Toxicity profiling and antioxidant activity of ethyl acetate extract of leaves of *Premna integrifolia* L. for its application as protective agent against xenobiotics

Chandrashekhar Singh, Kavindra Nath Tiwari, Pradeep Kumar, Anil Kumar, Jyoti Dixit, Rajesh Saini, Sunil Kumar Mishra

**ARTICLE INFO**

**Keywords:**
- *Premna integrifolia*
- Acute toxicity
- Biochemical parameters
- Hematological parameters
- Subacute toxicity

**ABSTRACT**

*Premna integrifolia* (Lamiaceae) is widely used in herbal formulation “Dashmoolarishtha” which is useful in postnatal care. Ethyl acetate extract obtained from the leaves was evaluated for phenolic content and its antioxidant activity. Acute and subacute toxicity of the extract was studied in mice of both sexes to get an idea about LD<sub>50</sub> value and assessed its safety profile before its application as a protective agent against different toxicities induced by xenobiotics. Phenol enriched extract (phenol content is 63.10 ± 1.26 mg/g of gallic acid equivalent and flavonoid content 75.33 ± 0.23 mg/g of rutin equivalent) showed good antioxidant activity. In acute toxicity studies it was observed that single different doses (300–5000 mg/kg b.wt.) of extract did not show any mortality of mice. Thus the LD<sub>50</sub> of the extract was determined, and it was higher than 5000 mg/kg. There was no major change in behavioral and general appearance of mice. External morphology of liver, kidneys, lungs, spleen and heart did not show any effect of treatment. In subacute toxicity no statistically significant change in body weight, relative organ weight, food intake and water uptake, hematological, biochemical parameters were reported after comparison with control. Extract did not show significant effect in the level of antioxidant enzymes in the liver of treated groups. Extract did not show any sign of toxic effects, when administered orally to male and female mice at dose level up to 1000 mg/kg. So, it can be utilized as protective agent against toxicity produced by different xenobiotics.

**1. Introduction**

With changing environmental conditions, living style and food habit of people caused several health complications. Xenobiotics entered in the body of human being affect the metabolism and resistance of the host [1]. Xenobiotics are exogenous chemicals or foreign substances such as drugs, pollutants, and some food additives etc. which was not recognized by the body. Decrease in the resistance caused the susceptibility for infection by several pathogens [2]. Presence of pathogens and xenobiotics in body accelerates the generation of free radicals [3] which attack on the biomolecules and leads to cell injury and death. Food rich with natural antioxidants scavenged the free radicals. The medicinal plants contribute major portion in complementary and alternative medicines. Pharmaceutical industries commonly used different parts of higher plants, because it contained valuable phytoconstituents. According to World Health Organization (WHO), 252 drugs are considered as basic and essential for food and health. Among these drugs 11 % are plant based. Recently demand of herbal drugs are increased many folds not only in developing countries but in highly developed countries also, due to its holistic way of recovery and least side effects [4]. Several phytoconstituents of medicinal plants contribute major role in scavenging reactive oxygen species (ROS), which protects the damages caused by xenobiotics. Crude extracts or purified compounds obtained from natural sources widely used as hepatoprotective [5],

**Abbreviations:** EAEPI, ethyl acetate extract of leaves of *P. integrifolia*; PC, polyphenol content; GAE, gallic acid equivalents; TFC, total flavonoid content; RE, rutin equivalents; DPPH, 1, 1-diphenyl-2-picrylhydrazyl; TBARS, thiobarbituric acid-reactive species; MDA, malondialdehyde; TBA, thiobarbituricacid; TCA, trichloroacetic acid; OECD, Organization for Economic Co-operation and Development.

* Corresponding author.

E-mail address: kntiwaribhu@gmail.com (K.N. Tiwari).

https://doi.org/10.1016/j.toxrep.2021.01.004

Received 14 March 2020; Received in revised form 30 December 2020; Accepted 4 January 2021

Available online 8 January 2021

2214-7500/© 2021 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
anti-inflammatory [6], antidiabetic [7], anti-nephrotoxic [8], antioxidant and anticancer [9,10] agent. In general perception, herbal drugs are safe and have no side effects. There is no sufficient evidence regarding the toxicity of these products to consumers. The limitation of plant based herbal formulations is unknown chemical composition and biological activity. Its constituents may be beneficial or harmful to consumers. So there is an urgent need to evaluate the safety and efficacy of these extracts for safe pharmaceutical applications. Without confirmation of its safety, its use in therapy is highly risky for the health.

Premna integrifolia L. (synonyms Premna serratifolia) commonly known as Agnimantha belongs to family Lamiaceae [11], distributed in tropical and subtropical regions of Asia, Africa and Australia [12]. In traditional medicines root of the plant is an important ingredient of ‘Dashmoolarishta’ which is well known for reconditioning of normal health of postpartum females [13]. The important phytochemicals of P. integrifolia are steroids, triterpenoids, alkaloids, phenolic, flavonoid, saponins, tannins, catechins and amino acids [14]. In traditional medicine different parts of the plant was used for the treatment of bronchitis, headache, liver disorder, piles, constipation and fever [15]. Leaves of the plant were used in diet by women of Indonesia to promote lactation [16]. Methanolic root extract (300 mg/kg b.wt.) of the plant was found effective for inhibition on carrageenan induced rat hind paw edema which confirmed its anti-inflammatory action [17]. Methanolic extract of root bark (500 mg/kg b.wt.) when administered to male albino rat for 30 days significantly decreased the serum total cholesterol, LDL, VLDL, triglycerides and increased the HDL level, it revealed the anti hyperlipidaemic action of the extract [18]. The substantial drop in elevated glucose in alloxan induced diabetic rat by methanolic extract of the bark (300 mg/kg b.wt.) of the plant proved its antidiabetic activity [19]. A report of the decreased Na -K- ATPase and Mg2+ATPase function as well as increased the Ca2+ATPase function by ethanol extract of stem bark and wood (200 mg/kg/b.wt.) in frog heart reflects the cardiotonic potential of the plant [20]. Hepatoprotective function of alcoholic [21], and ethyl acetate [5] leaf extracts of the P. integrifolia against carbon tetrachloride, cyclophosphamide and aflatoxin B1 induced toxicity in rodent model was reported respectively. Anti-ulcerogenic activity of ethanolic extract of the leaves of the plant against aspirin induced ulcer was visible by drastic reduction in the lesion index [14].

Despite wide uses its toxicity and safety profile has not been fully documented. So, the objective of the present work was to evaluate the antioxidant activity as well as phytochemical and toxicity profiling of ethyl acetate extract of leaves of P. integrifolia in mice for its further applications as protective agent against different xenobiotics.

2. Materials and methods

2.1. Plant material

The leaves of P. integrifolia were obtained in August 2015 from Ayurvedic garden, Institute of Medical Science, Banaras Hindu University, Varanasi, India. A voucher specimen was authenticated by Botanical Survey of India (BSI), Allahabad, India under the accession no.97879 and deposited in the herbarium of BSI (BSI/CRC/2016–17).

2.2. Preparation of plant extract and its administration

The leaves of the plant were washed thoroughly under running tap water, shade dried at room temperature and then powdered in a mechanical grinder. For preparation of ethyl acetate extract of leaves of P. integrifolia (EAPEI) powdered sample (100 g) was extracted in 250 mL of ethyl acetate by using a soxhlet extractor. Extract was filtered and evaporated to dryness at a 45 °C with rotatory evaporator. The dried extract (yield 5.2 %) was stored in air tight container at 4 °C for further use. The extract was administered orally by means of gavage for the acute and subacute toxicity studies.

2.3. Animals

The study was conducted on adult healthy albino mice of Swiss strain (20 ± 10 g) (both sexes), procured from the animal house of Institute of Medical Science, Banaras Hindu University, U.P., India. The animals were segregated according to gender to avoid any chance of mating. The mice were kept in polypropylene cages for seven days to acclimatize at 12 h light/dark cycle under controlled temperature (25 ± 2 °C) and relative humidity (70 %) before the commencement of the work. All mice were fed with commercially available standard mice pellet feed and water ad libitum. All procedures involving animals were performed in accordance with the Ethical Principles in Animal Research. Animals used for the experiments were approved by the animal ethical clearance committee of the Institute of Science, Banaras Hindu University, Varanasi, India (F. Sc./88/IAEC/2016–17/23).

2.4. In vitro antioxidant activity

2.4.1. Measurement of polyphenol content (PC)

The PC was determined by Folin-Cioalteu method as previously reported by McDonald et al. (2001) [22] with some modification. Briefly, 1 mL of distilled water, 0.1 mL of 1 mg/mL EAPEI, and 0.2 mL of Folin-Cioalteu reagent were added in test tube; then contents were mixed and allowed to stay for 5–8 min at room temperature. Further, solution was neutralized with 2 mL of 7% sodium carbonate solution, followed by maintaining the volume of reaction mixture up to 3 mL by adding 0.7 mL distilled water. Subsequently, solutions were mixed and allowed to stand at room temperature for 15 min, and then absorbance was noted at 750 nm. Phenolic contents were estimated by using a standard curve obtained from various concentration of gallic acid. The results were expressed as milligrams per gram of gallic acid equivalents (GAE).

2.4.2. Measurement of total flavonoid content (TFC)

Determination of TFC was done by AlCl3 colorimetric method [23]. Aliquots of EAPEI (0.1 mL of 10 mg/mL) in ethanol were mixed with equal volume of 2% AlCl3, 0.1 mL of 1 M potassium acetate, and 2.7 mL of ethanol. The reaction mixture was vigorously shaken, kept at room temperature for 30 min and absorbance was recorded at 415 nm. TFC was calculated using rutin as standard and expressed as milligram per gram of rutin equivalents (RE).

2.4.3. DPPH scavenging assay

The free radical scavenging activity of EAPEI, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by slightly modified method described by Brand-Williams et al. (1995) [24]. Different concentrations (25–500 μg/mL) of EAPEI was added to 3 mL of 0.004 % methanol solution of DPPH and incubated for 15 min in dark at room temperature, the absorbance was recorded at 517 nm against a blank by using conventional UV/Visible absorption spectrophotometer (Thermo Scientific UV1).

2.4.4. Lipid peroxidation assay

According to Ohkawa (1979) [25] modified method thiobarbituric acid-reactive species (TBARS) was used to measure the lipid peroxide formed. Egg-yolk homogenates was used as lipid-rich media. Malondialdehyde (MDA), a secondary product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA), yielding a pinkish red chromogen with an absorbance maximum at 532 nm. Egg homogenate (250 μL, 10 % in distilled water, v/v) and 50 μL of extracts were mixed in a test tube and maintained the volume to 500 μL by adding distilled water. At last, 25 μL FeSO4 (0.07 M) was added to the above mixture and incubated for 30 min to induce lipid peroxidation. Thereafter, 750 μL of 20 % acetic acid (pH 3.5) and 750 μL of 0.8 % TBA (w/v) (prepared in 1.1 % sodium dodecyl sulphate) and 25 μL 20 %
TCA were added, vortexed and then heated on boiling water bath for 60 min. After cooling, 3.0 mL of 1-butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured against 3 mL 1-butanol at 532 nm. For the blank 50 μL of distilled water was used in place of the extract.

2.4.5. Reducing power assay (RP)

The ability of EAEPI to reduce Fe$^{3+}$ was determined by the method described in earlier reports [26]. Different concentrations (25–500 μg/mL) of EAEPI were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and same volume of potassium ferricyanide $[K_3(Fe(CN))_6]$ (1%). The mixture was incubated at 37°C for 20 min further 2.5 mL of trichloroacetic acid (TCA, 10%) was added, then centrifuged at 1000 rpm for 10 min. 2.5 mL of the upper organic layer of above solution was mixed with distilled water (2.5 mL) and FeCl$_3$ (0.5 mL, 0.1 %), and the absorbance of reaction mixture was measured at 700 nm. Higher absorbance indicated high reducing power. Ascorbic acid was used as standard.

2.5. Toxicity studies

As per the guideline No. 425 [27] and 407 [28] of Organization for Economic Co-operation and Development (OECD) acute and subacute toxicity were performed respectively.

2.5.1. Acute toxicity studies

Sixty Swiss albino mice (aged 7–10 weeks) weighed between 20–30 g (half male and half female), were randomly divided into six groups (10 in each group, each group 5 per sex). Before administration of doses all the animals were maintained for overnight fasting with free access to water. Ethyl acetate extract of P. integrifolia was dissolved in distilled water and given to animals orally using gavage. Group 1 animals received distilled water only, and served as control, while groups II, III, IV, V and VI were administered with EAEPI single doses of 300, 1000, 2000, 3000 and 5000 mg/kg body weight respectively [29]. After oral extract administration, animals were maintained on standard animal diet and water. After treatment, animals were observed after each 30 min interval initially up to 4 h, then over a period 24 h, thereafter daily for 14 days for any toxicity. The animals were observed for general behavioral changes and other characteristics such as body weight, temperature, food intake, water intake, respiration, urination, diarrhea, general physique, sedation, drowsiness, tremor, change in skin hair and eye color as well as comma and mortality were recorded. Post mortem macroscopic and microscopic observation of animal’s vital organs (heart, liver, lungs, kidney and spleen) were performed as described previously by Ha et al.(2011) [30] after euthanizing by CO$_2$ (adjusting flow rate 3 L/min in mouse cage and continued until one min after breathing stop).

2.5.2. Subacute oral toxicity studies

Swiss albino mice were randomly divided into six groups of 10 animals, each group containing 5 male and 5 female mice separately. Group I mice considered as control administered with distilled water daily by gavage up to 28 days orally. Mice of the Group II to V were administered by different doses of the EAEPI (400, 600, 800 and 1000 mg/kg b.wt.) by gavage daily for 28 days [31]. Animals were observed daily for any adverse effect or toxic signs and behavioral changes, mortality and morbidity till the completion of the experiment. Additional 8 mice of 2 groups each group have 4 animals (2 females and 2 males) of different sexes. Each of which served as satellite groups (control and the highest dose of EAEPI 1000 mg/kg b.wt.) were prepared so as to monitor the recovery or reversibility, persistence or delayed occurrence of toxic effects of EAEPI 14 days after the 28th day administration (subacute treatment). Group I to V animals were sacrificed by cervical dislocation after euthanizing by CO$_2$ as in acute toxicity study on the 29th day after an overnight fast.

2.5.2.1. Body weight, food and water consumption.

Body weights of the mice in all groups were recorded before administration of doses, further body weight was taken once in a week during entire treatment and finally on the day of sacrifice. The amount of food and water intake was recorded daily. The amount of food and water consumption during experiments was calculated by deducting the remnants from the initial of each group. Food and water remnants were calculated next day to get the differences, and recorded as daily food (gm/mice/day) and water consumption (ml/mice/day).

2.5.2.2. Relative organ weight.

Animals of the all groups were sacrificed on 29th day, while mice of the satellite group were sacrificed on 42nd day. Before sacrifice, animals were maintained on fast for overnight. In case of acute toxicity, at the end of the experiments (15th day) animals were sacrificed and body organs (liver, kidneys, lungs, spleen and heart) were excised as well as examined microscopically for any change. Relative organ weight of each animal of each treatment group was calculated by this formula:

Relative organ weight (ROW) = absolute organ weight (g) × 100/body weight of mice on day of sacrifice (g).

2.5.2.3. Blood sample collection and analysis.

Blood samples were collected by puncturing the heart with disposable sterile syringe into heparinized and non-heparinized bottles for hematological and biochemical analyses respectively.

2.5.2.4. Hematological analyses.

The blood samples collected in heparinized tubes were used for the hematological analyses. The following parameters: red blood cell count (RBC), white blood cell count (WBC), neutrophils (NP), lymphocytes (LC), monocytes (MC), eosinophils (EP), hemoglobin (Hb), platelets (PL) and packed cell volume (PCV) by automated analyzer (KK-21-Hematology-analyzer, Sysmex Corporation, USA).

2.5.2.5. Biochemical analyses.

The blood collected in non heparinized tube was centrifuged at 3000 rpm for 10 min using a bench centrifuge (REMI C24 BL Mumbai, Maharashtra, India) to obtain serum which was stored at – 20°C until the measurement of biochemical parameters. The measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), total protein content and albumin content was performed to assess the liver function. The measurement of urea and creatinine concentrations reflects about kidney function. The amount of total cholesterol was determined to evaluate the effect of the extract on the lipid profile. The analysis was done using commercially available test kits, products of ARKRAY Healthcare Pvt. Ltd. (Surat, India).

2.5.2.6. Measurement of oxidative stress in liver tissues.

For measurement of oxidative stress induced after administration of extract to animals liver of each treatment group was excised immediately after sacrifice and collected in pre chilled phosphate buffer saline (PBS) of pH 7.4 after rinsing 2–3 times to avoid any blood residue. Tissues were homogenized in 10% PBS (w/v) in teflon homogenizer and then tissue homogenates were centrifuged for 15 min at 3500 rpm at 4°C in a centrifuge, then stored the supernatants of each sample at −80°C till next analysis. Supernatants of liver were used for analysis of super oxide dismutase (SOD) by the method described by Kono (1978) [32] and estimation of catalase activity was done by the method described by Aebi (1984) [33]. Reduced glutathione (GSH) content was estimated as described in previous report [5] while malondialdehyde (MDA) content was assayed by the method of Wills (1966) [34] with slight modification. The total protein was estimated by the method described by Lowry et al.(1951) [35].

2.5.2.7. Histopathological assessment.

Organs such as liver and kidney
were collected from the mice of each group, fixed in 10% neutral formalin, dehydrated in graded alcohol and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin-eosin for light microscopic analysis.

2.6. Statistical analysis

The result were exercised as mean ± standard error mean (SEM). Statistical analysis was done by one-way analysis of variance (ANOVA) using windows SPSS 16.0 software package followed by Dunnett’s test for inter-group comparisons. In all instances p values < 0.05 were considered statistically significant.

3. Results

3.1. In vitro antioxidant activity

In the EAEPI polyphenol (PC) and total flavonoid content (TFC) was 63.10 ± 1.26 (mg gallic acid equivalent) and 75.33 ± 0.23 (mg rutin equivalent) respectively (Table 1). A significant reduction in the amount of DPHH radical was observed due to the scavenging ability of EAEPI and that of standard. The EAEPI exhibited a higher scavenging effect (EC\textsubscript{50} = 342.12 ± 1.23) that was lower than that of standard (ascorbic acid) with an EC\textsubscript{50} of 26.96 ± 1.64. In LPO assay this extract exhibited EC\textsubscript{50} = 350.12 ± 0.13. In reducing power assay, the transformation of Fe\textsuperscript{3+} to Fe\textsuperscript{2+} by donating an electron indicates the presence of reducing constituents in the extract. It was observed that the reducing capability of EAEPI and ascorbic acid was increased with concentrations. The EAEPI (EC\textsubscript{50} = 373.19 ± 1.67 μg/mL) possess significant reducing power, although it was lower than ascorbic acid (EC\textsubscript{50} = 46.18 ± 2.10 μg/mL).

3.2. Acute toxicity study

The results of acute toxicity test revealed that upon single dose oral administration of EAEPI (300 mg/kg b.wt. up to 5000 mg/kg b.wt.) did not show any toxic effects on mice. The general behaviors of treated and control group animals were monitored daily up to 14 days after the administration of the EAEPI. EAEPI associated alterations in behavior, body weight, temperature, food intake, water intake, respiration, urination, diarrhea, general physique, tremor, change in skin hair and eye color as well as comma and mortality was not observed, while at doses of 3000 mg/kg and 5000 mg/kg b.wt. drowsiness, sedation and lethargy were observed in treated mice only (Table 2). Body weights of mice of both sexes of all groups were recorded on 1,7and 14 days. Results indicate that no significant differences were found in weight gain in treated group than control (Table 3). Post mortem microscopic observation of important organs e.g., heart, liver, lungs, kidney and spleen of the mice of treated groups did not show any change in external morphology and appearance than control. No mortality was recorded throughout the period of observation of acute toxicity. As there was no mortality and clinical sign of toxicity in all the tested doses, LD\textsubscript{50} value of EAEPI were found to be > 5000 mg/kg.

### Table 1

| Extract/standard | Polyphenolic content (mg/g gallic acid equivalent) | Total flavonoid content (mg/g rutin equivalent) | EC\textsubscript{50} Value |
|------------------|-----------------------------------------------|-----------------------------------------------|-------------------------|
|                  | DPPH scavenging effect (μg mL\textsuperscript{-1}) | LPO activity (μg mL\textsuperscript{-1}) | Reducing power (μg mL\textsuperscript{-1}) |
| EAEPI            | 63.10 ± 1.26                                  | 75.33 ± 0.23                                 | 342.12 ± 350.00         |
| Ascorbic acid    | –                                             | –                                             | 26.96 ± 1.64            |

3.3. Subacute toxicity

3.3.1. Effect of EAEPI on body weight

Oral administration of EAEPI at doses of 400 mg/kg, 600 mg/kg, 800 mg/kg and 1000 mg/kg b.wt. for 28 days did not show any mortality of treated animals. No observable symptoms of toxicity were detected during the experimental and recovery periods (satellite group). No significant difference in (p > 0.05) weight gain of EAEPI treated mice of both sexes has been observed in comparison to control group (Table 4). Mice of satellite group treated with maximum dose (1000 mg/kg b.wt.) of EAEPI, after 42 days of treatment also did not show significant change in body weight.

3.3.2. Effect of EAEPI on food intake and water consumption in mice

During dosing (28-day) and the recovery periods, there was no significant change in food and water intake in both the female and male mice at EAEPI treated groups as compared to their respective control group (Table 5). In satellite group, also mice treated with highest dose (1000 mg/kg b.wt.) did not show any significant change in food uptake and water intake.

3.3.3. Effect of EAEPI on organ weight

Relative organ (heart, kidney, liver, lungs and spleen) weights of EAEPI treated mice was not significantly different (p < 0.05) with the control even after 28 days of treatment (Table 6a) and similar results was also recorded with satellite group after 42 days of recovery period (Table 6b).

3.3.4. Effect of EAEPI on hematological parameters

Data of hematological analysis was given in Table 7. Results revealed that some parameters of hematological analysis showed a statistical difference in the treated groups, while other parameters were similar among the groups when compared with control. The difference in the values of hematological parameters has no biological significance because all values are within the normal range [36].

3.3.5. Effect of EAEPI on serum biochemical parameters

In Table 8, levels of some biochemical parameters in male and female mice were summarized. Results suggest that no significant change in the level of serum marker enzymes (ALT, AST and ALP) at the all treatment dose of EAEPI in both female and male mice in comparison with control group was observed. Effect of administration of EAEPI on serum total protein, albumin, bilirubin, creatinine, blood urea nitrogen, uric acid in mice of both sexes was not significant different when compared to their respective control group. Similar result was also recorded with cholesterol content in both sexes of the mice. Like hematological analysis value of biochemical parameters are within normal range so we can conclude that there is no biological significance of EAEPI on test organism.

3.3.6. Effect of EAEPI on antioxidant enzymes in liver tissues during subacute toxicity

Mice of both sexes when treated with EAEPI for 28 days did not induce any significant (p < 0.05) changes in level of enzymes SOD (Fig. 1A) and catalase (Fig. 1B) and LPO (Fig. 2A) and GSH content (Fig. 2B) in the liver tissue of treated group when compared with respective control.

3.3.7. Histopathological study of liver and kidney tissue

Histopathological observation of liver section of treated male mice exhibited normal histological appearances at all treated doses (400, 600 and 1000 mg/kg b.wt.) after comparison with control. There was no degeneration of hepatocyte in all sections. Obstruction in central vein, focal steatosis and bulging of central vein was not observed (Fig. 3). Similarly, kidney section of male mice did not exhibit any alteration in histology. In all sections of the mice continued with the normal glomeruli and tubular epithelium as compared to control group. On the
Effect of oral administration of ethyl acetate extract of leaf of *P. integrifolia* on body weight in subacute toxicity studies.

| Treatment | Main study | Sex | Initial | 7 days | 14 days | 21 days | 28 days | Weight gain (%) | Recovery period | 35 days | 42 days | Weight gain (%) |
|-----------|------------|-----|---------|--------|---------|---------|---------|-----------------|----------------|----------|----------|-----------------|
| Control   | Female     | 20.01 ± 1.34 | 21.7 ± 0.78 | 22.0 ± 1.34 | 23.0 ± 1.64 | 23.7 ± 1.11 | 17.56 | 24.00 ± 2.45 | 24.80 ± 1.37 | 23.93 |
|           | Male       | 26.10 ± 1.26 | 27.3 ± 2.18 | 28.33 ± 2.02 | 28.44 ± 1.67 | 30.2 ± 1.29 | 15.70 | 30.60 ± 1.69 | 31.50 ± 1.34 | 20.68 |
| 400 mg/kg | Female     | 25.9 ± 1.03 | 26.0 ± 2.20 | 27.8 ± 1.21 | 29.6 ± 0.34 | 29.8 ± 1.39 | 15.05 | 29.88 ± 1.39 | 24.39 |
|           | Male       | 23.06 ± 1.23 | 25.4 ± 1.22 | 26.0 ± 1.56 | 26.0 ± 1.54 | 27.2 ± 0.98 | 17.95 | 27.1 ± 2.45 | 12.91 |
| 600 mg/kg | Female     | 24.0 ± 1.45 | 24.3 ± 0.97 | 24.3 ± 1.21 | 24.9 ± 1.23 | 27.1 ± 2.45 | 12.91 | 28.6 ± 1.36 | 15.78 |
|           | Male       | 20.5 ± 2.35 | 22.8 ± 1.13 | 24.4 ± 2.10 | 25.5 ± 1.43 | 25.5 ± 2.67 | 24.39 | 26.8 ± 1.36 | 15.78 |
| 800 mg/kg | Female     | 24.2 ± 1.24 | 24.5 ± 3.01 | 26.0 ± 1.23 | 27.1 ± 1.03 | 27.3 ± 1.12 | 12.98 | 28.6 ± 1.36 | 15.78 |
|           | Male       | 24.7 ± 1.95 | 24.8 ± 1.23 | 26.6 ± 1.22 | 26.8 ± 2.12 | 29.1 ± 1.63 | 18.31 | 29.1 ± 1.63 | 18.31 |
| 1000 mg/kg| Female     | 20.2 ± 2.33 | 22.0 ± 2.23 | 19.7 ± 1.56 | 23.0 ± 1.54 | 23.9 ± 1.63 | 18.31 | 24.5 ± 1.45 | 24.25 |
|           | Male       | 23.9 ± 1.75 | 24.6 ± 1.26 | 25.0 ± 1.45 | 26.4 ± 2.35 | 28.7 ± 1.78 | 17.84 | 29.9 ± 1.47 | 25.10 |

Values expressed as mean ± SEM, n = 10 animals/group (5male and 5 female), p < 0.05 (ANOVA/Dunnett’s test).
Values expressed as mean ± SEM, n = 10 animals/group (Smale and 5 female), p < 0.05 (ANOVA/Dunnett’s test).

Table 7
Hematological parameters of mice treated with ethyl acetate extracts of leaf of *P. integrifolia* in subacute toxicity.

| Parameters | Normal range | Male | Female | Control (400 mg/kg) | 600 mg/kg | (800 mg/kg) | (1000 mg/kg) | Satellite |
|------------|--------------|------|--------|---------------------|-----------|-------------|-------------|----------|
| Hemoglobin (%) | 10.2–16.6 | 16.22 ± 0.21 | 15.37 ± 0.18 | 14.56 ± 0.26 | 14.11 ± 0.80 | 14.14 ± 1.65 | 15.06 ± 0.97 |
| Total RBC (10^6/μL) | 5–10 | 10.09 ± 0.60 | 9.41 ± 0.92 | 9.08 ± 0.56 | 9.08 ± 0.54 | 9.04 ± 0.41 | 9.48 ± 0.52 |
| WBC (10^3/μL) | 6–15 | 12.49 ± 1.47 | 11.14 ± 2.42 | 9.80 ± 0.26 | 9.58 ± 0.96 | 9.50 ± 0.61 | 10.22 ± 0.94 |
| Platelets (10^3 /L) | 782–985 | 908.00 ± 60.25 | 885.91 ± 49.36 | 920.37 ± 44.37 | 881.72 ± 46.33 | 831.18 ± 53.42 | 877.56 ± 76.56 |
| PCV (%) | 39–49 | 50.55 ± 1.43 | 49.09 ± 2.64 | 45.66 ± 3.31 | 43.45 ± 3.22 | 40.48 ± 2.65 | 44.50 ± 2.94 |
| LC (%) | 55–95 | 74.91 ± 2.96 | 75.54 ± 2.68 | 73.78 ± 1.92 | 74.98 ± 2.47 | 74.39 ± 2.13 | 71.03 ± 0.03 |
| NP (%) | 10–40 | 23.23 ± 1.84 | 23.52 ± 1.38 | 24.88 ± 2.60 | 23.72 ± 1.50 | 24.14 ± 3.06 | 23.06 ± 1.90 |
| MC (%) | 1–4 | 1.15 ± 0.11 | 1.21 ± 0.12 | 1.20 ± 0.20 | 1.33 ± 0.26 | 1.10 ± 0.39 | 1.40 ± 0.14 |
| EP (%) | 0–4 | 1.66 ± 0.18 | 1.73 ± 0.21 | 1.96 ± 0.21 | 1.89 ± 0.22 | 1.84 ± 0.33 | 1.74 ± 0.20 |

Values expressed as mean ± SEM, n = 10 animals/group (Smale and 5 female); significance p < 0.05 (ANOVA/Dunnett’s test).
Table 8
Biochemical parameters of mice treated with different doses of ethyl acetate extract of leaf of *P. integrifolia* in subacute toxicity.

| Parameters                     | Normal ranges | Sex    | Control    | 400 mg/kg   | 600 mg/kg   | 800 mg/kg   | 1000 mg/kg | Satellite |
|--------------------------------|---------------|--------|------------|-------------|-------------|-------------|------------|-----------|
| AST (U/L)                      | 54–298        | Female | 109 ± 3.23 | 106.76 ± 2.53 | 103.40 ± 2.95 | 96.71 ± 3.52 | 95.17 ± 2.87 | 99.20 ± 3.90 |
| Male                           | 99.65 ± 6.05  | Male   | 102.84 ± 3.23 | 107.46 ± 2.24 | 104.18 ± 6.39 | 106.94 ± 6.88 | 109.79 ± 3.32 |
| ALT (U/L)                      | 17–77         | Female | 47.50 ± 2.90 | 45.48 ± 2.67  | 46.64 ± 2.98  | 59.25 ± 5.03  | 49.44 ± 1.40  | 49.57 ± 0.90  |
| Male                           | 46.91 ± 2.94  | Male   | 46.91 ± 2.60  | 47.15 ± 2.91  | 48.34 ± 4.04  | 50.65 ± 2.01  | 49.09 ± 1.15  |
| ALP (U/L)                      | 64–128        | Female | 113.16 ± 2.7 | 114.61 ± 3.08 | 115.61 ± 3.61 | 110.57 ± 5.78 | 108.62 ± 5.78 | 113.97 ± 2.81 |
| Male                           | 114.53 ± 3.0  | Male   | 115.99 ± 3.19 | 108.33 ± 8.76 | 97.99 ± 4.04  | 111.29 ± 6.30 | 115.32 ± 2.53 |
| Total proteins (g/dl)          | 3.5–7.2       | Female | 4.3 ± 0.24  | 4.4 ± 0.25   | 4.4 ± 0.17   | 4.3 ± 0.36   | 4.7 ± 0.45   | 4.7 ± 0.17   |
| Male                           | 4.9 ± 0.34    | Male   | 4.4 ± 0.45   | 4.4 ± 0.29   | 4.5 ± 0.30   | 4.6 ± 0.21   | 4.7 ± 0.30   |
| Albumine (g/dl)                | 2.5–3         | Female | 3.92 ± 0.13 | 4.09 ± 0.16  | 4.14 ± 0.12  | 4.28 ± 0.22  | 4.23 ± 0.16  | 4.20 ± 0.17  |
| Male                           | 4.17 ± 0.20   | Male   | 4.03 ± 0.12  | 4.19 ± 0.18  | 4.16 ± 0.26  | 4.14 ± 0.12  | 4.38 ± 0.17  |
| Bilirubine (mg/dL)             | 0–0.9         | Female | 0.53 ± 0.06 | 0.71 ± 0.08  | 0.72 ± 0.10  | 0.71 ± 0.07  | 0.74 ± 0.07  | 0.70 ± 0.04  |
| Male                           | 0.68 ± 0.06   | Male   | 0.67 ± 0.07  | 0.67 ± 0.04  | 0.71 ± 0.04  | 0.80 ± 0.07  | 0.74 ± 0.08  |
| Creatinine (mg/dL)             | 0.2–0.9       | Female | 0.304 ± 0.04 | 0.301 ± 0.06 | 0.223 ± 0.01 | 0.233 ± 0.06 | 0.271 ± 0.03 | 0.268 ± 0.26 |
| Male                           | 0.307 ± 0.01  | Male   | 0.298 ± 0.01 | 0.300 ± 0.01 | 0.310 ± 0.02 | 0.302 ± 0.02 | 0.311 ± 0.01 |
| BUN (mg/dl)                    | 8–33          | Female | 8.95 ± 0.31 | 9.04 ± 0.17  | 9.15 ± 0.48  | 9.89 ± 0.48  | 9.42 ± 0.25  | 8.99 ± 0.47  |
| Male                           | 9.62 ± 0.52   | Male   | 9.46 ± 0.42  | 9.97 ± 0.46  | 9.11 ± 0.40  | 9.65 ± 0.54  | 9.60 ± 0.47  |
| Uric acid (mg/dL)              | 3–6.8         | Female | 5.15 ± 0.27 | 5.19 ± 0.16  | 4.97 ± 0.39  | 5.19 ± 0.24  | 5.53 ± 0.24  | 5.26 ± 0.42  |
| Male                           | 5.40 ± 0.10   | Male   | 5.04 ± 0.26  | 5.13 ± 0.38  | 5.29 ± 0.32  | 5.57 ± 0.18  | 5.52 ± 0.16  |
| Cholesterol (mg/dL)            |               | Female | 202.62 ± 4.95 | 197.46 ± 8.61 | 197.87 ± 4.83 | 202.42 ± 3.82 | 193.92 ± 6.38 | 202.54 ± 3.76 |
| Male                           | 194.01 ± 6.37 | Male   | 196.33 ± 4.13 | 191.73 ± 2.54 | 180.18 ± 13.25 | 182.74 ± 9.45 | 190.58 ± 8.43 |

Values expressed as mean ± SEM, n = 10 animals/group (5 male and 5 female), p < 0.05 (ANOVA/ Dunnett’s test).

---

**Fig. 1.** Effect of ethyl acetate extract of leaf of *P. integrifolia* on antioxidant enzymes superoxide dismutase (SOD) (A) and catalase (B) level in liver tissues during subacute toxicity studies (mean ± SE, n = 10/group (5 male and 5 female), P < 0.05 (ANOVA/ Dunnett’s test).

**Fig. 2.** Effect of ethyl acetate extract of leaf of *P. integrifolia* on MDA (A) and glutathione (B) content in liver tissues during subacute toxicity studies (mean ± SE, n = 10/group (5 male and 5 female), P < 0.05 (ANOVA/ Dunnett’s test).

Substances (xenobiotics) either drugs or plant extracts in the body of an organism generally metabolized by liver and waste byproducts excreted out by kidneys. Thus liver and kidney are first target of the toxic substances which caused the hepatic and renal injuries. Assessment of some
Fig. 3. Histology of liver after treatment with different doses of ethyl acetate extract of leaves of *P. integrifolia* during subacute toxicity study. A: control, B: 400 mg/kg b.wt, C: 600 mg/kg b.wt, D: 800 mg/kg b.wt. and E: 1000 mg/kg b.wt. H &E stain (400X) (letter ‘a’ and ‘b’ showing central vein and plates of hepatocyte respectively).

Fig. 4. Histology of kidney after treatment with different doses of ethyl acetate extract of leaves of *P. integrifolia* during subacute toxicity study. A: control, B: 400 mg/kg b.wt, C: 600 mg/kg b.wt, D: 800 mg/kg b.wt. and E: 1000 mg/kg b.wt. H &E stain (400X) (letter ‘a’ and ‘b’ showing glomeruli and renal tubules respectively).
serum biomarker enzymes (ALT, AST and ALP) gives the signal for the health status of liver and kidney [50]. Transaminases ALT and AST mainly present in hepatocyte of liver while ALP is major components of plasma membrane and endoplasmic reticulum of the tissues. Increased level of these enzymes is the indicator of the damage of liver tissues. It may be due to infection in these organs or damage due to toxic substances. EAEPI administration did not change significantly in the level of these enzymes in treated and satellite groups as compared to control. Other biochemical parameter like serum albumin and bilirubin contents reflects about hepatocellular function. Reduction in the level of these two parameters suggests the problem in normal function of liver. In this study difference in serum concentration of albumin and bilirubin in treated and control group is insignificant. Xenobiotics introduced in the body of organisms may cause generation of reactive oxygen species (ROS) which induced the change in cellular antioxidants levels. These stress caused the inactivation of antioxidant enzymes, denaturation of proteins, DNA damage and lipid peroxidation of cellular membrane [51]. Neutreuticals application helped in prevention and control of diseases. Plant extracts sometimes may be toxic to organisms by production of ROS and reduction of endogenous antioxidant enzymes. SOD, GSH and CAT level in tissues are reliable marker of antioxidant status, while MDA is a sensitive and reliable indicator for lipid peroxidation [5]. Oral administration of EAEPI did not alter the level of antioxidant enzymes (SOD, and CAT) as well as MDA and GSH content in treated and control group mice. Thus, it confirmed the non toxic effect of EAEPI in liver tissues. However, in previous reports plant extracts caused ROS production affecting antioxidant enzyme levels in tissues which leads to damage in liver tissues of the hosts. Kidney is an important organ involved in filtration of waste substances. Plant extract when administered in animals may create toxicity to kidneys. Measurement of creatinine, blood urea nitrogen (BUN) and uric acid level is good indicator of renal function. Increased levels of these parameters suggest the renal damage. In present findings after administration of EAEPI level of creatinine, BUN and uric acid in treated and satellite groups is not statistically different with control one. It suggests that EAEPI is not toxic to kidney of tested mice. The level of safety of EAEPI also confirmed by non significant change in cholesterol level of control and treated groups. Histological analysis of liver and kidney showed normal structure. Thus it can be concluded that EAEPI did not produce any toxic effects in albino mice. 

Non toxic effect of EAEPI may be due to presence of several compounds in the extract which have strong antioxidant and free radicals scavenging capacities. Polyphenols as well as other phytoconstituents also showed good antioxidant activity [52,53]. Strong in vitro antioxidant activity of the extract was confirmed by different assays. Pronounced antioxidant activity of the EAEPI, manifested as inhibition of lipid peroxidation, scavenging of free radical, it was possible due to its high phenolic content. This strong antioxidant reaction of EAEPI confirmed its application to tackle the problem generated by free radicals in host in response to xenobiotics.

5. Conclusion

On the basis of findings it can be concluded that the ethyl acetate extract of the leaves of \textit{P. integrifolia} is non toxic and safe on acute and subacute administration. Extract at the different doses did not exhibit any lethality or adverse effects on the mice. No significant alterations in the whole body and relative organ weights and histopathological, hematological, biochemical and morphological parameters were recorded. There was no significant change induced by the extract in the level of antioxidant enzymes in liver tissues of both sexes. In \textit{vitro} antioxidant activity of the extract confirmed its strong antioxidant nature. Thus, present study supports the safe application of leaf extract of \textit{P. integrifolia} up to 1000 mg/kg b. wt. against different xenobiotics.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors thankfully acknowledge to the Head, Department of Botany, MMV, BHU, Varanasi for providing laboratory facilities.

References

[1] N. Koppel, V.M. Rekdal, E.P. Balkus, Chemical transformation of xenobiotics by the human gut microbiota, Science 356 (6344) (2017) eaag2770.
[2] J.C. DeWitt, D.R. Germolec, R.W. Luebke, V.J. Johnson, Associating changes in the immune system with clinical diseases for interpretation in risk assessment, Curr. Protoc. Toxicol. (2016), 18.1.1-18.1.22.
[3] V. Mehrotra, S. Mehrotra, V. Kirar, R. Shyam, K. Misra, A.K. Srivastava, S.P. Nandi, Antioxidant and antimicrobial activities of aqueous extract of \textit{Withania somnifera} against methicillin-resistant Staphylococcus aureus, J. Microbiol. Biotechnol. Res. 1 (1) (2017) 40–45.
[4] A.A. Izzo, S. Hoon-Kim, R. Radhakrishnan, E.M. Williamson, A critical approach to evaluating clinical efficacy, adverse events and drug interactions of herbal remedies, Phytother. Res. 30 (5) (2016) 691–709.
[5] C. Singh, C. Prakash, K.N. Tiwari, S.K. Mishra, V. Kumar, \textit{Premna integrifolia} ameliorates cyclophosphamide-induced hepatotoxicity by modulation of oxidative stress and apoptosis, Biomed. Pharmacother. 107 (2018) 634–643.
[6] M. Sajid, M.R. Khan, S.A. Shah, M. Majid, H. Ismail, S. Maryam, R. Batoel, T. Younis, Investigations on anti-inflammatory and analgesic activities of \textit{Althaea rosea} Spach (Endl). stem bark in Sprague Dawley rats, J. Ethnopharmacol. 198 (2017) 407–416.
[7] D.-B. Aane, B. Elfah-Yeboah, P. Barnes, H.A. Abban, E.-O. Ameyaw, J. Boampong, E.G. Ofori, J.B. Dadzie, Antidiabetic effect of young and old ethanolic leaf extracts of \textit{Vernonia amygdalina}: a comparative study, J. Diabetes Res. (2016).
[8] A. Ferroni, M. del Mar Contreras, N. Talhouk, A.M. Gómez-Caravaca, A. Mazallón, A. Segura-Carretero, L. Ghazouani, A. El Feki, M.S. Allagui, Protective effect of \textit{Globularia alba} leaves against deltamethrin-induced nephrotoxicity in rats and determination of its bioactive compounds using high-performance liquid chromatography coupled with electrospray ionisation tandem quadrupole–time-of-flight mass spectrometry, J. Funct. Foods 32 (2017) 139–148.
[9] S. Mohammadi, A. Mahboubi, M. Mohammadi, H. Hedayati, A. Jalili, Assessing the anticancer effect of the \textit{Euphorbia кондюлорскарпа} plant on AGS gastric cancer cell line, Gene Cell Tissue 4 (1) (2017).
[10] S. Loganathan, M.S. Shivakumar, S. Karthi, S.S. Nathan, K. Selvam, Metal oxide nanoparticle synthesis (ZnO-NPs) of \textit{Knotia sumatranae} (Retz.) DC. aqueous leaf extract and its evaluation of their antioxidant, anti-proliferative and larvicidal activities, Toxicol. Rep. 8 (2021) 64–72.
[11] A.P. Group, An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III, Bot. J. Linn. Soc. 161 (2) (2009) 105–121.
[12] A.A. Munir, A taxonomic revision of the genus \textit{Premna} (Verbenaceae) in Australia, J. Adelaide Botanic Garden (1984) 1–43.
[13] M. Dwivedi, J. Saxtry, R.K. Rai, S. Vedula, Clinical evaluation of \textit{Dumortierilishta} (Ayurvedic formulation) in restoring normal health of postpartum females. J. Res. Tradit. Med. 2 (2016).
[14] R. Rehka, P.Richa, S. Bahu, M. Rao, A phytotoxicity of the genus \textit{premna}: a review, Int. J. Pharm. Chem. Sci. 4 (3) (2015) 317–325.
[15] L.S. Joshy, Z. Zakaria, V. Chen, Y.L. Lau, L.V. Latha, S. Saifuddian, Acute oral toxicity of methanolic seed extract of \textit{Carissa fusca} in mice, Molecules 16 (6) (2011) 5268–5282.
[16] R. de Kok, The genus \textit{Premna} (Lamiales) in the Flora Malesiana area, Kew Bull. 66 (1) (2012) 55–84.
[17] R.H. Gekani, S.K. Labidi, D.D. Santani, M.B. Shah, Evaluation of anti-inflammatory and antioxidant activity of \textit{Premna integrifolia} root, J. Complement. Integr. Med. 8 (1) (2011).
[18] R.H. Gekani, J. Patel, Evaluation of the anti-hyperlipidaemic activity of \textit{Premna integrifolia} on nicotine induced hyperlipidaemia in rats, Int. J. Pharma Biosci. 3 (2012) 226–232.
[19] R. Majumder, S. Akter, Z. Naim, M. A. Alin, M.B. Alam, Antioxidant and anti-diabetic activities of the methanolic extract of \textit{Premna integrifolia} bark, Adv. Biol. Res. 8 (1) (2014) 29–36.
[20] R. Rajendran, N.S. Basha, S. Ruby, Evaluation of in vitro antioxidant activity of stem-bark and stem-wood of \textit{Premna serratifolia} Linn., \textit{Verbenaceae}, Phytochemistry 1 (1) (2014) 11–14.
[21] R. Vadivu, A.J. Suresh, K. Girinath, P.B. Kannan, R. Vimala, N.S. Kumar, Evaluation of hepatoprotective and in-vitro cytotoxic activity of leaves of \textit{Premna serratifolia} Linn., J. Sci. Res. 1 (1) (2008) 145–152.
[22] S. McDonald, P.D. Prendergast, M. Antolovich, K. Roberts, Phenolic content and antioxidant activity of olive extracts, Food Chem. 73 (1) (2001) 73–84.
[23] C.-C. Chang, M.-H. Yang, H.-M. Wen, J.-C. Chern, Estimation of total flavonoid content in propolis by two complementary colorimetric methods, J. Food Drug Anal. 10 (3) (2002).
