Oral acute toxicity study extract ethanol of balakka fruit (*Phyllanthus emblica*)

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Abstract

*Phyllanthus emblica* (PE) is a plant widely found in Indonesia, especially in Sumatra island, and in India. This study followed the OECD No. 420. The groups were divided based on gender, male and female rats. Male rats were divided into 6 groups, which were male/female control, male/female 2000 mg/kgBW, and male/female 5000 mg/kgBW. This study revealed that the ethanol extract of *Phyllanthus emblica* (EEPE) doses of 2000 mg/kgBW and 5000 mg/kgBW did not cause histological changes in the heart, liver, testes, ovaries, and kidneys, and did not cause changes to the hematological parameters, kidney biochemical parameters, liver biochemical parameters, and electrolyte parameters both in male and female rats. The results show that the LD50 of EEPE is higher than 5000 mg/kgBW. In short, this study provides information regarding the antioxidant activity and the safe use of EEPE. The LD₅₀ of extract ethanol of *Phyllanthus emblica* is higher than 5000 mg/kgBW.

Keywords

*Phyllanthus emblica*, Acute Toxicity Study, LD₅₀, OECD

Introduction

*Phyllanthus emblica* (PE) is a plant widely found in Indonesia, especially in Sumatra island under the name Balakka, and in India, this plant, named Indian gooseberry, is also commonly found and widely used in Ayurvedic medicine. PE is traditionally used for daily treatment for hair growth, anti-constipation, and reducing fever and pain. *Phyllanthus emblica* belongs to the family Euphorbiaceae and is widely distributed in the subtropics and tropics, such as China, India, Malaysia, and Thailand. *Phyllanthus emblica* fruit is very popular because it contains vitamin C and high phenolic compounds. From various pharmacological activities reported, *Phyllanthus emblica* fruit has antioxidant, immunomodulatory, and anticancer activities (Khodpe et al. 2001; Anila and Vijayalakshmi 2003; Liu et al. 2012), analgesic, anti-pyretic, anti-diabetic, and antimicrobial. (Perianayagam et al. 2004; Liu et al. 2009; Sharma et al. 2020).

All parts of *Phyllanthus Emblica*, including fruits, flowers, seeds, leaves, and bark have been widely used in various traditional medicines, such as in Indian Medicine (Ayurveda), Chinese Traditional Medicine, Tibetan Medicine, and Greek Arabic Medicine. Southwest China's
minority residents apply *Phyllanthus emblica* root to treat Eczema and its fruit to treat jaundice and diarrhea. Besides, in Nepal, it is used as an astringent and hemostatic (Mirunalini and Krishnveni 2010; Gaire and Subedi 2014). The bark of *Phyllanthus emblica* has antioxidant activity and radical scavenging due to its polyphenol compounds (Liu et al. 2008; Charoenteerataboon et al. 2010). As a tannin-rich plant (21–33%), the bark of *Phyllanthus emblica* is used as a material for tannin extraction in China. Many pharmacological studies have identified *Phyllanthus emblica* but primarily focused on the fruit, whereas other parts, such as the bark, have rarely been examined.

PE is rich in metabolite compounds, including flavonoids, saponins, tannins, steroids, and glycosides. The flavonoid compounds contained in PE are kaempferol-3-O-α-L-(6'-methyl)-rhamnopyranoside, kaempferol-3-O-α-L-(6'-ethyl) rhamnopyranoside, and other compounds, such as Triacantanol, Triacantanoic acid, β-Amirny ketone, Betulonic acid, Daucosterol, Lupeol acetate, β-Amirny-3-palmitate, Gallic acid, Betulinic acid, Ursolic acid, Oleanolic acid, Quercetin, Rutin, and Bisabolane. Also, PE fruit is rich in vitamin C, luteolin, and corilagin (Habib et al. 2007; Poltanov et al. 2009; Luo et al. 2011; Pentaweerath et al. 2016).

Many studies have been conducted to determine the pharmacological activities of PE, including on immunomodulators, hepatoprotective, antiaging, anti-inflammatory, nephroprotective, anti-proliferative, anticancer, cardiac disorder, antibacterial, and antioxidant activities. A study conducted by Juree (2010) reported that water extract of PE had strong antioxidant activity using the DPPH method that yielded a value of 51.3 ± 16.5, a value of 295 ± 5.4 using ABTS, and a value of 0.65 ± 0.04 using DCF. PE also contains a total flavonoid of 389.33 ± 1.25 mg quercetin hydrate/g and a total phenol value of 99.52 ± 1.91 mg GAE/g; this proves that PE has strong antioxidants (Chaphalkar et al. 2007; Poltanov et al. 2009; Luo et al. 2011; Pientaweerath et al. 2016).

According to WHO, ensuring the safe use of herbs is very important to prevent toxic effects. Some toxicity tests must follow the standard guidelines from OECD (Organization for Economic Co-operation and Development) protocol guideline No. 420. The groups were divided based on gender, male and female rats. Male rats were divided into 3 groups, which were male control (MC), male 2000 mg/kgBW (M2000), and male 5000 mg/kgBW (M5000). Meanwhile, the female rats were divided into 3 groups, which were female control (FC), female 2000 mg/kgBW (F2000), and female 5000 mg/kgBW (F5000). All groups were given a single dose of ethanol extract of PE according to the dose using oral gavage on the first day. Then, clinical (dyspnea, dullness, abdominal cramp, diarrhea) and mortality observations were performed for 48 hours and continued for 14 days. At the end of the study, rats were fasted overnight and sacrificed by using diethyl ether inhalation. Blood was directly taken and the fruits were crushed until obtaining dry fruit powder. The solution was then filtered using 96% ethanol and macerated for seven days, and occasionally steered every day. The solution was then filtered using Whatman paper No 1, and the filtered result was evaporated using a rotary evaporator under reduced pressure until crude extract/ethanol extract of PE (EEPE) was obtained. Phytochemical screening (alkaloids, flavonoids, tannins, saponins, glycosides, steroids/triterpenoids) was then performed.

**Hematological analysis**

The hematological analysis was conducted using cell-dyn at Universitas Sumatera Utara Hospital. The parameters examined included red blood cells (RBC), white blood cells (WBC), hemoglobin, hematocrit, Mean Corpuscular Volume (MCV), mean cell hemoglobin concentration...
(MCHC), and mean cell hemoglobin (MCH) using a hematology analyzer (Roche Diagnostic, Switzerland).

**Biochemical analysis**

The parameters examined in this study were total protein, direct bilirubin, Alanine aminotransaminase (ALT), Aspartate aminotransaminase (AST), Alkaline Phosphatase (ALP), Urea, Creatinine, and Uric Acid using Cobas 6000 (Roche diagnostic, Switzerland). The measurement of sodium, chloride, and potassium levels was done using Cobas b221 (Roche diagnostic, Switzerland) (Van et al. 2008; Ameijeiras et al. 2010).

**Histopathology**

Heart, liver, kidney, pancreas, ovary, and testis were taken, and the samples were soaked with liquid paraffin at 60–70 °C for 2 hours. It was molded and allowed to freeze, then the paraffin blocks were cut using a microscope with a thickness of 5–7 μm and were attached to slides. The organ incision that has been attached to the slide was immediately placed on a heating surface at a temperature of 56–58 °C for approximately 10 seconds, so that the organ stretches and sticks to the slide; adjustments were made to avoid wrinkled or folded organs. Further, hematoxylin-eosin staining was carried out. First, the preparations were soaked in xylene solution for the deparaffination process for 12 minutes. Next, the dehydration process was carried out by soaking the preparations in 70%, 80%, 90%, and absolute ethanol for 5 minutes, and followed by washing using running water. Subsequently, the preparations were soaked with hematoxylin solution for 5 minutes, washed with running water, stained with eosin, and then dipped in ethanol 70%, 80%, 90%, and absolute ethanol for 10 minutes. Finally, the preparations were put in xylene for 12 minutes. A microscope (Thermo, German) at 100× magnifications was used to observe the preparations.

**Data analysis**

Data analysis in this study used SPSS (statistical program for social sciences) version 21 using the one-way ANOVA (Analysis of Variance) test. If the p-value was less than 0.05, there was a significant difference between groups, and if the p-value was higher than 0.05, there was no difference between groups.

**Results and discussion**

**Acute toxicity result**

The 48-hour observation showed no deaths from the rats either at the dose of 2000 mg/kgBW or 5000 mg/kgBW; thus, it can be concluded that the LD50 of EEPE is above 5000 mg/kgBW. During the 48 hours, there were no clinical symptoms, including dyspnea, dullness, abdominal cramp, and diarrhea in all treatment groups.

**Body weight, organ weight, and relative organ**

Data on body weight from each experimental group can be seen in Table 1. The data above shows that body weight, organ, and relative organ weight in all groups increased, seen from the initial body weight and the final body weight. The highest increase in body weight was in MC group while...
the smallest was in M2000. The heaviest heart in M2000 group was 0.668 g, the heaviest liver in M group was 7.63 g, and the heaviest left kidney was 0.674 g. Body weight, organ weight, and relative organ weight between the groups showed no statistically significant difference (p > 0.05).

**Hematological analysis**

The effect of EEPE on hematological parameters including WBC (white blood cells), RBC (red blood cell), hemoglobin, hematocrit, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) can be seen in Table 2.

Table 2 shows WBC, RBC, HGB, HCT, MCV, MCH, and MCHC levels from each treatment group. The values of WBC, RBC, and HGB did not differ significantly between treatment groups (p > 0.05). Meanwhile, there was a significant increase in the MCV value in M2000 group compared to normal MCV group (p < 0.05). The MCH value in the F2000 group showed a significant increase compared to FC group (p < 0.05). There was no difference between the MCHC values between groups (p > 0.05).

**Serum biochemical parameters**

The effect of EEPE on kidney biochemical parameters, including urea, creatinine, and uric acid, showed no significant difference (p > 0.05) between the groups.

Table 3 shows the urea, creatinine, and uric acid levels. The highest urea value was in M2000 group (31.3±0.37 mg/dL), while the lowest urea value was in F5000 group (22.3 ± 0.13 mg/dL). The highest creatinine value was in F2000 group (1.52±0.01 mg/dL), while the lowest creatinine value was in MC group (0.56±0.16 mg/dL). The highest uric acid value was in M2000 group (3.07±0.41 mg/dL), while the lowest uric acid value was in MC group (2.13±0.21 mg/dL).

**Hepar Biochemical Parameters**

Biochemical parameters of the liver are essential to determine whether EEPE has an effect on the liver. Blood taken from all groups on day 14 was analyzed for total protein, albumin, direct bilirubin, alanine aminotransferase (AST), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Complete data can be seen in Table 4.

Table 4 shows the value of liver biochemical parameters, that are total protein, albumin, direct bilirubin, AST, ALT, and ALP. The highest total protein value was in F2000 group (6.18 ± 0.05 g/dL) while the lowest protein value was in FC group (5.27 ± 0.14 g/dL). The highest albumin value was in FC group (4.67 ± 0.11 g%), while the lowest albumin value was in MC group (3.10±0.10 g%). Interestingly, the albumin value of the female groups was higher than that of all male groups. The highest direct bilirubin value was in F5000 group (0.06±0.068 mg/dL), while the lowest direct bilirubin value was in MC group (0.009±0.001 mg/dL); there was a statistically significant difference between the FC and F5000 groups (p < 0.05).
Histopathology of the organs

The histopathology results of the heart, kidneys, liver, testes and ovaries can be seen in figure 1.

Histopathology results of Organs including the heart, testis, ovary, liver, and kidney from CM, CF, M2000 (M2), F2000 (F2), M5000 (M5), and F5000 (F5) (H&E x20). Testis ST: seminiferous tubules; IT: Intestinal tissue. Heart MC: cardiac muscle cells; MC: muscle nucleus cell. Ovary O: Oocytes. Liver tissue PA: portal area; H: hepatocytes. Kidney tissue G: glomerulus; BS: Bowmen space, DT: Distal Convoluted tubule. Tissues from the liver, testes, ovaries, liver, and kidneys were observed using a x100 microscope (Thermo, Germany). The images above show that the heart tissue for the M5000 and F5000 mg/kgBW groups did not experience structural changes of the cardiac muscle cells compared to CM and FM groups as well as M2000 and F2000 groups. The liver tissue showed that the oocytes in F2000 and F5000 groups did not experience any structural changes compared to FM group. It indicates that EEPE groups did not have a toxic effect on the ovary. Testis tissue in M2000 and M5000 groups did not change compared to MC group. The liver tissue disclosed that the groups given EEPE showed no changes in the portal area and hepatocytes compared to normal group. Likewise, the kidney tissue also showed no changes on glomerulus, bowmen space, and also distal convoluted tubule in the groups given EEPE. Overall, all of the tissues in the heart, testis, ovary, liver, and kidney organs were normal after given EEPE doses of 2000 mg/kgBW and 5000 mg/kgBW.

Histopathology of Organs including the heart, testis, ovary, liver, and kidney from CM, CF, M2000 (M2), F2000 (F2), M5000 (M5), and F5000 (F5) (H&E x20). Testis ST: seminiferous tubules; IT: Intestinal tissue. Heart MC: cardiac muscle cells; MC: muscle nucleus cell. Ovary O: Oocytes. Liver tissue PA: portal area; H: hepatocytes. Kidney tissue G: glomerulus; BS: Bowmen space, DT: Distal Convoluted tubule. Tissues from the liver, testes, ovaries, liver, and kidneys were observed using a x100 microscope (Thermo, Germany). The images above show that the heart tissue for the M5000 and F5000 mg/kgBW groups did not experience structural changes of the cardiac muscle cells compared to CM and FM groups as well as M2000 and F2000 groups. The liver tissue showed that the oocytes in F2000 and F5000 groups did not experience any structural changes compared to FM group. It indicates that EEPE groups did not have a toxic effect on the ovary. Testis tissue in M2000 and M5000 groups did not change compared to MC group. The liver tissue disclosed that the groups given EEPE showed no changes in the portal area and hepatocytes compared to normal group. Likewise, the kidney tissue also showed no changes on glomerulus, bowmen space, and also distal convoluted tubule in the groups given EEPE. Overall, all of the tissues in the heart, testis, ovary, liver, and kidney organs were normal after given EEPE doses of 2000 mg/kgBW and 5000 mg/kgBW.

Histopathology of the organs

Table 5 above shows Na+, K+, and Cl- levels in all groups were done on day 14. Complete data can be seen in Table 5.

Table 5 above shows Na+, K+, and Cl- levels in all groups. The highest sodium value was in FC group (124 ± 2.915 mmol), while the lowest value was in F2000 group (121 ± 6.81 mmol). The highest potassium value was in M2000 group (7.58 ± 0.14 mmol), while the lowest potassium value was in MC group (3.65 ± 0.28 mmol); there was a statistically significant difference (p < 0.05) between the M2000 and MC groups. The highest chloride value was in M2000 group (85.24 ± 39 mmol), while the lowest chloride value was in F5000 group (75.88 ± 2.31 mmol).

| Parameters | Units | MC  | FC  | M2000 | F2000 | M5000 | F5000 |
|------------|-------|-----|-----|-------|-------|-------|-------|
| Sodium     | mmol  | 112.4 ± 2.41 | 124 ± 2.915 | 117.6 ± 2.40 | 121 ± 6.81 | 113.9 ± 4.50 | 104 ± 2.8 |
| Potassium  | mmol  | 3.65 ± 0.28 | 4.70 ± 0.08 | 7.58 ± 0.14 | 5.18 ± 0.04 | 5.91 ± 0.08 | 4.71 ± 0.22 |
| Chloride   | mmol  | 84 ± 2.23 | 85.36 ± 1.58 | 85.24 ± 39 | 84.36 ± 3.39 | 83.98 ± 3.33 | 75.88 ± 2.31 |

Data are presented Mean ± SEM. *(p < 0.05) significant different from normal group.
al. 2000). In this study, there was no increase in the parameters (Table 4). This is also highly correlated with the histological results of the liver, which showed no damage. Previous studies have revealed that PE has a hepatoprotective effect by significantly reducing AST, ALP, ALP, and total protein levels. Other studies have also proven that PE upregulates PI3K/Akt/GSK3β/β-catenin pathway (Thirunavukkarasu et al. 2015).

Acute kidney injury will occur if there is a toxic drug exposure that will generally increase the level of kidney biochemical parameters, such as urea, creatinine, and uric acid (Coca and Parikh 2012). In this study, the results showed that the levels of urea, creatinine, and uric acid did not increase in both male and female groups given EEPE, either the dose of 2000 mg/kgBW or 5000 mg/kgBW. It is also in line with the results of kidney histology, which showed no changes in the morphology of the kidney (Figure 1). Several studies revealed that *Phyllanthus emblica* has a nephroprotective effect by reducing levels of blood urea nitrogen (BUN), lipid peroxidation, and increasing levels of endogenous antioxidants, such as SOD, GPx, CAT, and GR in cisplatin-induced mice (Pinami 2008; Purena et al. 2018). The active compounds found in PE are apigenin-7-O-(6′-butyryl-β-glucopyranoside), gallic acid, and luteolin-4′-O-neohesperidoside (Liu et al. 2012). This compound has an antioxidant role; in this study, the scavenging ability of EEPE has an IC50 value of 7.626 ± 0.41 µg/dL (Table 1). Another study also revealed

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**Figure 1.** Histopathology of Organs including the heart, testis, ovary, liver, and kidney from CM, CF, M2000 (M2), F2000 (F2), M5000 (M5), and F5000 (F5) (H&E x20). Testis ST: seminiferous tubules; IT: Intestinal tissue. Heart MC: cardiac muscle cells; MC: muscle nucleus. Ovary O: Oocytes. Liver tissue PA: portal area; H: hepatocytes. Kidney tissue G: glomerulus; BS: Bowman space, DT: Distal Concoluted tubule.
that luteolin has several cardioprotective mechanisms by means of anti-calcium overload, other luteolin functions can also reduce radical compounds (O-, H2O2, and OH-), luteolin also has anticancer activity by stimulating pathway apoptosis (Xu and Jiang 2012). Moreover, Luteolin has LD50 higher than 5000 mg/kgBW, while Gallic acid has LD50 more than 2000 mg/kgBW (Seelinger et al. 2008; Variya et al. 2019). Research on Antioxidant activity of methanolic extract of Emblica fruit (Phyllanthus emblica L.) from six regions in China, Liu et al. (2007) showed that methanol extract of Phyllanthus emblica fruit has high antioxidant activity derived from phenolic compounds as much as 81.5–120.9 mg gallic acid equivalent (GAE)/g, flavonoid compounds as much as 20.3–3.7 mg quercetin equivalent (QE)/g and proanthocyanidin compounds as much as 3.7–18.7 catechin equivalent (CE)/g. The high levels of flavonoids, phenols, and antioxidant activity in Phyllanthus emblica offer benefits in their use as traditional therapy together with LD50 higher than 5000 mg/kgBW, no toxic effect on organs, and no effect on biochemical parameters, thus confirm that PE is safe to use.

**Conclusion**

In short, this study provides information that Phyllanthus emblica fruit has high antioxidant activities, LD50 higher than 5000 mg/kgBW, no histological changes in the organs, and does not cause significant changes in the biochemical parameters of the kidney, liver, and electrolyte. In future, a chronic toxicity study is recommended to confirm the safe use of Phyllanthus Emblica.

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