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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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ABSTRACT

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 nelausari, 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-chlro-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 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).

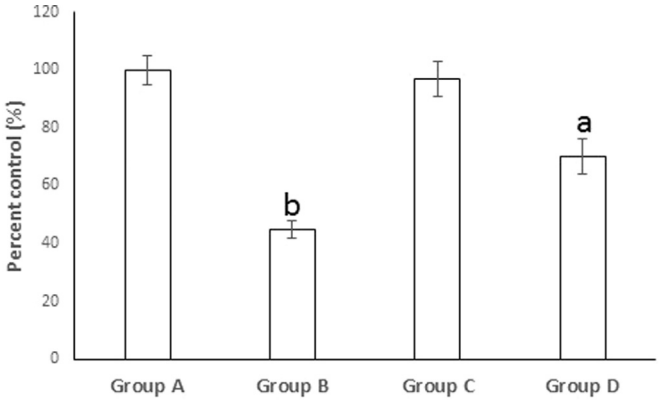

Fig. 1. Effect of administration of bromobenzene with or without the prior administration of aqueous extract of *Phyllanthus fraternus (AEPF)* on GST activity in the liver. Values are given as percent control, and are mean ± SD of 6 rats. GST activity was expressed as units per mg protein. $^{a}p < 0.05$ vs. control group (Group A); $^{b}p < 0.001$ vs. BB group (Group B). One unit of enzyme is defined as 1 µmol of thioether formed per minute. The control value of GST is 5.3 ± 0.14.

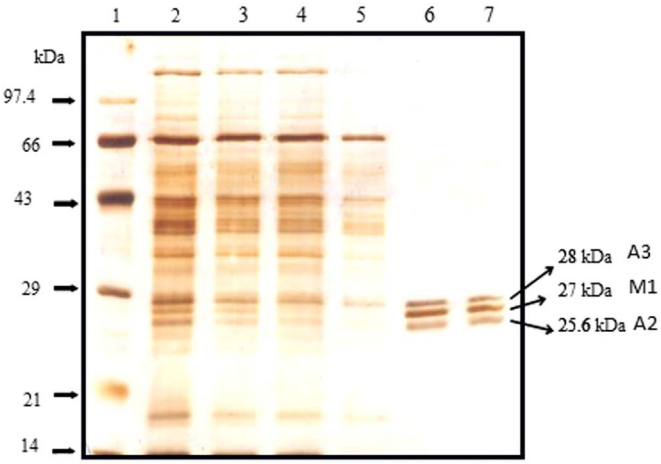

Fig. 2. 12% SDS PAGE analysis of affinity purified GSTs. Lane1: Marker, 2: Crude extract, 3: Dialyzed, 4: Flow through, 5: Wash, 6 and 7: Elutions.

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.