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# Geraniin and amariin, ellagitannins from *Phyllanthus amarus*, protect liver cells against ethanol induced cytotoxicity

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ABSTRACT

Ethanol mediated free radical generation plays an important role in the pathogenesis of liver injuries and alcoholic liver diseases. In the present study two ellagitannins namely geraniin and amariin isolated from *Phyllanthus amarus* were examined for their ability to protect mouse liver slices against ethanol induced toxicity and possible mechanism of its protection. Oxidative stress markers such as, lipid peroxidation, protein carbonyl formation, amount of 8-hydroxy-2-deoxyguanosine and antioxidant enzymes levels were measured using specific biochemical assays. Poly (ADP-ribose) polymerase (PARP), Bax and Bcl2 were checked to assess the induction of apoptosis using western blots. The results showed that geraniin and amariin protected mouse liver slices against ethanol induced cytotoxicity. Both compounds inhibited oxidation of lipid, protein and formation of 8-hydroxy-2-deoxyguanosine, all of which were found to be elevated on exposure to ethanol. These compounds restored the antioxidant enzymes altered on ethanol exposure. Compounds also inhibited the cleavage of PARP and bax and restored Bcl2, induced on exposure to ethanol. In summary, both ellagitannins effectively protected mouse liver slices against ethanol induced cytotoxicity and apoptosis by reducing oxidative damage to biological molecules and modulating Bax/Bcl-2 ratio respectively, thus minimizing liver injury.

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## 1. Introduction

Ethanol mediated reactive oxygen species (ROS) generation is shown to be involved in the pathogenesis and progression of both infectious and non-infectious liver diseases [1,2]. Alcohol is metabolized via three major pathways in the liver cells, each located in a different subcellular compartment; alcohol dehydrogenase in the cytosol, microsomal ethanol oxidizing system in the endoplasmic reticulum, and catalase located in the peroxisomes [3,4]. Metabolism by these three enzymes results in the generation of ROS, including superoxide, hydroxyl radical and hydrogen peroxide [3,4]. Alcohol-induced hepatic oxidative stress has been repeatedly demonstrated by detecting ROS or by measuring lipid peroxidation, a marker for oxidative stress in both alcoholic patients and animal models.

Since alcohol consumption is one of the leading causes of illness and death from liver disease, many agents have been evaluated for the prevention and treatment of alcoholic liver disease in experimental models or clinic trials [5]. Antioxidants are potential pharmacological agents for the treatment of alcoholic liver diseases. Several herbal preparations are already in use for years as hepatoprotectants. *Phyllanthus amarus* is one of the plants used as hepatoprotective and liver tonic for several years in Indian tradition as medicine [6]. It is a tropical medicinal plant, widely distributed, with many reported beneficial effects. These include antiviral [7], hypoglycemic, hypocholesteremic [8] and radioprotective [9] activities. The

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antioxidant activities of the methanolic extracts of *Phyllanthus* are also demonstrated [10]. *Phyllanthus* contains many phytochemicals comprising of lignin, alkaloids, flavonoids and hydrolysable tannins. Among them very few, namely gallic acid, ellagic acid, rutin, and quercetin [11,12] are studied extensively for their biological activities. Pharmacological actions of polyphenolic compounds may stem mainly from their free radical scavenging and metal chelating properties as well as their effect on cell signaling pathways and gene expression [13]. Recently one of the lignin, phyllanthin from *P. amarus* has been shown to protect rat hepatocytes from ethanol induced cytotoxicity by being antioxidant [14]. We have reported earlier strong in vitro antioxidant [15] and radioprotective [16] activity of the ellagitannins and flavonoids isolated from this plant. In the present study, we demonstrate the antioxidant effects of two ellagitannins namely; geraniin and amariin alleviating the ethanol mediated liver damage. The inhibition of apoptosis pathway, a probable mechanism of protection was also tested.

## 2. Materials and methods

### 2.1. Materials

Adult Swiss albino mice (6–8 week old) of either sex bred in the animal house of the Department of Zoology, University of Pune were used for the preparation of liver slices. Prior approval for the protocols used involving animals during this work was obtained from the Pune University Institutional Animal Ethical Committee.

All common chemicals used were from one of the following suppliers: SRL, India, Sigma Chemical (USA) and Merck (Germany). Ethanol was obtained from Fluka, (Switzerland). Antibody against Poly (ADP) ribose polymerase (PARP) was purchased from Cell Signaling Technology (USA). Enhance Chemiluminescence's kit was from Boehringer Mannheim, Roche (Germany). Geraniin and amariin from *P. amarus*, were purified by Dr. Foo [17–19] in his laboratory at the New Zealand Institute for Industrial Research and Development, Industrial Research Limited, Gracefield Research Centre, New Zealand.

### 2.2. Liver slice culture

Liver slice culture was maintained following the protocol developed by Wormser and Ben Zakine [20] and Invittox Protocol No. 42 [21]. Briefly, mice were dissected open after cervical dislocation, and liver lobes were removed and transferred to prewarmed Kreb's ringer HEPES buffer (KRH) (2.5 mM HEPES, pH 7.4, 118 mM NaCl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $KH_2PO_4$ 1.18 mM $MgSO_4$, 5 mM beta-hydroxybutyrate, and 4.0 mM glucose). The liver was then cut into thin slices using sharp scalpel blades. The slices, weighing between 4 and 6 mg, were used for the experiment. Each experimental system contained 20 to 22 slices. These slices were washed with 10 ml of KRH medium, every 10 min during a period of 1 h. These were then preincubated for 60 min in small plugged beakers containing 2 ml of KRH on a shaker water bath at 37 °C. At the end of preincubation, the medium was replaced by 2 ml of fresh KRH and incubated for 2 h at 37 °C with either 1.732 M ethanol or 0.25 mM geraniin or 0.25 mM amariin or with ethanol in the presence of 0.25 mM geraniin/amariin. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath at a tissue concentration of 100 mg/ml. Both the tissue homogenates and supernatants were collected and used for estimation of lactate dehydrogenase (LDH), which was used as a cytotoxicity marker using standard protocol.

### 2.3. Measurement of cytotoxicity to liver slices

#### 2.3.1. Lactate dehydrogenase measurement

LDH was estimated by the method of Wahlefeld [22]. Each unit of enzyme was calculated as 1 mol of NAD reduced per minute. Enzyme units in the medium and in tissue homogenate were estimated and percent release of enzyme from liver slices was calculated as the ratio of LDH activity found in the supernatant to the total LDH (supernatant + homogenate) activity.

### 2.4. Measurement of oxidative damage to liver slices

#### 2.4.1. Lipid peroxidation

Lipid peroxidation was estimated following the method of Devasagayam [23] and is expressed in terms of thiobarbituric acid reactive substances (TBARS). In brief, 0.5 ml of homogenate and 0.5 ml of TBA reagent were mixed and this was boiled in boiling water bath for 15 min. The pink colored chromogen formed was read at 532 nm.

#### 2.4.2. Estimation of protein carbonyl formed

Estimation of protein carbonyl is based on the reaction of protein carbonyl groups, with 2,4-dinitrophenylhydrazine (DNPH) that leads to the formation of 2,4 dinitrophenylhydrazone, which can be measured at 366 nm [24]. The experiment was carried out using a blank set of tubes for every corresponding experimental tube. The difference in absorbance between blank and corresponding experimental tube gives the amount of carbonyls formed. Protein carbonyls are expressed as nmol/mg protein.

#### 2.4.3. Measurement of 8-hydroxydeoxyguanosine (8-OHdG) level

8-Hydroxy-2-deoxyguanosine, marker for the oxidative DNA damage was estimated by competitive ELISA using monoclonal antibody [25].

### 2.5. Measurement of antioxidant enzymes

Superoxide dismutase was assayed spectrophotometrically according to Beauchamp and Fridovich [26]. The extent to which the enzyme decreases the reduction of nitroblue tetrazolium (NBT) by superoxide radical generated by riboflavin in the presence of light was monitored at 560 nm.

Assay for catalase was carried out according to the method of Aebi [27]. One unit was defined as the amount of the enzyme that converts 1 mmol of $H_2O_2$ to water in 1 min.

Glutathione peroxidase (GPX) activity was determined by means of a coupled reaction system assay [28]. One unit of GPX activity was defined as the amount of enzyme needed to catalyze the oxidation of 1 nmol NADPH per minute.

Glutathione reductase activity was determined by the protocol of Goldberg and Spooner [29]. One unit was defined

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