2.10. Statistics

Statistical analysis was carried out using one-way analysis of variance (ANOVA) taking significant at $P \leq 0.05$ followed by student's *t*-test taking significant at $P \leq 0.01$ and $P \leq 0.05$ (Snedecor and Cochran, 1994). Tukey's honestly significant difference (HSD) *post hoc* test was used for comparison among different treatment groups ($P \leq 0.05$).

3. Results

3.1. Biochemical analysis

Table 1 represents the effect of propolis extract on APAP induced blood biochemical alterations. Significant decrease was observed in hemoglobin ($P \leq 0.01$), blood sugar ($P \leq 0.05$) and albumin ($P \leq 0.01$) after APAP administration. Propolis therapy for 5 days recovered hemoglobin ($P \leq 0.05$) and albumin significantly towards control ($P \leq 0.01$). More than 60% protection was found in above-mentioned parameters and ANOVA was found to be significant at 5% level. The APAP administration significantly increased serum bilirubin and protein contents ($P \leq 0.01$). Propolis and silymarin down regulated the bilirubin and protein contents significantly