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Premna integrifolia ameliorates cyclophosphamide-induced hepatotoxicity by modulation of oxidative stress and apoptosis

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ABSTRACT

The present study was designed to evaluate the ameliorative effect of ethyl acetate extract of *Premna integrifolia* L. (EAEPI) leaves in cyclophosphamide (CP)-induced hepatic injury in mice. Mice were intoxicated with CP (200 mg/kg b. wt., i.p.) for 5 weeks or EAEPI (400 and 600 mg/kg b. wt., orally) in combination with CP. The results demonstrated that EAEPI exerts protective effect against CP induced hepatotoxicity, as evident from restoration of altered haematological parameters and alleviations of liver marker enzymes in serum. EAEPI also attenuated oxidative stress and antioxidant markers as evident from reversal of lipid peroxidation, glutathione levels as well as activities of catalase and superoxide dismutase enzymes. Moreover, EAEPI attenuated apoptosis and histopathological liver tissue damage in CP-intoxicated mice. In conclusion, EAEPI could protect mice liver against cyclophosphamide toxicity by inhibiting oxidative stress and apoptosis.The protective activity of EAEPI may be due to presence of polyphenolic compounds as identified by UHPLC–Q-TOF-MS/MS.

1. Introduction

Cyclophosphamide (N, N-*bis* (2-chloroethyl) tetrahydro-2H-1, 3,2-oxazaphosphorin-2-amine 2-oxide; CP) is an antineoplastic chemotherapeutic agent extensively used for the treatment of various kind of cancers including solid tumours and lymphomas. It has also been used to treat some non-neoplastic diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus [1]. Despite its broad spectrum applications, there are some proven deleterious side effects in human and experimental animals [2]. Previous studies have shown that liver toxicity is a major problem associated with cyclophasphamide application [3,4]. Apart from liver, use of CP caused disorder in other organs like kidney, lung, bone marrow and reproductive organs [5–8]. Continuous application of CP causes DNA damage leading to genotoxicity [9].

Once inside the body, bioconversion of CP took place through microsomal cytochrome P450 oxidase, which produces two metabolites namely phosphoramide mustard and acrolein [3]. Phosphoramide mustard was reported to be effective against tumor, while acrolein was responsible for CP-induced liver injury [10]. Accumulation of CP inside the cell, declines cellular antioxidant defence, and increases the generation of reactive oxygen species (ROS) [11]. This leads to oxidative stress and caused oxidative damage to cellular macromolecules such as lipids, proteins and nucleic acids. Earlier reports have shown that CP intoxication causes lipid peroxidation and protein oxidation in the liver of experimental animals [12,13]. Oxidative stress is regulated through antioxidant mechanism of cells and triggers apoptotic cell death. Both mitochondria-dependent and mitochondria-independent apoptotic pathways are activated by CP. Caspases, execute mitochondria dependent apoptosis by releasing cytochrome c, from mitochondria to cytosol [14]. Bax, a pro-apoptotic factor facilitates the transport of cytochrome c through the mitochondrial outer membrane to cytosol, while an anti-apoptotic protein Bcl-2, responsible for release of cytochrome c [15].

Plant derived natural antioxidants are the topic of interest for current researchers because they have potential compounds responsible for free radical scavenging activity [16]. Recently, several studies have shown that natural antioxidants are potent to minimize the ROS induced side effects in liver tissue against chemotherapeutic drugs

Abbreviations: CP, cyclophosphamide; EAEPI, ethyl acetate extract of *P. integrifolia*; ROS, reactive oxygen species; RBC, red blood cells; WBC, white blood cells; PL, platelets; Hb, haemoglobin; PCV, packed cell volume; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GSH, glutathione; TBARS, thiobarbituric acid reactive substance; MDA, malondialdehyde; SOD, superoxide dismutase

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[17,18]. Moreover, plant extracts enriched with antioxidant compounds were shown to be effective against CP induced hepatic disorders [13].

P. integrifolia (Family-Lamiaceae) is a medicinal plant, distributed in the tropical and subtropical regions of Africa, Asia and Australia [19]. In traditional medicine it has been used as antimicrobial, antioxidant, antipyretics, hypoglycaemic, cardiotonic and diuretic agents [20]. In Indonesia, mixture of its leaves and roots has been used to control fever. The leaves of the plant are also used by women for lactation [21]. It leaves contains various phytoconstituents like phenolics, flavonoids [22], saponins and tannins [23], which might be responsible for its antioxidant activity.

Ethanolic extract of *P. integrifolia* has significant analgesic, antidiabetic, antiulcer, antimicrobial, and antioxidant activity [24]. Hepatoprotective action of *P. integrifolia* leaves extracts against carbon tetrachloride [25] and paracetamol [26] induced liver toxicity was reported. However, no study evaluated the role of this plant against cyclophasphamide induced liver toxicity. Hence, the present study was executed to evaluate the protective effect of *P. integrifolia* leaves extract against CP-induced toxicity in mice liver and to identify the bioactive compounds may be responsible for protective action.

2. Materials and methods

2.1. Materials

The chemicals CP, thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), bovine serum albumin (BSA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), Taq polymerase and dNTPs were purchased from Sigma-Aldrich, St Louis, USA. The rest of the chemicals were purchased from Fischer Scientific, Mumbai, India. Fresh leaves of *P. integrifolia* were collected from Ayurvedic Garden, Institute of Medical Science, Banaras Hindu University, Varanasi, India. A voucher specimen (BSI/CRC/2016-17) was deposited in the herbarium of Botanical Survey of India (BSI), Allahabad, India after authentication under the accession number 97,879.

2.2. Preparation of ethyl acetate extract of P. integrifolia (EAEPI)

P. integrifolia leaves were washed thoroughly under running tap water, dried in shade at room temperature and pulverized in a mechanical grinder. For the preparation of ethyl acetate extract of *P. integrifolia* (EAEPI) powdered sample (100 g) was extracted in 250 ml of ethyl acetate by using a Soxhlet extractor [27]. A greenish colour extract was filtered and evaporated to dry at 45 °C with the rotatory evaporator. The dried extract (yield 5.8%) was stored in a sealed container at 4 °C for further use.

2.3. Profiling of bioactive compounds using UHPLC–Q-TOF-MS/MS

Leaves extract was separated on an Acquity UPLC system (Waters, Milford, MA, USA) equipped with Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm) and the column temperature was maintained at 25 °C. The mobile phase consisted of 0.1% formic acid (A) acetonitrile (B) and methanol (C). The flow rate of the mobile phase was 300 µl/min and the injection volume was 5 µl. Gradient elution of the analyte was carried out using the following program: initial composition of B:C was 90:10% and increased to 80:20% in 2 min, 50:50% for 1–3 min, 30:70% for 3–6 min, then 10:90% for 1 min and finally, decreased quickly to 90:10% for 7–10 min. The Mass spectroscopic analysis of phenolic compounds in the sample was performed in both positive and negative ion modes equipped with electrospray ionization (ESI) source. The MS parameters were as follows: cone and desolvation gas flow was 52 l/hr and 647 l/hr, respectively; source and desolations temperature was 40 °C and 450 °C, respectively. The capillary and cone voltage was set at 2.72 Kv and 40 eV, respectively. Micro-channel plates (MCPs) were functional at 1750 V and Q-TOF mass spectrometer was conducted in MSE mode with low collision energy set at 6 eV in the first function and a collision energy ramp from 20 to 40 eV in the second function. Centroid mode data was collected over the m/z range 100–1000 in both functions, and the scan time was 1 s with an interscan delay of 0.024 s. The accurate mass and molecular formula denomination were acquired with the Mass Lynx 4.1 software (Waters MS Technologies).

2.4. Animals and their care

Thirty adult healthy in-bred male albino mice of Swiss strain (25 ± 5 g) were procured from the animal house of Institute of Medical Science, Banaras Hindu University, U.P., India. All mice were fed with standard pellet feed and water *ad libitum*. Mice were kept in polypropylene cages at 12 h light/dark cycle under controlled temperature (25 ± 2 °C) and relative humidity (70%). All procedures involving animals were performed by following the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, India. Studies were conducted after obtaining prior consent by Ethical Committee of the Institute of Science, Banaras Hindu University, Varanasi, India (F. Sc./88/IAEC/2016-17).

2.5. Experimental design

After 10 days of acclimatization, animals were randomly divided into five groups (6 mice per group).

Group I: Mice treated as control, received normal saline via intraperitoneal (i.p.) injection, once in a week for 5 weeks.

Group II: Mice received CP (200 mg/kg b. wt., i.p., dissolved in saline) once in a week for 5 weeks.

Group III: Mice received CP (200 mg/kg b. wt., i.p., dissolved in saline) and EAEPI (400 mg/kg b. wt., orally, dissolved in saline) both once in a week for 5 weeks.

Group IV: Mice received CP (200 mg/kg b. wt., i.p., dissolved in saline) and EAEPI (600 mg/kg b. wt., orally, dissolved in saline) both once in a week for 5 weeks.

Group V: Mice received EAEPI (600 mg/kg b. wt., orally, dissolved in saline) alone once in a week for 5 weeks.

After the completion of 5 weeks of the treatment period, the mice were anaesthetized, and sacrificed by decapitation after euthanizing by CO_2 (flow rate was adjusted at 3 l/min in mouse cage and continued until one min after breathing stopped). After sacrifice liver was removed and rinsed in ice-cold physiological saline.

2.6. Preparation of tissues homogenate and blood collection

Liver tissue was homogenized in phosphate buffer saline using glass homogenizer to prepare 10% tissue homogenate. Tissue homogenate was centrifuged at 5000 × g for 15 min and stored at −80 °C until further analysis. For haematological and biochemical analysis blood was collected in EDTA coated tubes by cardiac puncture. Serum was prepared by the centrifugation of blood at 3000 × g for 20 min and was stored at −20 °C till further use.

2.7. Hematological examination

Red blood cells (RBC), white blood cells (WBC), platelets (PL) count, haemoglobin (Hb) content, and packed cell volume (PCV) was analyzed in the blood sample by an automated analyzer (KX-21-Hematology-analyzer, Sysmex Corporation, USA).

2.8. Serum biochemical analysis

Activities of biomarker enzymes for liver functions including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and