



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

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ABSTRACT

Premna integrifolia L. (Lamiaceae) is widely used in herbal formulation “Dashmoolarishta” 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 LD₅₀ 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 LD₅₀ 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 mice of treated groups. No histopathological changes were observed in liver and kidney tissues. Thus, 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, thiobarbituric acid; TCA, trichloroacetic acid; OECD, Organization for Economic Co-operation and Development.

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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 blood 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 $\text{Na}^+ - \text{K}^+$ ATPase and Mg^{2+} ATPase function as well as increased the Ca^{+2} ATPase function by ethanol extract of stem bark and wood (200 mg/kg/b.wt.) in frog heart reflects the cardiotoxic 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* (EAEPI) 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 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-Ciocalteu 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 EAEPI, and 0.2 mL of Folin-Ciocalteu 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 AlCl_3 colorimetric method [23]. Aliquots of EAEPI (0.1 mL of 10 mg/mL) in ethanol were mixed with equal volume of 2% AlCl_3 , 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 EAEPI, 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 EAEPI 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 extract were mixed in a test tube and maintained the volume to 500 µL, by adding distilled water. At last, 25 µL FeSO_4 (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 μ L 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 μ g/mL) of EAEPI were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and same volume of potassium ferricyanide [$\text{K}_3(\text{Fe}(\text{CN})_6)$] (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 I 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) \times 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 (KX-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 \pm 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 DPPH radical was observed due to the scavenging ability of EAEPI and that of standard. The EAEPI exhibited a higher scavenging effect ($\text{EC}_{50} = 342.12 \pm 1.23$) that was lower than that of standard (ascorbic acid) with an EC_{50} of 26.96 ± 1.64 . In LPO assay this extract exhibited $\text{EC}_{50} = 350.12 \pm 0.13$. In reducing power assay, the transformation of Fe^{+3} to Fe^{+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 ($\text{EC}_{50} = 373.19 \pm 1.67$ $\mu\text{g}/\text{mL}$) possess significant reducing power, although it was lower than ascorbic acid ($\text{EC}_{50} = 46.18 \pm 2.10$ $\mu\text{g}/\text{mL}$).

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 1,7 and 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_{50} value of EAEPI were found to be > 5000 mg/kg.

Table 1

In vitro antioxidant activity and total phenol, total flavonoid content of ethyl acetate extract of *P. integrifolia*.

Extract/ standard	Polyphenolic content (mg/ g gallic acid equivalent)	Total flavonoid content (mg/g rutin equivalent)	EC_{50} Value		
			DPPH scavenging effect (μg mL^{-1})	LPO activity (μg mL^{-1})	Reducing power (μg mL^{-1})
EAEPI	63.10 ± 1.26	75.33 ± 0.23	342.12 ± 1.23	350.00 ± 0.13	373.19 ± 1.67
Ascorbic acid	–	–	26.96 ± 1.64	85.6 ± 0.29	46.18 ± 2.10

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 (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

Table 2
Behavioral responses and general appearance of mice during acute toxicity studies treated with different single dose of ethyl acetate extract of leaf of *P.integrifolia*.

Observation	Control	300 mg/kg	1000 mg/kg	2000 mg/kg	3000 mg/kg	5000 mg/kg
Temperature	Normal	Normal	Normal	Normal	Normal	Normal
Change in skin	No effect	No effect	No effect	No effect	No effect	No effect
Eye color change	No effect	No effect	No effect	No effect	No effect	No effect
Food intake	Normal	Normal	Normal	Normal	Normal	Normal
General physique	Normal	Normal	Normal	Normal	Lethargy	Lethargy
Diarrhea	Not present	Not present	Not present	Not present	Not present	Not present
Coma	Not present	Not present	Not present	Not present	Not present	Not present
Drowsiness	Not present	Not present	Not present	Not present	Present	Present
Breathing difficulty	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Sedation	No effect	No effect	No effect	No effect	Observed	Observed
Tremor	Not present	Not present	Not present	Not present	Not present	Not present
Death	Alive	Alive	Alive	Alive	Alive	Alive

Table 3
Body weight (g) of mice during acute toxicity study treated with different doses of ethyl acetate extract of leaf of *P.integrifolia*.

Treatment	Sex	Initial	7days	14days	Weight gain (%)
Control	Female	23.38 ± 1.95	25.18 ± 1.93	26.32 ± 2.03	9.57
	Male	27.16 ± 1.86	28.23 ± 2.36	29.44 ± 2.20	8.39
300	Female	24.47 ± 2.09	25.82 ± 2.13	26.76 ± 2.41	9.35
	Male	27.16 ± 1.86	29.66 ± 1.94	30.61 ± 1.77	12.70
1000	Female	23.82 ± 2.76	25.15 ± 2.92	26.11 ± 3.01	9.61
	Male	24.19 ± 1.19	24.80 ± 2.01	26.19 ± 1.76	8.26
2000	Female	23.67 ± 2.16	24.95 ± 2.32	25.97 ± 2.22	9.71
	Male	22.17 ± 0.81	23.41 ± 0.84	24.74 ± 0.86	10.85
3000	Female	24.50 ± 1.06	25.66 ± 1.02	27.33 ± 2.44	11.55
	Male	24.50 ± 1.06	25.66 ± 1.02	26.91 ± 1.11	9.83
5000	Female	24.61 ± 2.06	25.63 ± 2.33	26.55 ± 2.34	7.88
	Male	23.59 ± 1.88	24.50 ± 1.83	26.97 ± 1.10	12.50

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

basis of above observations it can be concluded that liver and kidney did not show any sign of abnormalities, after treatment with different doses of EAEPI (Fig. 4).

Table 4
Effect of oral administration of ethyl acetate extract of leaf of *P. integrifolia* on body weight in subacute toxicity studies.

Treatment	Main study							Recovery period		
	Sex	Initial	7 days	14 days	21 days	28days	Weight gain (%)	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			
	Male	23.06 ± 1.23	25.4 ± 1.22	26.0 ± 1.56	26.0 ± 1.54	27.2 ± 0.98	17.95			
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			
	Male	20.5 ± 2.35	22.8 ± 1.13	24.4 ± 2.10	25.5 ± 1.43	25.5 ± 2.67	24.39			
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			
	Male	24.7 ± 1.95	24.8 ± 1.23	26.6 ± 1.23	26.8 ± 2.12	28.6 ± 1.36	15.78			
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	25.1 ± 1.56	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	29.7 ± 2.16	25.10

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

4. Discussion

To standardize the safety dose of natural products, it is essential to study its various toxicities in animal first, before its utilization as an alternative medicine for human health care. In some reports extracts of *Rutachalepensis* [37] and *Alibertiaedulis* [38] when administered, the animal exhibited a mild acute and subacute toxicity. *Premna integrifolia* has great importance in traditional medicine worldwide due to its use for the treatment of rheumatism, asthma, dropsy, beri-beri and vaginal irritation [39]. There is no report on acute and subacute toxicity study of *P. integrifolia*. Acute toxicity study provides the information about the dose of drug which produces maximum adverse effects and to decide the dose dependant minimum lethality [40]. During acute toxicity study it

Table 5
Effect of ethyl acetate extract of leaf of *P.integrifolia* on food intake and water consumption by mice during 28 days treatment and recovery period (satellite group).

Treatment	Sex	Average food intake (g/day/mice)	Average water intake (ml/day/mice)
Control	Female	4.92 ± 1.19	4.18 ± 1.02
	Male	5.60 ± 1.16	4.25 ± 1.13
400 mg/kg	Female	4.25 ± 1.16	3.18 ± 1.49
	Male	4.59 ± 1.29	4.04 ± 0.80
600 mg/kg	Female	4.98 ± 1.13	4.62 ± 1.29
	Male	5.39 ± 1.25	4.34 ± 1.01
800 mg/kg	Female	3.82 ± 1.20	4.62 ± 1.86
	Male	5.38 ± 1.58	4.61 ± 1.25
1000 mg/kg	Female	4.42 ± 1.88	4.56 ± 1.32
	Male	4.93 ± 1.49	4.38 ± 1.35
Satellite control	Female	4.32 ± 1.65	4.21 ± 1.18
	Male	5.39 ± 1.87	4.72 ± 1.26
Satellite (1000 mg/kg)	Female	4.54 ± 1.17	4.19 ± 1.11
	Male	4.94 ± 2.18	4.49 ± 1.49

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

Table 6aEffect of leaf extract of *P. integrifolia* on relative organ weights (per 100 g body weight) of mice in subacute toxicity studies.

Treatment	Sex	Liver	Kidney	Heart	Spleen	Lung
Control	Female	3.72 ± 0.32	0.857 ± 0.23	0.474 ± 0.36	0.271 ± 0.17	0.607 ± 0.23
		4.40 ± 0.42	0.836 ± 0.28	0.434 ± 0.18	0.280 ± 0.32	0.680 ± 0.12
400 mg/kg	Female	4.33 ± 0.65	0.748 ± 0.27	0.419 ± 0.18	0.244 ± 0.29	0.634 ± 0.18
		3.84 ± 0.18	0.863 ± 0.65	0.498 ± 0.73	0.294 ± 0.18	0.479 ± 0.19
600 mg/kg	Female	4.76 ± 0.17	0.783 ± 0.28	0.467 ± 0.18	0.294 ± 0.17	0.619 ± 0.19
		3.91 ± 0.19	0.766 ± 0.27	0.479 ± 0.54	0.294 ± 0.19	0.667 ± 0.18
800 mg/kg	Female	4.43 ± 1.34	0.793 ± 0.23	0.413 ± 0.19	0.269 ± 0.13	0.653 ± 0.18
		4.38 ± 0.32	0.787 ± 0.28	0.470 ± 0.18	0.312 ± 0.29	0.634 ± 0.19
1000 mg/kg	Female	4.67 ± 0.13	0.753 ± 0.65	0.484 ± 0.23	0.269 ± 0.61	0.648 ± 0.17
		3.89 ± 0.32	0.753 ± 0.17	0.425 ± 0.27	0.314 ± 0.19	0.564 ± 0.28

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

Table 6bRelative organ weights (per 100 g body weight) of mice treated for 28 days with ethyl acetate extracts of leaf of *P. integrifolia* after the recovery period.

Treatment	Sex	Liver	Kidney	Heart	Spleen	Lung
Satellite control	Female	4.33 ± 0.18	0.725 ± 0.19	0.426 ± 0.16	0.271 ± 0.29	0.619 ± 0.28
		4.25 ± 0.17	0.776 ± 0.21	0.446 ± 0.32	0.315 ± 0.35	0.635 ± 0.19
Satellite (1000 mg/kg)	Female	4.45 ± 0.54	0.721 ± 0.21	0.419 ± 0.18	0.278 ± 0.29	0.610 ± 0.26
		4.39 ± 0.23	0.790 ± 0.51	0.449 ± 0.21	0.330 ± 0.17	0.625 ± 0.17

Expressed as mean ± SEM, n = 4 animals/group (2male and 2 female), p < 0.05 (ANOVA/Dunnett's test).

has been found that oral administration of EAEPI up to the doses of 5000 mg/kg b.wt. did not produce any sign of toxicity or death in animal of both sexes. It suggests that the LD₅₀ of EAEPI is above 5000 mg/kg b.wt. According to the OECD recommendation for the chemical labeling and

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

Parameters	Normal range	Sex						Satellite
			Control	(400 mg/kg)	600 mg/kg)	(800 mg/kg)	(1000 mg/kg)	
Hemoglobin (%)	10.2–16.6	Female	16.22 ± 1.19	15.37 ± 1.84	14.56 ± 1.27	14.11 ± 1.80	14.14 ± 1.65	15.06 ± 0.97
		Male	13.53 ± 0.93	14.38 ± 1.42	15.48 ± 1.27	14.56 ± 1.48	13.95 ± 1.12	14.77 ± 1.69
Total RBC (10 ⁶ /μL)	5–10	Female	10.09 ± 0.70	9.41 ± 0.92	9.08 ± 0.56	9.08 ± 0.54	9.04 ± 0.41	9.48 ± 0.52
		Male	9.38 ± 0.72	9.39 ± 0.55	9.39 ± 0.74	9.09 ± 0.28	9.22 ± 0.70	9.45 ± 0.56
WBC (10 ³ /μL)	6–15	Female	12.49 ± 1.47	11.14 ± 2.24	9.80 ± 2.69	9.58 ± 0.96	9.50 ± 0.61	10.22 ± 0.94
		Male	13.48 ± 1.37	10.86 ± 1.23	8.71 ± 1.82	11.36 ± 0.94	9.92 ± 1.00	10.40 ± 1.81
Platelets (10 ³ /L)	782–985	Female	909.03 ± 22.44	822.70 ± 34.82	862.60 ± 51.50	959.67 ± 63.22	895.71 ± 80.52	900.85 ± 84.99
		Male	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	Female	50.55 ± 1.43	49.09 ± 2.64	45.66 ± 3.31	43.45 ± 1.32	44.08 ± 2.65	44.50 ± 2.94
		Male	45.90 ± 2.44	45.64 ± 3.38	45.53 ± 2.87	47.17 ± 1.29	48.32 ± 1.45	46.82 ± 2.45
LC (%)	55–95	Female	73.09 ± 2.52	74.10 ± 2.68	76.38 ± 2.36	73.43 ± 1.58	74.14 ± 1.96	71.59 ± 2.55
		Male	74.91 ± 2.96	75.54 ± 2.68	73.78 ± 1.92	74.98 ± 2.47	74.39 ± 2.13	71.03 ± 3.03
NP (%)	10–40	Female	23.23 ± 1.84	25.32 ± 1.38	24.48 ± 2.60	23.72 ± 1.50	24.14 ± 3.06	23.00 ± 1.90
		Male	23.41 ± 1.54	23.66 ± 1.42	26.15 ± 2.66	23.34 ± 2.59	23.58 ± 2.16	22.89 ± 2.28
MC (%)	1–4	Female	1.15 ± 0.11	1.21 ± 0.12	1.20 ± 0.20	1.33 ± 0.26	1.10 ± 1.39	1.40 ± 0.14
		Male	1.33 ± 0.27	1.25 ± 0.10	1.23 ± 0.16	1.36 ± 0.20	1.22 ± 0.14	1.19 ± 0.14
EP (%)	0–4	Female	1.66 ± 0.18	1.73 ± 0.21	1.96 ± 0.21	1.89 ± 0.22	1.84 ± 0.33	1.74 ± 0.20
		Male	1.62 ± 0.30	1.87 ± 0.23	1.99 ± 0.18	1.85 ± 0.68	1.62 ± 0.21	1.69 ± 0.14

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

classification of acute systemic toxicity, the EAEPI will be considered in category 5 (LD₅₀ > 5000 mg/kg), which states the lowest toxicity class. Doses tested could be considered safe in mice because EAEPI showed LD₅₀ higher than 5000 mg/kg b.wt. via oral is generally considered practically non-toxic [41,42].

The importance of subacute toxicity lies on the evaluation of effects of long term exposure of plant products. This study clearly gives the idea about safety level of the tested drugs [43]. Based on the subacute toxicity study, no deaths were recorded. There was no significant difference in the body weights at all doses in the mice of both sexes when compared with control. Although the mean intake differed statistically but no biological importance was assigned to this, since the body weight gain values did not differ among groups. After 28 days oral administration, the food intake and water consumption was not affected by the administration of EAEPI. It indicates that extract did not induce appetite suppression and deleterious effects on growth of mice. Additionally, no delayed occurrence of toxic effect was observed in satellite even after 42 days of observation. Normal health of mice suggested that there was no disturbance in carbohydrate, protein or fat metabolism [15] in the body. Similarly, in other cases [38] after subacute toxicity test no difference in body weight, food intake and water uptake was reported. In some reports reduction in body weight, food intake and water uptake was observed during the end of subacute toxicity [44]. Some times during subacute toxicity lethality of animals took place [45]. Negative effect of subacute toxicity and lethality of mice confirmed the toxic nature.

Changes in organ weights are indices of toxicity in animals [46]. The possibility for change in organ weight may be due to effects on important vital organs, such as the kidney, liver, spleen, heart and brain which play diverse roles in the body of organism.

The study of hematological parameters provides information about toxic effects of extract on blood of mice. Hematological parameters of any organism clearly indicates its physiological and pathological status [47]. Blood is the main medium for transport of nutrients and foreign bodies in the body of animals. If toxic substances were present in blood, it directly affects its components such as red blood cells, white blood cells, platelets and hemoglobin. The measurement of hemoglobin, total RBC, WBC, platelets, PCV, LC, NP, MC, EP significantly affected by the level of toxicity caused by the compounds. Toxicity of drugs affects the bone marrow, which results the damage of blood cells and ultimately normal body function [48]. The administration of EAEPI did not cause any significant change in the hematological parameters.

The liver and kidney are two very important vital organs, its proper function is essential for the survival of the organism [49]. Toxic

Table 8Biochemical 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	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	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	115.65 ± 6.31	100.57 ± 5.78	108.62 ± 5.78	113.97 ± 3.81
		Male	114.53 ± 3.0	115.09 ± 3.19	108.33 ± 8.76	97.39 ± 4.04	111.29 ± 6.30	115.32 ± 2.33
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	4.4 ± 0.17
		Male	4.9 ± 0.34	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	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	0.67 ± 0.07	0.67 ± 0.04	0.71 ± 0.07	0.80 ± 0.07	0.80 ± 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	0.298 ± 0.01	0.330 ± 0.021	0.317 ± 0.03	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 ± 0.35	8.99 ± 0.47
		Male	9.62 ± 0.52	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	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	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 (5male and 5 female), p < 0.05 (ANOVA/ Dunnett's test).

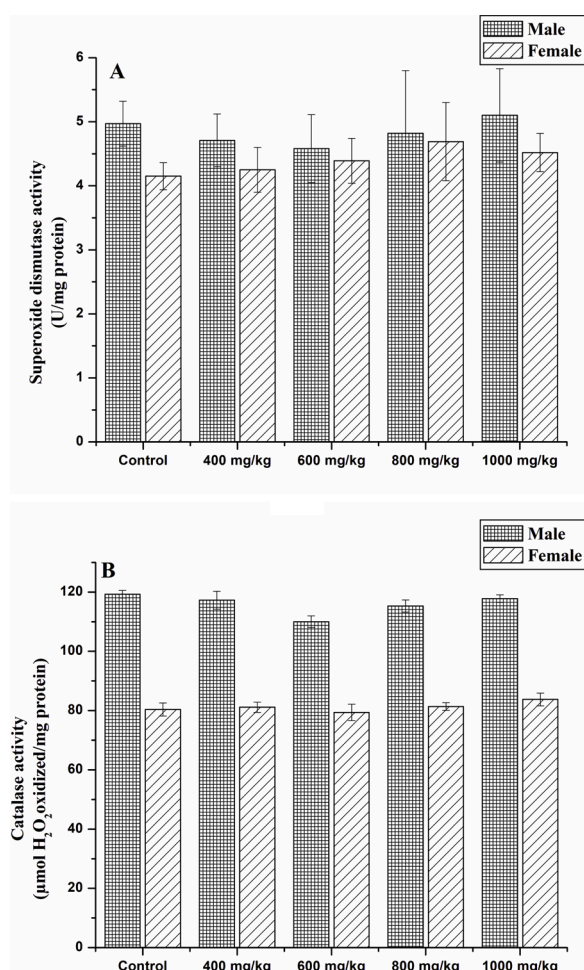


Fig. 1. Effect of ethyl acetate extract of leaf of *P. integrifolia* on antioxidant enzymes super oxide dismutase (SOD) (A) and catalase (B) level in liver tissues during subacute toxicity studies (mean ± SE, n = 10/ group (5male and 5 female), P < 0.05 (ANOVA/ Dunnett's test).

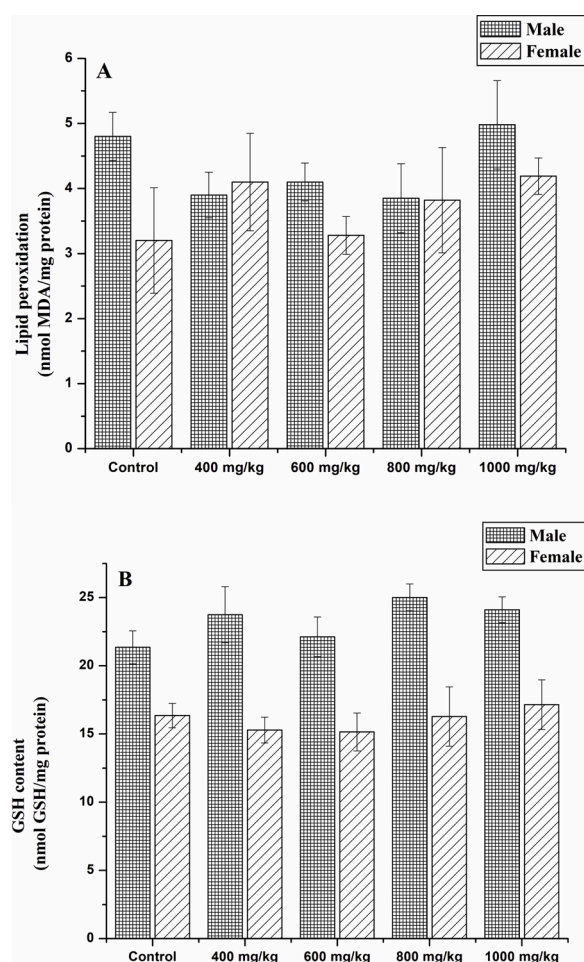


Fig. 2. Effect of ethyl acetate extract of leaf of *P. integrifolia* on MDA (A) and glutathione (B) content in liver tissues during subacute toxicity studies (mean ± SE, n = 10/ group (5male and 5 female), P < 0.05 (ANOVA/ Dunnett's test).

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

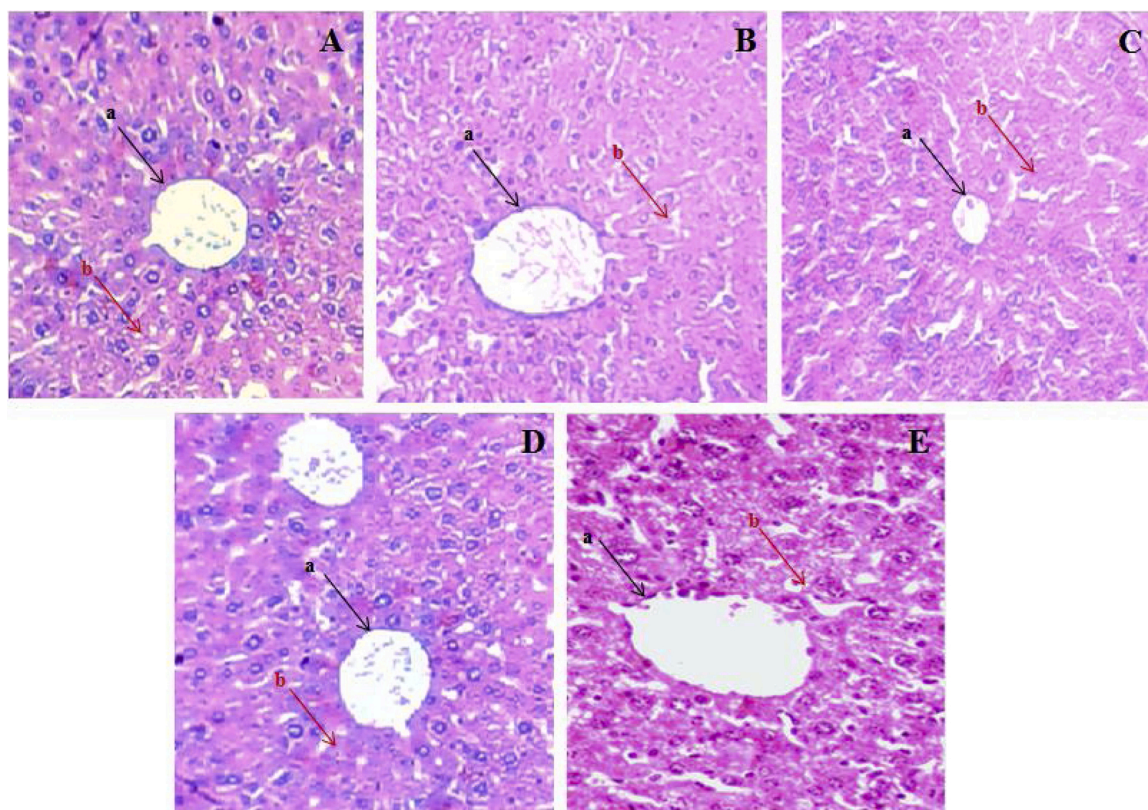


Fig. 3. Histology of liver after treatment with different doses of ethyl acetate extract of leaves of *P. integrifolia* during subacute toxicity study. A: control, B: 400 mg/kg b.wt, C: 600 mg/kg b.wt, D: 800 mg/kg b.wt. and E: 1000 mg/kg b.wt. H &E stain (400X) (letter 'a' and 'b' showing central vein and plates of hepatocyte respectively).

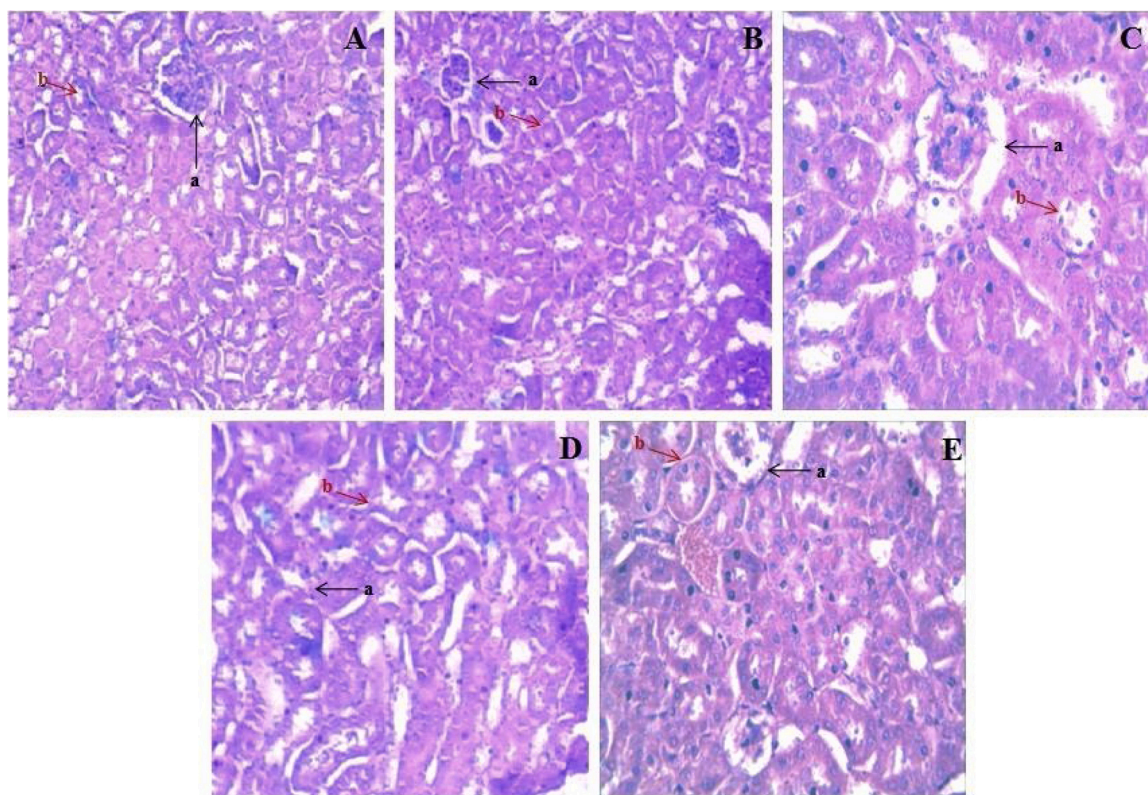


Fig. 4. Histology of kidney after treatment with different doses of ethyl acetate extract of leaves of *P. integrifolia* during subacute toxicity study. A: control, B: 400 mg/kg b.wt, C: 600 mg/kg b.wt, D: 800 mg/kg b.wt. and E: 1000 mg/kg b.wt. H &E stain (400X) (letter 'a' and 'b' showing glomeruli 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. EAPEI 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]. Nutraceuticals 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 markers of antioxidant status, while MDA is a sensitive and reliable indicator for lipid peroxidation [5]. Oral administration of EAPEI 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 EAPEI 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 EAPEI level of creatinine, BUN and uric acid in treated and satellite groups is not statistically different with control one. It suggests that EAPEI is not toxic to kidney of tested mice. The level of safety of EAPEI 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 EAPEI did not produce any toxic effects in albino mice.

Non toxic effect of EAPEI 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 this extract was confirmed by different assays. Pronounced antioxidant activity of the EAPEI, manifested as inhibition of lipid peroxidation, scavenging of free radical, it was possible due to its high phenolic content. This strong antioxidant reaction of EAPEI 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 *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 vitro* antioxidant activity of the extract confirmed its strong antioxidant nature. Thus, present study supports the safe application of leaf extract of *P. integrifolia* up to 1000 mg/kg b. wt. against different xenobiotics.

Declaration of Competing Interest

The authors report no declarations of interest.

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