Pharmacy Education (2021) 21(2) 201 - 204
https://doi.org/10.46542/pe.2021.212.201204

IAI CONFERENCE

RESEARCH ARTICLE

Acute toxicity test of 96% ethanol extract of *Syzygium myrtifolium* leaves in white mice (*Mus musculus*)

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Keywords
Acute toxicity test
Lethal dose 50% (LD_{50})
Syzygium myrtifolium leaves

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Abstract

Introduction: Acute toxicity effects appear within a short time following the oral administration of either a single dose or repeated doses of toxin within 24 hours. Acute toxicity testing involves the administration of a range of doses across several groups of experimental animals with one dose administered per group, followed by the observation of toxic effects and mortality. **Aims**: The purpose of this study was to determine the lethal dose 50 (LD_{50}) and acute toxicity of an ethanol extract of *Syzygium myrtifolium* leaves in white mice. **Methods:** Exposed groups consisted of a negative control group (carboxymethylcellulose sodium) and four treatment groups (500, 1000, 2000, and 4000 mg/kg body weight (bw)). Mortality was observed for 14 days following oral administration. **Results:** The results demonstrated an LD_{50} of 1995 mg/kg bw, categorised as moderately toxic. Observed toxic effects included white lesions in the lungs, blackened liver, organ swelling, and fluid accumulation in the abdominal cavity and thorax.

Introduction

Toxicity tests are classified as acute, subchronic, or chronic. They are designed to determine the safety of traditional medicines by detecting the toxic effects of a substance on a biological system and obtaining typical dose-response data. These data can provide insight into the potential human toxicity of the doses used in the test to determine a safe dosage in humans (Depkes RI, 2015).

Acute toxicity tests determine the lethal dose 50 (LD_{50}) of a compound. They are carried out by either single or repeated administration of the chemical compound tested, followed by observation of the test subjects for 24 hours.

One of the plants currently used as traditional medicine is the red shoot plant (*S. myrtifolium*). This plant contains flavonoids, phenols, and terpenoids, which have anti-tumour and anti-angiogenesis activities (Aisha *et al.*, 2013). According to Liniawati (2019), the *n*-hexane extract of red shoot leaves has triterpenoid compounds.

Toxicity testing of the 96% ethanol extract of red shoot leaves has been carried out *in vitro* with the Brine Shrimp Lethality Test (BSLT) method; the LC_{50} results were 171.59 ppm, indicating that the red shoot leaves were toxic (Haryati *et al.*, 2015). Research of *in vivo* toxicity is necessary to determine the safety of red shoot leaves. Therefore, an acute toxicity test was carried out *in vivo* by administering 96% ethanol extract of red shoots in experimental animals to determine the LD_{50} value and the maximum tolerated dose. These results may be an indication of potential toxicity in humans by extrapolation. This test generally uses two experimental animal models, with two administration routes and a single dose (Priyanto, 2010).

The LD_{50} value was determined using the Thompson and Weil formula. This method was chosen because it has a reasonably high level of confidence, is frequently used, and does not require a large number of

experimental animals. This method also uses a list of LD_{50} calculations to improve the accuracy of results. This study was conducted to determine the lethal dose 50 (LD_{50}) and the acute toxicity of a 96% ethanol extract of red leaves in white mice.

Methods

Materials

The tools used in this study included aluminium foil, glassware, funnels, filters, analytical scales, vacuum tray dryer, oral syringes, watch glasses, mouse cages, mortar, pestle, dropper, vial, spatula, ovens, plates, crucibles, animal scales, and stainless-steel surgical instruments. The materials used included *S. myrtifolium* leaves, distilled water, carboxymethylcellulose sodium (CMC-Na), 96% ethanol, white mice (*Mus musculus*), Mayer's reagent, Bouchardat's reagent, Dragendorff's reagent, gelatin, 10% sodium chloride, 1% and 3% ferric chloride, 80% methanol, 10% acetic acid, 2N hydrochloric acid, sulfuric acid, and anhydrous acetic acid.

Preparation of dried plants (Simplicia) and extract

A total of 3500 g of fresh *S. myrtifolium* leaves was cleaned of impurities, dried, and mashed. Then, 800 g of Simplicia powder was extracted using the maceration method. The Simplicia powder was placed into a dark bottle or macerator, then soaked with 96% ethanol in a 1:10 ratio of powder to solvent until the powder was completely immersed. The soaking process was carried out for 24 hours with occasional stirring. Macerate was then filtered, and the maceration process was repeated on the resulting pulp up to three times. The filtrate obtained was collected and evaporated using a vacuum tray dryer until a thick extract was obtained (Depkes RI, 2015). The viscous extract was stored in a tightly closed container and protected from light.

Preparation of experimental animals

This study utilized about 25 *Mus musculus* male mice, weighing between 25 and 40 g. First, the coefficient of variation (CV) of mouse body weight was calculated. Mice were then randomly divided into five treatment groups with five mice per group, then acclimatized for seven days in a cage. The mice were provided with standard food and drink (ad libitum) and individually weighed again after seven days. Furthermore, the final CV of mouse body weight was calculated to obtain relatively homogeneous mice (CV<15%).

Treatment of experimental animals

Mice were fasted for 24 hours prior to exposure. The experimental animals in each group were administered 0.4 mL of the following treatments:

1. Group I: 0.5% CMC-Na (the negative control)
2. Group II: 500 mg/kg body weight (bw) of *S. myrtifolium* leaves extract
3. Group III: 1000 mg/kg bw of *S. myrtifolium* leaves extract
4. Group IV: 2000 mg/kg bw of *S. myrtifolium* leaves extract
5. Group V: 4000 mg/kg bw of *S. myrtifolium* leaves extract

Mortality among experimental animals within 14 days post-exposure was determined. The deceased mice were then dissected to observe the effects of acute toxicity on their internal organs. The research protocol has been approved by the Ethical Committee for the Use of Experimental Animals, Faculty of Mathematics and Natural Sciences, Pakuan University (No.66/KEPHP-UNPAK/8-2019).

Calculation of LD_{50}

The LD50 value was calculated using the Thompson and Weil formula and the number of observed animal deaths in this study, as per the following equation:

$$\textbf{Log m = Log D + d (f + 1)}$$

Notes:

M: LD_{50} value
D: The lowest dose used
d: Log of the multiplier between dose concentrations
F: A value in the Weil table, determined by the specific mortality rate (r)

Results

Preparation of Simplicia and extract

The Simplicia has a characteristic greenish-red colour with a distinctive aroma and a slightly chewy taste. In the present study, 3500 g of fresh *S. myrtifolium* leaves provided a Simplicia powder yield of 27.14%. This result is consistent with previous research, which produced a 25% yield (Indriani *et al.*, 2020). Extraction of 800 g of *S. myrtifolium* leaves powder using 8 L ethanol 96% resulted in a thick extract of up to 339.58 g (42.45%).

Acclimatisation result

The CV of mouse weight was calculated, followed by acclimatization for seven days to allow for the adaptation of the mice to their new environment. Following acclimatisation, the CV of body weight was

recalculated. The CV value obtained is 10.176%, with average body weight as high as 30.56 g. CV value is considered homogeneous if it has a value <15% (Montgomery, 1991). The CV value provides a measure of the homogeneity of experimental animals based on body weight. It will also affect the quality of the data distribution: the smaller the CV values, the more homogeneous the data. After acclimatisation, mouse body weight increased, with no accompanying mortality, indicating that the mice had adapted to their environment.

Acute toxicity test results

This test uses the Thompson and Weil method. Qualitative data were obtained by observing whether mortality was present among the experimental animals in each treatment group, and quantitative data were obtained from the number of deaths in each group. Calculation of the LD_{50} value is based upon mortality among the experimental animals after 14 days of observation (Table I).

Table I: Number of deceased mice following 14 days of observation

Dose (mg/kg bw)	Number of mice	Number of deceased mice
500	5	2
1000	5	1
2000	5	2
4000	5	3

The results in Table I show two deaths in the 500 mg/kg bw group, one death in the 1000 mg/kg bw group, two deaths in the 2000 mg/kg bw group, and three deaths in the 4000 mg/kg bw group. The *r* values obtained from the number of deaths among mice given the ethanol extract of *S. myrtifolium* leaves were 2, 1, 2, and 3, respectively. Based on Thompson and Weil's LD_{50} calculation table, the *r* values of 2, 1, 2, and 3 have an *f* value of 1.0, which is then used to calculate the LD_{50}. The resulting LD_{50} value is 1,995 mg/kg bw, categorised as moderately toxic with a dosage ranging from 0.5 to 5 g/kg bw (Priyanto, 2010).

The administration of 96% ethanol extract of *S. myrtifolium* induced acute toxicity marked by mortality in experimental animals. Internal organ morphology was examined in the deceased mice (Figure 1), and toxic effects on the organs were demonstrated after administration of the thick extract. At doses of 500 mg/kg bw and 1000 mg/kg bw, white lesions on the lungs were observed along with a blackened liver, while doses of 2000 mg/kg bw and 4000 mg/kg bw caused almost all organs to swell and blacken alongside fluid accumulation in the abdomen and thorax.

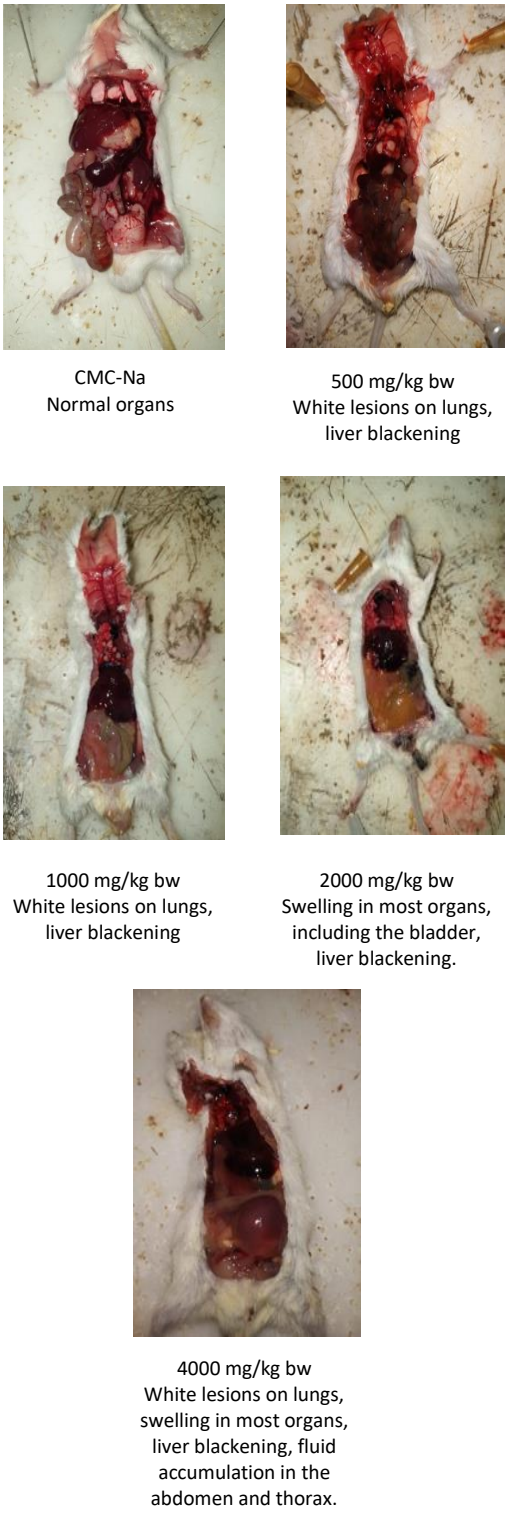

Figure 1: The effects of acute toxicity in internal organs of mice

Discussion

White lesions and swelling of the internal organs of mice are thought to be caused by necrotic damage to the tissue. Cells that experience necrosis can no longer revert to a healthy state and will proceed to die (Kumar *et al.*, 2007). The blackened liver observed in experimental mice is believed to be caused by disruption of the hepatocytes undergoing pycnosis. Damage to the lymph vessels causes fluid accumulation in the abdomen and thorax of mice. Lymph vessels play a role in the absorption of fluids and macromolecules from the tissues in the body and removing toxic substances after tissue damage occurs (Banks, 1993).

Toxic effects arise when absorbed toxins are transferred through the circulatory system to receptors. Oral administration of ethanol extract of red shoot leaves causes the active substances present in the red shoot leaves to be absorbed through the digestive tract, and these active substances then undergo distribution and metabolism (Katzung, 2002). Secondary metabolites of the red shoot leaves used in this study may be potentially responsible for the observed toxicity. These alkaloids and flavonoids act as stomach irritants and therefore cause digestive disturbance upon entering the body. According to Rita and the authors (2008), flavonoids are plant defence compounds that may be toxic. These compounds must first be isolated then tested to determine the toxicity of alkaloids. Saponin content in red shoot leaves is also believed to cause death and damage to the digestive organs of experimental animals. Saponins contain glycosides and can dissolve in water, thereby reducing activity within the digestive system. Decreased absorption will result in disruption of iron transport through mucosal cells. Red shoot leaves also contain tannin compounds known to cause toxic effects such as necrosis and bleeding. The higher the dose of extract administered, the greater the damage incurred to the organs of the experimental animal. In the present study, the 4000 mg/kg bw treatment group showed the most severe organ damage (Yunita, 2009).

Conclusion

The administration of ethanol extract of *S. myrtifolium* leaves showed a toxic effect and had an LD_{50} value of 1995 mg/kg bw and was categorised as moderately toxic. The toxic effect on the internal organs of mice is characterised by the presence of white lesions on the lungs, blackened liver, swelling of the organs, and fluid accumulation within the abdominal cavity and thorax.

Acknowledgement

Thank you to Lembaga Penelitian dan Pengabdian pada Masyarakat (LPPM) Pakuan University, who had supported this study.

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