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)

Mycotoxin Patulin (PAT) is a secondary metabolite produced by certain species of *Penicillium*, *Byssoschlamys* and *Aspergillus* with diverse toxic effects both in humans and animals.¹ Patulin is a contaminant of food stuffs, fruits and vegetables, especially apple and apple based products² 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 by⁹ 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, epigallocatechin 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 undertaken 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 ($25 \pm 2^\circ\text{C}$) 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 intraperitoneal 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 method¹⁸ 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 MgCl₂, 20 mM NaH₂PO₄, 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 chameleonTM 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

stained with hematoxylin and eosin for histopathological observations. The hepatic tissue morphology was observed under light microscope (Olympus, Japan) and photographs were taken with Cool SNAP[®] Pro color digital camera.

Immunoblotting

Liver tissue was homogenized with cold lysis buffer, pH 7.4 with protease inhibitor cocktail and the protein contents were determined as mentioned earlier. Samples were (50 µg of proteins) separated by 10–15% SDS-PAGE followed by transferred to nitrocellulose membrane using an electro blotting apparatus (Cleaver Scientific Ltd, UK). The membranes were then blocked overnight at 4 °C with 5% (v/v) non-fat dry milk in Tris-buffered saline with Tween-20 (TBS-T) (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20, pH 7.5) and incubated with primary antibodies namely GAPDH (sc-5286), SOD(sc-8637), CAT (sc-34280), caspase-3 (sc-C8487), and p53 (sc-55476) (Santa Cruz Biotechnology, CA, USA) and caspase-3 (C8487, Sigma, St. Louis, MO, USA) at 1:1000 dilution and incubated at room temperature for 3 h with shaking. The membranes were washed in TBST followed by incubation for 2 h at room temperature in dark with horseradish peroxidase (HRP) conjugated rabbit anti-goat, goat anti mouse and goat anti-rabbit secondary antibodies (DAKO, Denmark) at 1:10,000 dilutions. The membranes were washed again and the immune reactivity was detected by using the enhanced chemiluminescence peroxidase substrate kit (CPS-160, Sigma, St. Louis, MO, USA) and the band intensity was measured using NIH image J software.

Statistical Analysis

The results were analyzed by one-way ANOVA followed by a Tukey's HSD-post hoc test. Significance was set at 0.05 and all comparisons were made against the control group.

RESULTS

Effect of PAT on Serum Aminotransferases

Serum aminotransferase such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly used as an indicator for liver disease. In the present study a significant elevation of serum ALT and AST activities ($P < 0.05$) were observed in PAT treated mice. However, 50, 100 and 200 mg GTL administration for 7 consecutive days prevented these elevations in a dose-dependent manner. Mice treated with 200 mg/kg b.wt GTL group with PAT group, showed a significant reduction in serum ALT and AST to 19% and 85% respectively, we found in our preliminary study that GTL treatment alone has no significant effect on ALT and AST activities (Figure 1).

Protective Effect of GTL Against Oxidative Stress

Effect of PAT on the levels of hepatic antioxidant enzymes namely SOD, CAT, GPx, GR and GSH were analyzed. A decrease was observed in antioxidant enzyme levels such as SOD, CAT, GPx, GR and also in the content of GSH in PAT challenged mice ($P < 0.05$). However a significant recovery ($P < 0.05$) in antioxidant enzyme activities was observed with 100 and 200 mg/kg b.wt GTL supplementation (Table 1). The mice treated with 200 mg/kg b.wt GTL group with PAT group, showed a significant recovery.

GTL Inhibits Lipid Peroxidation

The patulin induced ROS generation disturbs membrane lipids and causes lipid peroxidation which is measured by the malondialdehyde formed in oxidative damage of tissues, whereas GTL supplemented mice decreased lipid peroxidation with maximum decrease with 100 and 200 mg/kg b. wt GTL treatment ($P < 0.05$) which suggests that GTL exerts protective effects against free radicals mediated lipid damage (Table 1).

GTL Inhibits Protein Carbonyls

The oxidative modification of amino acid side chains in proteins to aldehydes or ketones gives rise to protein carbonyls. The protein carbonyl content was significantly elevated to 246% in PAT treated mice liver homogenates ($P < 0.05$) compared to untreated control animals which is considered as 100%. However a significant reduction in protein carbonyls was observed dose dependently with PAT treatment and up to 141 and 111% decrease in protein carbonyls ($P < 0.05$) was found in animals administered with 100 and 200 mg/kg b.wt GTL respectively (Figure 2A). In a recent study the protective effect of *Asparagus racemosus* was also recorded against t-BHP induced protein carbonyls in hepatic tissue damage.²

GTL Inhibits ROS Generation

Free radicals such as hydroxyl radical (HO), hydroxyl anion (HO⁻) and superoxide anion (O₂⁻), are collectively called reactive oxygen species (ROS), the elevation in ROS leads to cellular damage. Hence estimation of ROS is used as a marker to study the oxidative stress. In the present investigation the ROS generation in hepatic tissue challenged with PAT was estimated using fluorescent probe DCFH₂DA. 288% increase ($P < 0.05$) in fluorescence was observed in PAT treated liver homogenates which decreased dose dependently with GTL treatment to 171 and 140% decrease ($P < 0.05$) with 100 and 200 mg/kg b.wt GTL treatment respectively (Figure 2B).

Figure 1 (A) Effect of GTL on serum ALT level. Mice were treated with different doses of GTL followed by PAT and the serum content of ALT was measured ($n = 8$, $P < 0.05$, significantly different from the control group and homogenous sub groups share common letter). (B) Effect of GTL on serum AST level. Mice were treated with different doses of GTL followed by PAT and the serum content of AST was measured ($n = 8$, $P < 0.05$, significantly different from the control group and homogenous sub groups share common letter).

Protective Effect of GTL Against Hepatic Damage

Control mice showed normal structure and compactly arranged hepatocytes. Sinusoids were scattered randomly all over the hepatocytes, and they had uniform morphology along with the central vein. However, PAT treated mice showed focal hepatocellular vacuolation and necrosis of hepatocytes with inflammatory cell infiltration and mild hemorrhage. However the mice treated with 200 mg/kg

GTL showed better preservation of liver tissue histology against PAT induced hepatic damage (Figure 3).

Protective Effects of GTL on Oxidative Stress and Apoptotic Biomarker

The protective effect of GTL against PAT induced oxidative stress and apoptotic damage was evaluated by Western blotting. Enzymes such as SOD, CAT and GAPDH and apoptotic enzymes p53 and caspase-3 the antioxidant

Table 1 Effect of GTL on Hepatic Antioxidant Markers such as SOD, CAT, GPx, GR, GSH and Lipid Peroxidation. Mice were Treated with Different Doses of GTL Followed by PAT and the Liver SOD, CAT, GPx and GR were Measured ($n = 8$, $P < 0.05$, Significantly Different from the Control Group and Homogenous Sub Groups Share Common Letter).

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GR (U/mg protein)	GSH (μ M/mg protein)	MDA (M/mg protein)
Control	1.82 ± 0.69^a	24.61 ± 1.42^a	26.55 ± 1.42^a	33.17 ± 1.62^a	9.24 ± 0.62^a	30.14 ± 1.5^a
GTL 200	2.06 ± 0.82^a	27.15 ± 1.52^a	28.37 ± 1.64^a	36.54 ± 1.94^a	10.65 ± 0.82^a	28.11 ± 1.2^a
PAT	0.58 ± 0.26^b	11.10 ± 0.45^b	16.17 ± 0.8^b	16.14 ± 1.23^b	3.25 ± 0.19^b	53.16 ± 2.3^b
GTL 50 + PAT	0.98 ± 0.43^a	18.11 ± 0.82^a	19.34 ± 1.18^a	25.22 ± 1.83^a	5.19 ± 0.14^a	41.49 ± 2.1^a
GTL 100 + PAT	1.38 ± 0.54^a	20.52 ± 1.12^a	22.76 ± 1.24^a	29.56 ± 1.56^a	6.89 ± 0.29^a	39.61 ± 1.72^a
GTL 200 + PAT	1.44 ± 0.68^a	22.31 ± 1.29^a	24.82 ± 1.32^a	31.65 ± 1.72^a	8.11 ± 0.41^a	35.12 ± 1.67^a

Figure 2 (A) Effect of GTL on hepatic protein carbonyl content. Mice were treated with different doses of GTL followed by PAT and the liver protein carbonyl content was measured ($n = 8$, $P < 0.05$, significantly different from the control group and homogenous sub groups share common letter). (B) Effect of GTL on hepatic ROS generation. Mice were treated with different doses of GTL followed by PAT and the liver ROS generation was measured using spectrofluorimeter ($n = 8$, $P < 0.05$, significantly different from the control group and homogenous sub groups share common letter).

biomarkers SOD and CAT were down-regulated with PAT treatment and expression of p53 and caspase-3 were increased. Whereas GTL pretreatment significantly restored the SOD and CAT and the apoptotic enzymes p53 and caspase-3 normalized the protein expression (Figure 4).

DISCUSSION

Patulin is a fungi produced by *Aspergillus*, *Penicillium expansum* and other *Penicillium* spp., *Aspergillus* species and are classified as an emerging infectious pathogen by the U.S Department of Health and Human Services, National

Figure 3 Effect of GTL on PAT induced liver damage of mice. Mice were treated with different doses of GTL followed by PAT and the liver morphology was observed by hematoxylin/eosin staining. Scale bar represents 500 μm ; magnification 400 $\times$. (A) Vehicle control group (B) 2 mg/kg b.wt PAT treated group (C) 100 mg/kg b.wt GTL + PAT treated group (D) 200 mg/kg b.wt GTL + PAT treated group.

Figure 4 The protective effect of pre-treatment of GTL on PAT induced expression of oxidative stress marker proteins SOD, CAT and apoptosis marker proteins caspases-3 and p53 were analyzed by Westernblotting. (b–e) The band intensity is calculated by Image-J software. The data are represented as mean $\pm$ SD of three independent experiments. * $P < 0.05$ versus control group, # $P < 0.05$ versus PAT treated group.

Institute of Allergy and Infectious Diseases. Patulin commonly contaminates food grains and fruits especially apple and apple based products.² The in vitro and in vivo studies observed that patulin is found to be toxic to humans and animals. Patulin was reported to induce micronuclei and chromosomal aberrations and it also induces genotoxic risk and oxidative DNA damage in mammalian cells, cell cycle arrest and apoptosis through modulation of Bax, p53 and p21/WAF1 proteins in skin and also it is neurotoxic to mice. Further it has been reported to induce hepatotoxicity in mice and also has cytotoxic effects in HepG2 cell lines.^{5,17,8}

There are several reports on herbs/plants or the phytochemical compounds inhibit the mycotoxin induce toxicity. Rosmarinic acid has been shown to protect against

aflatoxin B1 and ochratoxin-A-induced cell damage in a human hepatoma cell line (Hep G2), α -tocopherol and retinol and catechins against ochratoxin A cytotoxicity Red ginseng extract protects against aflatoxin B 1 and fumonisins-induced hepatic pre-cancerous lesions in rats.^{22,23} Therefore the present study evaluates the protective effects by herbal plants, for the patulin there is less reports for the protective effects against patulin induce toxicity that is 6-Gingerol shows the protective effects patulin induce genotoxicity in HepG2 Cells and selenium shows protective effects against patulin-induced brain damage in mice.^{6,17}

Green tea leaf extract (*Camellia sinensis*) is rich in antioxidant mainly catechins, epicatechin, epigallocatechin gallate, epicatechin gallate, epigallocatechin, etc. The green tea leaf

has been reported to possess antidiabetic, antibacterial, anti-inflammatory, neuroprotective and hepatoprotective properties.^{24,25} The green tea leaf inhibits the oxidative stress induced by toxic compounds or mycotoxins.^{26,27} Therefore the present study was carried out to evaluate protective effects of green tea extract against patulin induced oxidative stress, apoptotic damage and hepatotoxicity.

Antioxidant enzymes such as glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase inhibits the oxidation of other molecules. Generation of reactive active species or in the toxic condition the antioxidant enzymes get imbalanced. The supplementation of herbal plant shows that the antioxidant enzymes restored when it reduced by oxidative stress^{36,37} lipid peroxidation (LP) – oxidative degradation of polyunsaturated fatty acids caused by ROS – is responsible for degradation of membrane lipids resulting in cell damage and formation of many toxic products and which can be measured by MDA. The herbal plants *Terminalia arjuna*, *Coriandrum sativum* which prevents the lipid peroxidation induced by carbon tetrachloride similarly the green tea also have rich antioxidants hence which is able to inhibit the lipid peroxidation induced by patulin.²⁸

Histology involves the examination of sampled whole tissues under the microscope in order to study the manifestations of disease. Histology of liver tissue with patulin treated group showed focal hepato cellular vacuolation and necrosis of hepatocytes with inflammatory cell infiltration and mild hemorrhage. But in the green tea treated group the hepatic lesion and cell infiltration was reduced, there are a several reports that the plant extracts can protect the liver damage induced by carbon tetrachloride acetaminophen. Hence the green tea can protect the liver damage induced by patulin.²⁹ ROS and protein carbonyls are the markers of oxidative stress these markers will increase when cell undergoes to toxic condition and this can be inhibited by plants³⁰ and Dkhar et al.³⁸ reports that *Punica granatum* and curcumin protects the tissue from the oxidative stress similarly the green tea extract also protects from the ROS generation and oxidation of proteins.

Western blotting also known as immunoblotting, is a technique used to detect the presence of a specific protein in a complex mixture extracted from tissue extract. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is one of the key enzymes involved in glycolysis. It is one of the so called housekeeping proteins and GAPDH is constitutively expressed in almost all tissues in high amounts. For this reason, GAPDH is widely used as a loading control for protein normalization in Western blotting.

The antioxidant enzyme such as SOD is the first line of defense against ROS and is active in catalyzing detoxification of superoxide radical (O_2^-). Generated H_2O_2 in this reaction is restored to water in the presence of CAT. When the cell undergoes oxidative stress these enzymes plays a significant role in cell defense against generation of

radicals. In the protein targeting technique the patulin treated group expression was decreased but the green tea treated group significantly increased the antioxidant expression. These protein expressions were increased by plant extracts or some phytochemicals. The purple sweet potato, pea root and salicylic acid reports that the antioxidant enzymes expression increased when it is treated with plant extracts. In the same way the green tea also proves that which can increase the protein expression.³¹

Apoptosis is programmed cell death there are so many genes involved in the apoptosis. One of the most important p53 functions is its ability to activate apoptosis, and disruption of this process can promote chemoresistance and tumor progression. p53 apparently promotes apoptosis through transcription-dependent and -independent mechanisms that act in concert to ensure that the cell death program proceeds efficiently.³⁸ This balance is the proportion of proapoptotic homodimers that form in the outer-membrane of the mitochondrion. The proapoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators. Caspases proteins are two types initiator and effector. The active effector caspases proteolytically degrade a host of intracellular proteins to carry out the cell death program. Hence the decrease expression of p53 and caspase can protect against apoptosis. There are several reports that inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death.³⁹ These findings suggested the inhibitor effect of green tea leaves (GTL) against patulin induced antioxidant, hepatotoxicity and gene expression.

CONCLUSION

The present study was designed to evaluate the modulation of antioxidant, hepatotoxicity and gene expression by GTL against PAT induced damage in mice. Overall results of the present research demonstrate the protective effect of GTL and its application to treat stress associated maladies.

CONFLICTS OF INTEREST

The authors have none to declare.

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