Protective effect of aqueous extract of *Phyllanthus fraternus* against bromobenzene induced changes on cytosolic glutathione S-transferase isozymes in rat liver

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The aim of this study was to investigate beneficial effect of aqueous extract of *Phyllanthus fraternus* (AEPF) on bromobenzene (BB) induced changes on cytosolic glutathione S-transferase (GST) isozymes in rat liver. Administration of BB significantly decreased the activity of GST, however, prior administration of AEPF prevented the BB induced decrease in GST activity. Further the cytosolic GSTs were purified from 3 groups of animals (control, BB and AEPF+BB administered) and resolved into three protein bands on SDS-PAGE. Densitometric analysis showed a significant decrease in BB group compared to control. Further, 2D PAGE analysis resolved these proteins into 8 bands which were identified as five isozymes of alpha, two of Mu and one of theta by MALDI-TOF MS and also observed decreased levels of isozymes in BB group. However, on prior administration of AEPF significantly prevented the BB induced decrease in GSTs and restored to normal levels.

1. Introduction

Glutathione S-transferases (GST) (EC 2.5.1.18) are ubiquitous multifunctional enzymes and represent 10% of cytosolic proteins and catalyze the conjugation of toxic xenobiotics and oxidatively produced compounds, and thus facilitate their metabolism, removal and provide protection against oxidants \([1,2]\). There are three major families that exhibit GST activities are cytosolic, mitochondrial and microsomal \([3]\). Cytosolic GSTs comprise a large family of detoxification enzymes that function as hetero or homodimers and are classified into seven classes termed as alpha (α), mu (μ), theta (θ), pi (π), zeta (ζ), chi (χ) and sigma (σ) \([4–6]\). The most abundant classes expressed in mammalian tissues are Alpha (GSTA), Mu (GSTM) and Pi (GSTP) \([7,8]\). The pattern of expression of these isozymes are specific for species, age and organs \([9]\). These enzymes also show variable expression towards certain highly reactive chemical contaminants and remarkable affect on resistance/sensitivity to chemical toxicities. Variations in GST isozyme expression have profound effects on health. For example, low expression of human GSTM is associated with an increased incidence of bladder \([10]\), and colon cancer \([11]\). Therapies that increase the expression of GST isozymes may be useful in disease prevention.

Medicinal plants are used to prevent many diseases since ancient days, and there is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine. *Phyllanthus fraternus* (Euphorbiaceae), a medicinal herb commonly known as nelusari, distributed in India, Pakistan, South Arabia, Africa and West Indies \([12]\). It is widely used in traditional and folk medicine for the treatment of various diseases of liver \([13]\) and also as natural remedy for a number of viral infections \([14]\). The aqueous and alcoholic extracts were reported to have an antidiabetic activity in alloxan induced diabetes \([15]\).

Bromobenzene (BB) is a toxic chemical that is converted to bromobenzene 3, 4-oxide in liver, which binds GSH and they depletes it. This leads to an impaired protection against reactive oxygen species (ROS) \([16]\) which leads to lipid peroxidation and altered calcium homeostasis that damage the cell and cell organelles \([17]\). Earlier studies from this laboratory have shown that mitochondrial dysfunction in liver caused by the administration of thioacetamide \([18]\) or carbon tetrachloride \([19]\) or alcohol \([20]\) or allyl alcohol \([21]\) or bromobenzene \([22]\) could be prevented by prior administration of AEPF.

**Abbreviations:** AEPF, aqueous extract of *Phyllanthus fraternus*; BB, bromobenzene; CDNB, 1-chloro-2, 4-dinitrobenzene; CHAPS, (3-[3-cholamidopropyl]-dimethylammino)-1-propane sulfonate); 2DE, two dimensional gel electrophoresis; DTT, dithiothretol; GSTs, glutathione S transferases; IPG, immobilized pH gradient; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; PMF, peptide mass fingerprint; ROS, reactive oxygen species

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Bromobenzene induced mitochondrial dysfunction was also detected in rat kidney and efficiently protected by prior administration of AEPF [23]. Not much information is available on BB induced changes on the pattern of cytosolic GST isozymes and protective effect of *Phyllanthus fraternus* extract in rat liver. The present study is aimed to isolate and characterize the GST isozymes in BB administered rat liver and protective role of *Phyllanthus fraternus* extract.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 100 ± 40 g were used in this study. They were kept in the animal house facility of University of Hyderabad in polypropylene cages at an ambient temperature of 25 ± 2°C and humidity of 45–60% with 12 h day – night cycles. They had free access to standard rodent pelleted food (Hindustan lever Ltd., India) and water ad libitum. The growth of the rats was monitored for at least one week before starting the experiment. This study was carried out with approval from the Institutional Animal Ethics Committee (IEAC) of University of Hyderabad.

2.2. Chemicals

Epoxy activated sepharose 6B and glutathione (GSH) were purchased from sigma chemical co. (St. louis, MO, USA). IPG strips, IPG buffer, DTT, CHAPS and urea were obtained from Amersham biosciences, NJ, USA. Rabbit polyclonal anti-GST antibody, Goat anti-rabbit IgG ALP conjugate secondary antibody, BCIP-NBT substrate for alkaline phosphatase and protein molecular weight marker were obtained from Bangalore Genei, India. All other chemicals were of analytical grade and were obtained from local firms.

2.3. Plant material and preparation of AEPF

Plants were collected from their natural habitat in the University of Hyderabad, Hyderabad-500046, India and voucher specimen with a number OHS-SG-1005, has been deposited at the Herbarium, University of Hyderabad, Hyderabad, India [22]. The whole plant including roots, stems and leaves were cleaned with water, air dried and powdered using mortar and pestle. 60g of this powder was mixed with 300 ml of double distilled water followed by centrifugation at 3000×g for 10 min. The supernatant was collected, filtered (by using cheese cloth) and used as an aqueous extract in this study. The dry weight was determined gravimetrically by drying the extract in hot air oven for 4 h. The yield of the extract was 5% (w/w).

2.4. Experimental design

The animals were divided into 4 groups of 6 rats in each.

**Group A:** Rats received a single dose of 0.1 ml of coconut oil through intragastric tube and sacrificed after 19 h. This is control group.

**Group B:** Received a single dose of 10 mmol of bromobenzene in 0.1 ml of coconut oil through intragastric tube and sacrificed after 19 h. This is BB treated group.

**Group C:** Received AEPF orally (100 mg/kg body wt.) for a period of 8 days and sacrificed 19 h after the last dose. This is AEPF treated group.

**Group D:** Received AEPF orally (100 mg/kg body wt.) for 8 days and then after 24 h of last dose a single dose of 10 mmol of bromobenzene in 0.1 ml of coconut oil was give through intragastric tube and sacrificed after 19 h. This is BB + AEPF treated group.

All 4 groups were fasted for 43 h before sacrifice (24 h before and 19 h after the plant extract/toxin/coconut oil administration).

2.5. Estimation of flavonoids, total phenols and tannins

The presence of secondary metabolites from AEPF was quantitatively determined by adopting standard protocols. Flavonoids were estimated by Swain and Hillis [24], tannins by Vanillin–HCl method of Price et al. [25] and total phenols by Folin-Ciocalteu method of Singleton et al. [26]. The results were expressed as mg/gm dry weight.

2.6. Preparation of the glutathione – affinity matrix

Affinity matrix was prepared according to Simmons and Vander Jagt [27]. Epoxy activated Sepharose 6B (16.3 g) was washed with 2 l of Milli Q water on Buchner funnel, followed by 200 ml of 44 mM phosphate buffer pH 7.0. The slurry was transferred to a side armed conical flask and the volume was adjusted to 100 ml with the same buffer and nitrogen gas was passed through for 5 min. To this, 325 mM GSH (100 mg/ml) of pH 7.0 was added and coupling was allowed to proceed for 24 h at 37°C with gentle shaking on a Dubnoff metabolic incubator. The coupled gel was washed with 400 ml of Milli Q water and the remaining active groups were blocked by allowing the gel to stand in 1 M ethanolamine for 4 h. Then the gel was washed sequentially with 400 ml each of 0.5 M KCl in 0.1 M sodium acetate (pH 4.0), 0.5 M KCl in 0.1 M sodium borate (pH 8.0) and with starting buffer of the column.
2.7. Preparation of tissue homogenates

Rat liver was homogenized in 50 mM Tris-HCl buffer (pH 8.0) containing 0.25 M sucrose and 1 mM phenyl methanesulphonyl fluoride (PMSF) using a glass homogenizer which was immersed in an ice bottle and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheese cloth and centrifuged at 100,000 \( g \) at 4 °C for 30 min. The resulting supernatant is used as crude cytosolic fraction.

2.8. GSTs assay

GST activity was measured at 25 °C using CDNB (1-chloro-2, 4-dinitrobenzene) as a substrate to determine total GST activity according to Habig et al. [28].

2.9. Purification of GSTs from rat liver cytosol

The crude cytosolic fraction was subjected to dialysis at 4 °C in 10 mM potassium phosphate buffer without KCl to remove the endogenous GSH, which may interfere with binding of GSTs to affinity column. Then the dialyzed sample was eluted with equilibration buffer (10 mM potassium phosphate buffer with KCl pH 7.0) and loaded on to the GSH-Sepharose 6B affinity column which was previously equilibrated with equilibration buffer and washed with the same buffer. The bound GSTs were then eluted with elution buffer (50 mM potassium phosphate buffer pH 7.5 and 50 mM GSH) and eluted fractions in 2 ml volume were collected and measured the protein and enzyme activity. Active fractions were pooled and then concentrated by using amicon filters (cut off 5 kDa, Millipore). Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm in the chromatographic fractions and the protein in the crude cytosol and purified GSTs were measured according to methods of Gornall et al. [29] and Bradford [30], respectively.

2.10. SDS–PAGE and Western blotting analysis

Proteins were separated on 12% polyacrylamide gels by a modified method of Laemmli, [31] and were visualized either by silver or coomassie staining [32]. Western blotting analysis using rabbit anti-GST polyclonal antibody against the three subunits was carried out according to Towbin et al. [33]. Densitometric analysis was performed using image J software (NIH, USA).

2.11. Two dimensional gel electrophoresis (2DE)

Purified cytosolic GSTs were rehydrated for 1 h in a volume of 350 μl in a reswelling tray on immobiline dry strip (IPG) of 18 cm pH 6–11 in 8 M Urea, 2% (v/v) CHAPS, 15 mM DTT, 2% (v/v) IPG buffer pH 6–11% and 1% bromophenol blue. Isoelectric focusing of samples was performed on a Multiphor II Electrophoresis unit for 60,000 V h using the following programme: 12 h at 20 V; 500 V for 30 min (gradient); 500 V for 30 min (step); 8000 V for 3 h (gradient); 8000 V to reach 60,000 V h. Subsequently, Immobilized pH gradient (IPG) strips were equilibrated for 30 min in equilibration buffer (containing 6 M urea, 29.3% (w/v) glycerol, 2% SDS in 75 mM Tris-HCl buffer pH 8.8, containing 0.001% (w/v) bromophenol blue, 0.2 g acrylamide and 0.1 g DTT). Second dimension electrophoresis was performed according to the method of Gorg et al. [34] on 12.5% polyacrylamide gels using the DALT multiple casting chamber (Amersham Biosciences). Molecular masses were determined by running standard protein markers (Bangalore Genei). Gels were silver stained for visualization of proteins. Scanning of the gels was done with Typhoon Trio + (GE health care) and the images were stored in tiff formats.

2.12. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS analysis)

The GST spots separated on 2D Electrophoresis were excised, destained with 1:1 mix of 30 mM potassium ferricyanide and 100 mM...
sodium thiosulphate and incubated at room temperature for 30 min followed by 5 min in 100% acetonitrile. After drying, the spots were incubated with 10 mM DTT at 56 °C for 1 h followed by 55 mM iodoacetamide at room temperature in the dark for 45 min. Furthermore, after the spots had been washed and dried with 25 mM ammonium bicarbonate (NH₄HCO₃) and 100% acetonitrile (CH₃CN) respectively, they were incubated with 10 ng of trypsin at 37 °C overnight. The digested peptides were extracted by adding acetonitrile:trifluoroacetic acid (TFA):water (50:1:49) and vortex the tubes for 5 min. The extracted peptides were mixed with an equal volume of HCCA matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% (v/v) CH₃CN, 0.1% TFA, 2:1 ratio) and 2 μl of this mixture was applied onto a MALDI target plate. MALDI-TOF-MS peptide mass spectra were recorded on a Bruker Daltonics Autoflex, Bremen, Germany. PMF

Fig. 4. Representative images of one of the two dimensional gel electrophoretic analyses of rat liver cytosolic affinity purified GSTs from four independent experiments. 40 μg proteins were resolved on a pH 6–11 IPG strip in the first dimension and separated on a 12.5% SDS-PAGE in the second dimension and silver stained as described in Section 2. The spots were identified by MALDI TOF-MS. Panels I: Control, II: BB administered, III: AEPF + BB administered.

Fig. 5. A representative of mass spectra of spot1 of the control. The data represent the peptide mass fingerprints generated by MALDI TOF MS analysis. The figure represents a corresponding protein to GST-A4.
2.13. Statistical analysis

All values were expressed as mean ± standard deviation (SD). "Student's" t-test was used for statistical analysis and statistical significance was defined as P < 0.05 or P < 0.001.

3. Results

The protective effect of prior administration of an AEPF against BB induced changes on the levels of cytosolic GST isozymes in rat liver was studied. For calculating the protective effect, the results of all groups were first normalized relative to control, which was taken as 100. The percent protective effect was calculated as \((100/100 - B \text{ group value}) \times (\text{value of group } D - \text{value of group } B)\). In other words a 100% protection (on a given parameter) means that the value is back to the control level which is normalized as 100.

Initially to know the hepatotoxicity of aqueous extract of *Phyllanthus fraternus* (AEPF) increasing dose of 20, 50, 100, 200 and 400 mg/Kg body weight was administered to male wistar rats. The protection observed was concentration dependant and saturated (i.e. 90–100% protection) at the dose of 100 mg/Kg body weight. Administration of the AEPF up to 400 mg/Kg body weight did not show any toxic effects on liver as compared with controls. Lipid peroxidation was used as a parameter for this study. Hence, 100 mg/Kg body weight was chosen for all our studies to demonstrate the protective effect against the hepatotoxicity induced by thioacetamide [18] or carbon tetrachloride [19] or alcohol [20] or allyl alcohol [21] or bromobenzene [22] and including for current study. So in the present study also, a dose of 100 mg AEPF was used.

3.1. Effect of administration of AEPF on the BB induced decrease GST activity

The activity of crude cytosolic GST was significantly decreased (46%) due to administration of BB (group B) when compared to control (group A). However, on prior administration of AEPF (group D) offered a significant protection of 74% (Fig. 1). Control group and AEPF extract administered groups were not different when compared with each other and hence only one group i.e. control group was used for all further studies.

3.2. Purification of cytosolic GSTs from rat liver

Liver cytosolic GSTs were purified through GSH-Sepharose affinity column to homogeneity from groups A, B and D. The extent of purification fold obtained was 40 (with a yield of 78%), 15 (with a yield of 69%) and 23 (with a yield of 76%) in the groups of A, B and D, respectively.

Affinity chromatography purified cytosolic GSTs were separated on 12% SDS-PAGE and observed as three protein bands with molecular weights of 25.6, 27 and 28 kDa which were designated as A2, M1 and A3 (Fig. 2, lanes 6 and 7) respectively.

3.3. AEPF prevents BB induced cytosolic GST degradation in the rat liver

Puriﬁed GSTs and crude cytosolic GSTs isolated from the groups A, B and D were separated on SDS-PAGE and analyzed on western blotting. The results were depicted in Fig. 3. Densitometric analysis showed a significant decrease on the levels of A3, M1 and A2 subunit proteins in the group B there were additional degraded products of subunits A3 and M1 (panel D). These were further identified through MALDI-TOF. However, on prior administration of AEPF increased the levels of these three subunits to control level and offered significant protection (p < 0.001).

3.4. AEPF suppresses the BB induced decreased levels of expression of GST isozymes and their degradation

The purified GSTs were resolved into eight spots on a pH scale of 6–11 by 2D PAGE. Panels I, II and III in Fig. 4 shows 2D gel electrophoretic pattern of afﬁnity puriﬁed cytosolic GSTs of groups A, B and D respectively. Each spot was analyzed on MALDI-TOF MS to generate peptide mass fingerprint (PMF) data (Fig. 5A, a representative of spot 1 and others were given as Fig. 5B–H in Supplementary data). Based on peptide mass fingerprints, spectra (speciﬁc peaks from PMF spectra) and MASCOT search, the spots were identiﬁed as five isozymes of alpha (GSTA1 to A5) and two of Mu (GSTM1 and M2) and one of theta (GSTT1) in rat liver as shown in Table 1. The isozymes of GSTs

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### Table 1
MALDI-TOF MS analysis of affinity purified cytosolic GSTs of rat liver separated on 2DE.

| Spot no. (gGST) isozymes | Swiss accession number | pI (theoretical) | Molecular weight (theoretical), Da |
|--------------------------|-----------------------|------------------|-----------------------------------|
| 1 (A4) | P14942 | 6.77 | 25,550 |
| 2 (M2) | P08010 | 6.90 | 25,837 |
| 3 (T1) | Q03579 | 7.12 | 27,735 |
| 4 (A1) | P00502 | 8.87 | 25,388 |
| 5 (A5) | P46418 | 8.42 | 25,347 |
| 6 (M1) | P04905 | 8.27 | 25,360 |
| 7 (A3) | P04904 | 8.78 | 25,657 |
| 8 (A2) | P04903 | 8.89 | 25,558 |

Values are given as mean ± SD of 5 samples.

### Table 2
Densitometric analysis of GST isozymes from groups control, BB and AEPF + BB.

| Spot no. | Control (A) | BB (B) | AEPF + BB (D) |
|----------|-------------|--------|---------------|
| 1 (A4) | 100% | 27\(a\) | 150\(b) |
| 2 (M2) | 100% | 44\(b) | 100\(b) |
| 3 (T1) | 100% | 25\(b) | 55\(b) |
| 4 (A1) | 100% | 15\(b) | 120\(b) |
| 5 (A5) | 100% | 20\(b) | 97\(b) |
| 6 (M1) | 100% | 19\(b) | 36\(b) |
| 7 (A3) | 100% | 10\(b) | 100\(b) |
| 8 (A2) | 100% | 10\(b) | 100\(b) |

Values are given as percent control.

*\(a\) p < 0.05 vs. Group A.
*\(b\) p < 0.05 vs. Group B.

### Table 3
Levels of flavonoids, polyphenols and tannins in aqueous extract of *Phyllanthus fraternus* (AEPF).

| S. no | Constituent of PF extract | Amount (mg/g dry wt) |
|-------|---------------------------|---------------------|
| 1. | Flavonoids | 1.84 ± 0.1 |
| 2. | Total phenols | 2.16 ± 0.3 |
| 3. | Tannins | 1.56 ± 0.15 |

Values are given as mean ± SD of 5 samples.
were quantitated respectively from the groups of A, B and D by densitometric analysis (Table 2). In group B there was a significant decrease in the levels of all the isoforms of the GSTs i.e. A4, M2, T1, A1, A5, M1, A3 and A2 (spots 1–8) by 27%, 44%, 25%,15%, 20%, 19%, 10% and 10% respectively compared with group A. In addition, two more spots (9 and 10) appeared in group B which were identified by MALDI-TOF MS analysis as the degraded products of A3 and M1 (Panel D in Fig. 3). However, prior administration of AEPF (group D) significantly increased the levels of all the isoforms of GSTs like A4, M2, T1, A1, A5, M1, A3 and A2 and offered good protection of 150%, 100%, 55%, 120%, 97%, 36%, 100% and 100% respectively compared to group B and the spots 9 and 10 (degraded products of A3 and M1) were not detected.

3.5. AEPF extract exhibited higher levels of phytochemicals

The quantity of secondary metabolites like flavonoids, phenolics and tannins in the AEPF extract were measured and shown in Table 3. AEPF showed high levels of total phenolics and flavonoids and offered significant protection against bromobenzene induced oxidative stress which further effects on GST degradation and expression.

4. Discussion

GSTs represent one of the major cellular defense mechanisms against electrophilic xenobiotics and their metabolites. In this study, we demonstrated the beneficial effect of AEPF on BB induced changes on cytosolic GSTs. The total GST activity in rat liver cytosol was significantly decreased in the BB administered compared to control. This effect can lead to toxic consequences, because the detoxification activity will be less and other electrophilic compounds will not be detoxified leading to the damage of DNA, proteins and lipids. However, prior administration of AEPF increased the GST activity which indicates the induction of the detoxifying system to enhance the conjugational capacity for inactivation of electrophiles. Similar enhanced activity of GST with methanol extract of Alisma orientale rhizome was reported by Hur et al. [35].

In rat liver, the GST isozymes are mainly made up of three major subunits, the GSTA3, M1 and A2. Each subunit has specific function [36]. Previous studies had shown the role of alpha class GSTs in the defense against oxidative stress [37]. In this study, administration of BB induced a significant decrease on the levels of these 3 subunits in crude cytosolic as well as affinity purified GSTs. However, in the affinity purified GSTs two extra bands were seen at the lower end on the blot (Fig. 3 panel D) as well as on 2DE (Fig. 4 panel II) which are identified and confirmed by MALDI-TOF MS analysis as the degraded products of the GST A3 and M1 subunits indicating the toxic effects of BB. However, prior administration of AEPF showed a significant increase in the levels of these GST subunits and no degraded products of alpha subunits were identified. These results suggested that AEPF role in countering the oxidative stress induced by BB.

Recent studies indicated that antioxidant response element (ARE), a cis-acting regulatory element in the gene promoter, plays a key role in regulating mRNA expression of phase II drug metabolizing enzymes such as GSTs, NAD(P)H: ubiquinone oxidoreductase and UDP-glucuronosyltransferase [38]. Natural compounds like flavonoids (quercetin and flavone), isocoumarins, orthoephens and sulforaphane have the ability to induce enzymes of phase II metabolism through a mechanism involving antioxidant response element (ARE). Most naturally occurring phenolic acids like protocatechuic and tannic acids are known to modulate the activity of GSTs (mGSTA, mGSTP, mGSTM and mGSTT) in hepatic tissues and exhibit antioxidant activity [39,40]. In this study, presence of high phenolics and flavonoids in AEPF appear to be responsible for the up-regulation of the isoenzyme of GSTs and thus offer protection against oxidative stress. Increased mRNA expression by quercetin was reported in rat liver for GSTA3, GSTP1 and GSTT3 and also observed moderately increased GSTM (Odbayer et al. [40]). Diet-derived compounds like ortho-phenols [41], butylated hydroxyanisole (BHA) [42], sulforaphane [43] and flavone [44–46] reported to up-regulate the GST activity.

In conclusion, our findings indicated that the prior administration of phenolic rich AEPF showed beneficial effect against the BB induced changes on cytosolic GST isozymes and thus prevented BB induced oxidative stress. These results indicate that AEPF may serve an effective chemopreventive agent against oxidative stress induced hepatic diseases.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.abbrep.2017.04.001.

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