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Hepatotoxicity of tubers of Indian Kudzu (*Pueraria tuberosa*) in rats

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ABSTRACT

Methanolic extract of tubers of *Pueraria tuberosa* Linn. (*Fabaceae*) (PTME) has been tested for hepatoxicity in rats. In acute study, PTME (100–400 mg/100 g BW, given orally) showed LD_{50} at 227.5 mg. For sub-chronic study, its repeated doses (5–100 mg/100 g BW, for 30 days), significantly increased hepatic enzymes in blood, sinusoidal congestion, disruption of central vein, inflammatory cell infiltration and hepatocellular necrosis in liver in dose dependent manner, with increase in NO, iNOS and ROS levels. In a kinetic study (single dose 227.5 mg/100 g BW), there was sequential decrease in GSH and enhanced NO suggesting free-radical generation as the primary cause of cell damage. It is concluded that the higher dosing of PTME or its continuous use for longer period (even in low doses) is hepatotoxicity by inducing oxidative stress.

1. Introduction

Herbal remedies are rapidly gaining popularity throughout the world because of adverse effect of conventional medicines. Herbal preparations are considered harmless because of their "natural" origin (Bateman et al., 1998). However, several herbal medicines are also reported to have hepatotoxic effects (Stickel et al., 2000, 2005). Although the toxicity could be due to contaminants and adulterated ingredients, but the safety of any herbal preparation can not be predicted unless it is tested scientifically. In view of wide application of herbal medicines on health. In the present communication we planned to explore a systematic study on safety profile of a popular herbal preparation, which is in clinical use in Ayurveda as tonic, aphrodisiac, demulcent, lactogogue, purgative and cholagogue (Pandey and Chunekar, 1998) in various formulations. The powder of tubers of *Pueraria tuberosa* Linn. (*Fabaceae*) (PT tubers), commonly known as Indian Kudzu or Vidarikand in Hindi (Anon, 2006), is recommended for clinical use in the dose of 2–6 g/ adult-person (Pandey and Chunekar, 1998). It is a perennial woody Lianas, producing underground tubers up to 20 kg at a depth of 1–2 m. PT tubers are rich in isoflavonoides and terpenes (Handa and Kaul, 1996; Koul and Sumbali, 2008). Its important Phyto-compounds are daidzein, puerarin (Handa and Kaul, 1996), puetubersoanol (Khan et al., 1996) and tuberosin (Joshi and Kamat, 1973). Its various formulations are used as nutritive, diuretic, expectorants, and for management of rheumatism, fever and bronchitis (Gupta, 2003; Pandey and Chunekar, 1998). Some of its important biological properties are anti-hyperglycemic (Hsu et al., 2003), anti-hyperlipidemic (Tanwar et al., 2008), anti-fertility in male rats (Gupta et al., 2004), hepato-protective (Shukla et al., 1996).

We have also reported the antioxidant property of various fractions of PT tuber (Pandey et al., 2007). In the recent communication, the antioxidant and anti-inflammatory potential of this material have been reported from our laboratory (Pandey and Tripathi-under revision, Journal of Inflammation, 2009). Despite of multifactorial beneficial effects, knowledge about its toxic effects is scarcely reported except a report is available towards its toxicity (Shukla, 1995), In sequel to this study, we investigated the role of its total methanolic extract on free-radicals, liver function, renal function and histopthological changes in acute and sub-chronic conditions in rats.

2. Materials and methods

2.1. Preparation of extract and characterization

The tubers of *P. tuberosa* were collected from local market and its authenticity was confirmed by comparison with the fresh sample collected from Ayurvedic garden, Department of Dravya Guna, Institute of Medical sciences, Bananas Hindu

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHU, Banaras Hindu University; BT, bilirubin total; BD, bilirubin direct; BUN, blood urea nitrogen; BW, body weight; CRE, creatinine; CAT, catalase; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; GLU, glucose; GSH, reduced glutathione; iNOS, inducible nitric oxide; LPO, lipid peroxidation; LD_{50}, median lethal dose; MDA, malondialdehyde; NBT, nitro blue tetrazolium; NO, nitric oxide; PTME, methanolic extract of tubers of *Pueraria tuberose* Linn.; PT tubers, powder of *Pueraria tuberosa*; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substance; TLC, thin layer chromatography; TP, total protein; TNF-α, tumor necrosis factor- α; IL, interleukin; IF, interferon.

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University and also by direct comparison with the sample preserved in our Department (voucher # YBT/MC/12/1-2007). The dried tubers were coarsely powdered and extracted with methanol in a continuous soxhlet apparatus. The yield of solvent free methanolic extract (PTME) was found to be 12–16% in our experimental conditions. It was characterized on TLC finger printing in various solvent systems. It produced three spots with R_f value 0.13, 0.38, 0.51 in Benzene:Ethyl acetate (8:2), four spots with R_f value 0.11, 0.23, 0.51, 0.68 in Benzene:Ethyl acetate (6:4) and four spots with R_f value 0.26, 0.47, 0.72, 0.87 in Benzene:Ethyl acetate (2:8).

2.2. Experimental design

The protocol of this study was approved by Institutional Animal Ethical Committee of Institute of Medical Sciences (BHU, INDIA). The inbreed albino rats of Charles foster strain (both sexes, 150–200 g) were purchased from the Central Animal Facility of our Institute and acclimatized in our laboratory conditions for 7 days with free access to normal standard chow diet and tap water. The PTME was dissolved in drug vehicle (10% aqueous solution of tween-20). The experiments were divided into acute and sub-chronic toxicity study. In acute toxicity study, single dose (100–400 mg/100 g BW) of extract (PTME) was given to each group ($n = 8$ in each group) of animal and they were observed for change in food and water intake and general behavior for 1 h after dosing and then intermittently for 4 h and thereafter over a period of 24 h (Twaij et al., 1983). The rate of mortality was finally monitored after 72 h. The LD_{50} (lethal dose that killed 50% of the rat) was determined according to the method of Miller and Tainter (1944). In sub-chronic study, rats were randomly divided into six groups, having six rats in each. The drug vehicle was given in experimental control group-I and different dose of PTME was given in drug treated, experimental groups (II–VI). Extract was orally given to each rat daily in the morning. Change in body weight was noted and blood samples were collected from each rat on 7th, 15th and on 30th day. Blood was collected from retro-orbital puncture (Waynforth, 1980) in a plain tube for serum biochemistry. For mechanistic study, single dose (100 mg/100 g BW) was daily given for 7 days and liver antioxidant enzymes and GSH in liver homogenate, iNOS expression and histopathological changes in T.S. of liver and concentration of nitric oxide and other hepatocellular markers in serum were estimated. A short term kinetic study was also done by administering single dose of PTME (227.5 mg/100 g BW) and GSH and NO content were measured in blood at the interval of 24, 48 and 72 h.

2.3. Assessment of antioxidant status

Liver was excised, rinsed with ice-cold normal saline (0.9% NaCl) containing 0.16 mg/ml heparin, blotted dry and homogenized in 0.1 M phosphate buffer (pH 7) containing protease inhibitor in a Teflon homogenizer to get 10% homogenate (w/v). In this sample, level of lipid peroxides was measured as thiobarbituric acid reacting substance (TBARS) and is expressed as equivalent of malondialdehyde (MDA) using 1′1′3′3′-tetramethoxypropane as standard (Ohkawa et al., 1979; Tripathi and Sharma,1998), superoxide dismutase (SOD) activity was assessed in terms of its ability to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide which was instantly generated in the reaction system through a photosensitive reaction in presence of riboflavin (McCord and Fridovich, 1969; Tripathi and Singh, 1996). Catalase (CAT) activity was determined from the rate of decomposition of H_2O_2 monitored by decrease of 240 nm following the addition of tissue homogenate (Aebi, 1983). Reduced glutathione (GSH) level was measured colorimetrically as protein-free sulfhydryl content using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Boyne and Ellman, 1972; Tripathi et al., 1995). Total protein content was determined by Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

2.4. Measurement of biochemical parameters in blood

For hepatic function, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total and direct bilirubin (BT and BD), total protein (TP) and albumin were determined, while for renal functions blood urea nitrogen (BUN) and serum creatinine (CRE) were evaluated. Serum glucose was assessed for carbohydrate metabolism. In addition to these serum NO was also estimated. All these biochemical parameters were determined using auto analyzer using different methods viz kinetic rate method for ALT and AST (Schumann et al., 2002), total and direct bilirubin by Jendrassik and Grof method (Garber, 1981), total protein (Doumas et al., 1971a) and albumin (Doumas et al., 1971b) by biuret and BCG method, urea by BUN (Fawcett and Scott, 1960), the modified rate jaffe's kinetic method for creatinine (Larsen, 1972), glucose by GOD–POD method (Trinder, 1969). Nitrite (NO_2^-), a stable metabolite of NO, in the serum was determined by the method of Green et al. (1982).

2.5. Histopathological examination

Animals were sacrificed at different time intervals by anesthetizing them with diethyl ether followed by decapitation. The liver was carefully dissected out, washed with isotonic saline and then a small piece of each tissue was fixed in 10% neutral buffered formalin for 24 h and rinsed with 70% ethanol, dehydrated in serial dilutions of ethanol before embedding in paraffin wax. Paraffin blocks of the tissues were sectioned at 5–6 μm thickness by microtome, taken stained with haematoxylin- eosin and examined under a light microscope (Nikon, Japan). Sections from all animals were scored and best sections were photographed for documentation.

2.6. Immunohistochemistry of iNOS

Immunohistochemistry was carried out as described (McNaughton et al., 2002) by using an antigen-retrieval technique. In brief, isolated liver tissues were fixed by immersion in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded sections were cut, deparaffinized in 100% xylene, dehydrated by graded alcohols and water, followed by microwave antigen-retrieval treatment (two cycles of 10 min each at 95° in 0.01 mol/L trisodium citrate buffer (pH 6.0)). The sections were then washed with TBS (pH = 7.6) three times for 5 min each. Next, sections were incubated in 3% H_2O_2 in TBS for 15–20 min to block endogenous peroxidase activity. Sections were again washed with TBS three times for 5 min each and non-specific binding was blocked for 1 h with 10% goat serum (serum in which secondary antibody has been raised) in humid chamber. The sections were then incubated with polyclonal rabbit anti-goat iNOS (dilution 1: 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C in moist chamber, washed three times with TBS for 5 min each and incubated with HRP linked goat anti-rabbit secondary antibody (dilution 1: 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. The antibody binding sites were visualized by incubation with a diaminobenzidine–H_2O_2 solution for 3–5 min at room temperature. The sections were incubated with PBS instead of the primary antibody were used as negative controls. Brown–yellow granules in cytoplasm were recognized as positive staining for iNOS. Lastly, the slides were stained with hematoxyline, rehydrated, cleared with xylene, mount in DPX and examined under a light microscope (Nikon, Japan).

2.7. Statistical analysis

All results were expressed as mean ± standard deviation (S.D.). The significance of difference among groups using one way ANOVA followed by Post Hoc Dunnett test. Probability level of less than 5% ($p < 0.05$) was considered insignificant.

3. Result

3.1. Effect of PTME on acute toxicity

The animals receiving single dose of PTME showed low intake of food and water. The physical activity was significantly reduced and the response was dose dependent resulting in death on higher doses. The calculated LD_{50} was 227.5 mg/100 g B.W. in rats.

3.2. Effect of PTME on sub-chronic toxicity

3.2.1. Clinical observation and body weight changes

In sub-chronic study, oral administration of PTME for 30 days continuously in different doses (100, 50, 30, 15, 5 mg/100 mg B.W.) showed dose dependent mortality in rats. There was 100% mortality at 100 mg dose, within 10 days and at 50 mg dose it was within 20 days along with gradual reduction in body weight in early days of treatment. However, at very low doses, an increasing trend in BW was noted, without any mortality. Though, it was not statistically significant ($p > 0.062$) (Table 1).

3.2.2. Biochemical parameters

In biochemical test, the PTME significantly raised AST, ALT, BT and BD along with decrease in albumin, TP and glucose (Table 2A) at higher doses, suggesting its hepatotoxicity. The animals, who survived up to 30 days (30 and 15 mg), showed time and dose dependent increase in serum AST, ALT but no significant change was noted in levels of BT, BD, albumin, TP (Table 2B). However, 5 mg dose of PTME was found to be totally safe over the span of 30 days treatment showing no change in all above described parameters. Contrary to this, it did not show any change in renal function test even on higher doses (100 mg/7 days, data not shown).