Patulin Induced Oxidative Stress Mediated Apoptotic Damage in Mice, and its Modulation by Green Tea Leaves

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Background: **The present study demonstrates the antioxidant and hepatic protective effects of Green tea leaves (GTL).** ***Methods:*** **The serum level of aspartate aminotransferase and alanine aminotransferase was analyzed. The liver antioxidant enzymes such as SOD, CAT, GPx, GR, GSH, lipid peroxidation and protein carbonyls, ROS content were estimated. The histology of liver tissue was observed and the protein expression of SOD, CAT, Caspase-3 and p53 was investigated by Western blotting.** ***Results:*** **Effectiveness of GTL extract in preventing patulin induced liver damage showed significant reduction in serum ALT and AST to 19% and 85% respectively, the increase in antioxidant levels and lipid peroxidation products with patulin treatment were also reduced with GTL supplementation. The patulin induced increase in hepatic protein carbonyls was significantly reduced by 141–111% with 100 and 200 mg/kg b.wt GTL and in ROS was significantly reduced by 171–140% with 100 and 200 mg/kg b.wt GTL administration respectively. Also showed protection against hepatic tissue damage and protein expression in mice.** ***Conclusion:*** **This study showed remarkable antioxidant and hepatic protective effects of GTL. (J Clin Exp Hepatol 2017;7:127–134)**

Hepatoprotection

Mycotoxin Patulin (PAT) is a secondary metabolite produced by certain species of *Penicillium*, *Byssochlamys* and *Aspergillus* with diverse toxic effects both in humans and animals.[1] Patulin is a contaminate of food stuffs, fruits and vegetables, especially apple and apple based products[2] World Health Organization (WHO) and European Union recommended 50 ppb in food stuffs.[3] Patulin has been shown to induce carcinogenesis, mutagenesis, and teratogenesis properties.[4] Several studies have been undertaken to demonstrate mutagenic activity through generation of micronuclei and chromosomal aberrations in mammalian cells,[5] quick depletion of GSH and increased generation of reactive oxygen species (ROS).[6,7] Patulin mediates toxicity via oxidative damage pathway.[8]

Patulin induce genotoxicity in HepG2 cells was also demonstrated by[9] as an evidence of oxidative damage. Patulin mediated toxicity seems to be targeting cellular components by formation of covalent adduct by electrophilic reactivity and interaction with electrophilic chemicals with free cysteine, cysteine-containing tripeptide glutathione, and it also react with lysine-, histidine-containing proteins.[10]

Several studies have reported that phytochemicals and polyphenols protect against the toxic effects induced by these mycotoxins.[11] Recent studies showed that silymarin could control PAT-induced hepatotoxicity and genotoxicity by antioxidant pathway.[12] Epidemiological, clinical and biological studies indicated that the consumption of these phytochemicals or polyphenols is associated with reducing oxidative damage and provides health benefits to humans and animals.[13] Green tea contains several polyphenols, epigallocatachine gallate is the major constituent of these green tea polyphenols and is known to have anti-carcinogenic, anti-tumor, anti-mutagenic activity.[14] Further green tea has significant antioxidant properties and exhibits protective role in the development of cardiovascular disease and other pathologies.[15,16] Although study was under taken by Song et al.[17] on the modulation of patulin induced hepatotoxicity and genotoxicity by green tea polyphenols there is lacunae in the study such as modulating protein expression responsible to oxidative and apoptosis damage. Hence the present study was undertaken to evaluate patulin induced oxidative stress and apoptotic damage in mice and their amelioration by green tea leaves (GTL).

MATERIALS AND METHODS

Chemicals and Reagents

Patulin (PAT, 4-hydroxy-4H-furo(3,2-C)pyran-2(6H)-one, purity >98.0%) were obtained from Sigma (Bangalore,

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Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; GTL: green tea leaves; LP: lipid peroxidation
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India), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and HiSep LSM LS001 were procured from Hi-Media (Bangalore, India). The other chemicals used were of high purity grade and were procured from Merck (Bangalore, India).

Plant Material

Green Tea Leaves were purchased from the local market of Mysore in the month of September 2013, Karnataka, India and washed with water and shade dried for six days till the moisture gets evaporated completely.

Preparation of Ethanolic Extract

The shade dried GTL were powdered and extracted with ethanol; 200 g of GTL were immersed in ethanol solution in 1000 ml flat bottom flask and was macerated for one week. The collected extracts were filtered and concentrated to dryness under reduced pressure and controlled temperature using rotary flash evaporator. The yield obtained was 0.9%.

Experimental Design

Animal experiments were performed according to the guidelines from the Institute Animal Ethical Committee and Committee for the Purpose of the Control and Supervision of Experiments on Animals; NO: 28/IAEC/CPCSEA. Male Balb/c mice weighing 30 ± 5 g were selected from the stock colony Defence Food Research Laboratory, Mysore, India. The animals were housed in acrylic fiber cages, temperature (25 ± 2 °C) and maintained in 12 h light/dark cycle. Food and water were provided ad libitum.

After one week's acclimatization, forty-eight male Balb/c mice weighing 30 ± 5 g were randomly divided into the following six experimental groups with eight animals in each group. (I) Control group (Saline), (II) GTL (GTL extract) 200 mg/kg b.wt (body weight) (III) PAT 2 mg/kg b.wt treated group (IV) GTL 50 mg/kg b.wt + PAT (V) GTL 100 mg/kg b.wt + PAT (VI) GTL 200 mg/kg b.wt + PAT. GTL extract was administered by gavage for one week before PAT treatment. After one week the PAT 2 mg/kg b.wt was administered by intraperitonial for three days and left for one week. Control mice were fed orally with an equal amount of saline. Body weights were recorded during the experimental period. After the experiments the mice were sacrificed and serum, blood and hepatic tissues were collected for further analysis.

Serum Biochemical Markers

Estimation of ALT and AST

To determine the hepatic damage, the serum biochemical markers such as ALT and AST were determined according to the kit suppliers protocols (Cat no. 11409003 and 11408002 Canada).

Tissue Biochemical Markers

Estimation of Hepatic Antioxidant Enzymes

SOD, GPx and GR were the hepatic antioxidant enzymes were determined according to the protocols of kit supplier (Randox, Cat no. SD. 125, RS 504 and GR 2368, Canada). The CAT enzyme content was estimated manually by spectrophotometric method[18] and the results were expressed in U/g tissue. Glutathione content was determined by DTNB method.[35] The reaction products were spectrophotometrically measured at 412 nm and results were expressed as mM/g of tissue. Protein concentration was determined by Bradford method and the results were expressed as U/mg of protein.

Estimation of Hepatic Lipid Peroxidation

Lipid peroxidation was measured by the Thiobarbituric acid reactive species (TBARS) method described by.[19] Liver tissues were homogenized in phosphate buffer (2 mL, pH 7.0). 10% TCA (0.5 mL) and 2 mL of TBA mixture were added to the homogenate (0.5 mL). The TBA mixture contained TBA (0.35%), FeCl3 (0.05 mM), SDS (0.2%) and BHT in glycine-HCl buffer (100 mM, pH 3.6). The above reaction mixture was boiled for 30 min at 100 °C, then cooled and centrifuged at 8000 rpm (revolutions per minute) for 10 min, absorbance was measured at 532 nm and the results were expressed as moles/mg of protein.

Estimation of Protein Carbonyls

The protein carbonyl content of mice liver homogenates were evaluated by the method described by Levine et al.[20] Briefly, one ml of 10 mM DNPH in 2 N HCl was added to the liver homogenates and samples were incubated for 1 h at room temperature. To this 1 ml of trichloroacetic acid (10%) was added and centrifuged at $3000 \times g$ for 10 min and protein pellets were washed thrice with 2 ml of ethanol/ethyl acetate (1:1, v/v) and were dissolved in 1 ml of guanidine hydrochloride (6 M, pH 2.3). The samples were incubated for 10 min at 37 °C and the absorbance was recorded at 370 nm.

Estimation of ROS

ROS generation was detected in liver fractions using a fluorescent probe 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA).[21] Briefly, the assay buffer contained 20 mM Tris–HCl, 130 mM KCl, 5 Mm $MgCl_2$, 20 mM NaH_2PO_4, 30 mM glucose and 5 µM DCFH-DA. Samples were incubated for 15 min at 37 °C and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 525 nm using Hidex plate chameleon™ V (Finland).

Histopathology

Liver tissues were collected and immediately fixed in 10% formalin in saline. The tissues were processed, and embedded in paraffin wax; 5 mm thick sections were taken and

