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RESEARCH ARTICLE

SUBCHRONIC TOXICITY TEST OF ETHANOLIC BAY LEAVE EXTRACT ON LUNG HISTOPATHOLOGY OF FEMALE DDY MICE

(UJI TOKSISITAS SUBKRONIS EKSTRAK ETANOL DAUN SALAM TERHADAP HISTOPATOLOGI PARU MENCIT BETINA GALUR DDY)

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ABSTRAK

Bay leaf (*Syzygium polyanthum*) is widely used in Indonesia as a traditional medicine. Despite its many pharmacological benefits, prolonged use at high doses can lead to toxic effects. Therefore, its safety should be evaluated through toxicity testing. This study aimed to evaluate lung histopathological changes in female DDY mice following subchronic administration of ethanolic bay leaf extract. This experimental study employed a post-test only control group design. Twenty-eight female DDY mice were divided into four groups and treated for 28 days. One control group and three treatment groups receiving ethanolic bay leaf extract (EEDS) orally at doses of 250, 500, and 1000 mg/kg BW. Using Hematoxylin–Eosin (HE) staining, lung histopathology was examined semi-quantitatively based on the degree of inflammatory cell infiltration and alveolar septal damage under 400× magnification. The results of the study showed that subchronic administration of ethanolic bay leaf extract at all treatment doses did not produce significant differences in lung histopathology compared with the control group. The lung histopathological appearance across all groups was similar, with mild changes that remained within normal limits and did not indicate significant toxic effects. These findings may be attributed to the bioactive compounds present in bay leaves, including flavonoids, tannins, saponins, polyphenols, essential oils, and vitamin C, which exhibit antioxidant and anti-inflammatory activities. In conclusion, the administration of ethanolic bay leaf extract at doses of up to 1000 mg/kg BW during the subchronic period appears to be relatively safe for the lungs of female DDY mice.

Keywords: bay leaves, female mice, histopathology, lung, subchronic toxicity

ABSTRAK

Masyarakat Indonesia banyak menggunakan daun salam (Syzygium polyanthum) sebagai obat tradisional. Meskipun memiliki banyak manfaat farmakologis, penggunaan jangka panjang dan dosis tinggi dapat menyebabkan efek toksik. Oleh karena itu, evaluasi keamanan diperlukan melalui uji toksisitas. Tujuan penelitian ini adalah untuk mengidentifikasi perubahan histopatologi paru mencit betina galur DDY setelah diberikan ekstrak etanol daun salam secara subkronis. Studi eksperimental ini menggunakan desain post-test only control group. Selama 28 hari, 28 ekor mencit betina galur DDY dibagi menjadi 4 kelompok. Satu kelompok kontrol dan tiga kelompok perlakuan masing-masing diberi ekstrak etanol daun salam dengan dosis 250 mg/kgBB, 500 mg/kgBB, dan 1000 mg/kgBB secara oral. Dengan pewarnaan Hematoksilin–Eosin (HE), histopatologi paru diperiksa secara semi-kuantitatif berdasarkan tingkat infiltrasi sel radang dan destruksi septa alveolar pada perbesaran 400×. Hasil penelitian menunjukkan bahwa pemberian ekstrak etanol daun salam secara subkronis pada seluruh dosis perlakuan tidak menimbulkan perbedaan gambaran histopatologi paru yang bermakna dibandingkan dengan kelompok kontrol. Gambaran histopatologi paru pada seluruh kelompok relatif serupa, dengan perubahan ringan yang masih berada dalam batas normal dan tidak menunjukkan efek toksik yang signifikan. Tidak ditemukannya perubahan bermakna diduga berkaitan dengan kandungan senyawa bioaktif daun salam, seperti flavonoid, tanin, saponin, polifenol, minyak atsiri, dan vitamin C, yang memiliki aktivitas antioksidan dan antiinflamasi. Dapat disimpulkan bahwa pemberian ekstrak etanol daun salam hingga dosis 1000 mg/kgBB selama periode subkronis relatif aman terhadap paru-paru mencit betina galur DDY.

Kata kunci: daun salam, histopatologi, mencit betina, paru, toksisitas subkronis

INTRODUCTION

The use of herbal plants as traditional medicine remains an important part of public health practices in Indonesia.[1] One of the most widely utilized plants is the bay leaves (*Syzygium polyanthum*), which has been traditionally been used as antihypertensive, antidiabetic, antihyperlipidemic, and anti-inflammatory agents.[2,3] Its bioactive compounds such as flavonoids, alkaloids, saponins, tannins, and essential oils contributes to these various pharmacological activities.[4,5,] However, despite its therapeutic benefits, prolonged use at high doses may cause toxic effects that are not yet fully understood.[6,7] The evaluation of the safety of an herbal ingredient is an important step before it can be widely recommended for use.[6] Toxicity tests, including subchronic toxicity tests, are necessary to assess the effects of repeated exposure over a specific period of time on target organs.[8,9] Subchronic exposure can lead to the accumulation of active substances or metabolites that may trigger oxidative stress, inflammation, and tissue damage.[10] Therefore, toxicity studies should not only focus on acute effects but also evaluate histopathological changes that may occur due to repeated exposure.[8]

Most previous studies on the toxicity of bay leaf extract have primarily assessed liver and kidney function parameters, as these organs are the primary sites of detoxification.[11,12] However, the lungs are also a vital organ susceptible to systemic toxic effects, particularly through oxidative stress and inflammatory mechanisms.[13] Because the lungs have a large alveolar surface area and a thin epithelium, they are particularly sensitive to disturbances in oxidative balance.16 Damage to lung tissue can be characterized by the infiltration of inflammatory cells, thickening of the alveolar septa, congestion, and even destruction of the alveolar structure.[14,15]

Female DDY mice were chosen as the experimental model due to their relatively stable genetic characteristics and their frequent use in toxicology research. [16] Additionally, the selection of female mice was based on hormonal sensitivity and biological responses to chemical exposure. Histopathological examination using Hematoxylin-Eosin (HE) staining enables the evaluation of microscopic changes in lung tissue , particularly in the alveoli, which are the main functional units responsible for gas exchange. [14]

Based on this background, this study aimed to evaluate the histopathological changes in the lungs of female DDY mice after subchronic administration of ethanolic bay leave extract . This research is expected to provide scientific data on the safety of bay leave extract, particularly regarding its potential toxic effects on lung tissue, thereby providing a scientific basis for its rational and safe use . Scientifically, this study contributes to the understanding of herbal toxicology, particularly its effects on the lungs, which remain relatively under-researched compared to the liver and kidneys. Practically, the findings may serve as a reference for the development of safer phytopharmaceuticals and support the principles of evidence-based herbal medicine.

MATERIALS AND METHODS

This laboratory experimental study employed a post-test-only control group design using a Completely Randomized Design (CRD to evaluate changes in lung histopathology following subchronic administration of the ethanolic extract of bay leaves (*Syzygium polyanthum*) in female DDY mice. [8,9,12]

The experimental animals used were female DDY mice (*Mus musculus*), aged 2–3 months and weighing 20–30 grams, obtained from the Animal Laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi.[16,17] The inclusion criteria included healthy, active mice that had not previously received any drug treatment. Mice that died or became ill

during the adaptation period, as well as those that experienced significant weight loss before the start of the treatment were excluded from the study. Before treatment, all mice underwent a 7-day acclimatization period in a controlled environment with a temperature of 22 ± 3°C, humidity of 30–70%, and a 12-hour light/12-hour dark light cycle. Standard pellet feed and water were provided *ad libitum*.[16]

The sample size was determined using the Federer formula $(n - 1)(t - 1) \geq 15$, with a total of four groups, resulting in a minimum sample size of six mice per group. To compensate for potential dropouts, the initial sample size of 24 mice was increased to 28 mice.[8] The mice were randomly divided into four groups: a negative control group that received only standard feed and drinking water, and three treatment groups that received ethanolic bay leave extract of (EEDS) at doses of 250, 500, and 1000 mg/kg BW, respectively.[8,12]

The extract was administered orally using a gastric tube once daily for 28 consecutive days, at the same time each day.[8] During the treatment period, the mice were observed for changes in general condition, including activity, behavior, and body, with body weight measured every seven days.[16]

Bay leaves were obtained from the Manoko Experimental Plantation, Lembang, and selected based on the following criteria: fresh, green, free from pest infestation, and free from physical damage. The leaves were then dried and ground into powder.[3,4] a total of 300 g of dried simplicia was extracted using 96% ethanol by the maceration method for five days at room temperature in a closed container. The filtrate was then evaporated using a rotary evaporator at 50°C until a thick greenish-brown extract was obtained.[4]

On day 29, the mice were anesthetized and euthanized by cervical dislocation in accordance with ethical guidelines for animal research. The lungs were harvested and fixed in 10% formalin. Histopathological specimen preparation included dehydration through a graded ethanol series (70%, 80%, 90%, 96%, and absolute ethanol), clearing with xylene, paraffin embedding, sectioning using a microtome, and staining with Hematoxylin-Eosin (HE), which stains cell nuclei bluish-purple and the cytoplasm pink to orange.[14]

The histological sections were observed using a slide scanner at 400× magnification, with five randomly selected fields of view. The assessment was conducted semi-quantitatively to evaluate the infiltration of inflammatory cells and/or destruction of the alveolar septum using a scoring system: score 0 (no damage), score 1 (damage <1–33% of the field of view), score 2 (damage 34–66%), and score 3 (damage 67–100%) (Table 1). The

histopathological findings were confirmed by a specialist in Anatomical Pathology and analyzed descriptively by comparing the degree of tissue damage among the experimental groups. [15]

This study received ethical approval from the Research Ethics Committee under approval number 021/UH2.08/2025 and was conducted in accordance with the 3R principles (Replacement, Reduction, Refinement) and the Four Freedoms of Animal Welfare.[18] The study was conducted at the Animal Laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi, with an experimental treatment period of 28 consecutive days.[8,19]

RESULTS AND DISCUSSION

This study aimed to evaluate the subchronic toxic effects of ethanolic bay leave extract (*Syzygium polyanthum*) on the lung histopathology of female DDY mice following administration of graded doses for 28 days. The observed parameters included inflammatory cell infiltration and alveolar septal destruction, which were assessed using a semi-quantitative scoring system across five fields of view at 400× magnification.[14,15]

The histopathological examination, as presented in Table 1, showed that the alveolar structure remained generally intact in all groups. In the control group, mild inflammatory cell infiltration characterized a small number of lymphocytes, and mild destruction of the alveolar septum involving less than 33% of the alveolar tissue area were observed, thus falling into the category of score 1.[18] A similar histopathological appearance was also observed in the treatment groups receiving ethanolic bay leaf extract at doses of 250 , 500 , and 1000 mg/kg BW. All groups demonstrated the same average histopathological score of 1, with no increase in the degree of tissue damage as the extract dose increased. The absence of a dose-dependent increase in histopathological changes indicate that the administration of ethanolic bay leave extract at doses up to 1000 mg/kg BW for 28 days did not produce significant subchronic toxic effects on the lung tissue of female DDY mice. In toxicological evaluation, the presence of a dose-response relationship is an important indicator of toxic effects.[8,9] Therefore, the lack of an increase in histopathological scores at higher doses further supports the finding that the extract did not induce damage to the lung organs under the conditions of this study.

Mild lymphocyte infiltration was observed in all groups, including the control group, and remained within the range of a physiological immune response as shown in Figures 1-4. The presence of a small

number of lymphocytes in lung tissue may represent a nonspecific response to environmental factors, mild stress during animal maintenance, or an adaptive psychological process.[13] Similarly, the mild alveolar septal destruction observed in the control group indicates that these changes were unlikely due to extract exposure, but rather reflected mild histological variations or technical factors associated with tissue preparation process. [14]

Biologically, the findings of this may be attributed to the presence of bioactive compounds in bay leaves, such as flavonoids, tannins, polyphenols, and vitamin C, which possess antioxidant and anti-inflammatory properties.[4,5] Flavonoids are known to inhibit the Reactive Oxygen Species (ROS) formation and suppress the production of inflammatory mediators such as TNF-α, IL-6, and COX-2.10,19 This activity plays a role in maintaining the integrity of alveolar structure and protecting against oxidative stress that can trigger lung tissue damage. These mechanisms can explain why no significant histopathological changes were observed, despite the administration of the extract at a broad dose range during the subchronic period.

The results of this study indicate that lung tissue was able to maintain homeostasis despite 28 days of exposure to ethanolic bay leave extract . Thus, based on the observed histopathological parameters, extract at doses ranging from 250 mg/kgBW to 1000 mg/kgBW did not produced significant subchronic toxicity effects on the lungs of female DDY mice. However, further research involving longer treatment durations and the evaluation of additional parameters, such as oxidative stress biomarkers, is required to provide a more comprehensive safety profile.[20]

Table 1 Percentage of histopathological changes and scores of lung tissue in female DDY mice treated with EEDS

Group	EEDS Dose (mg/kg BW)	Histopathological Change (%)	Score Category	Mean Score
K(-)	Control	<33%	1	1
K(1)	250	<33%	1	1
K(2)	500	<33%	1	1
K(3)	1000?	<33%	1	1

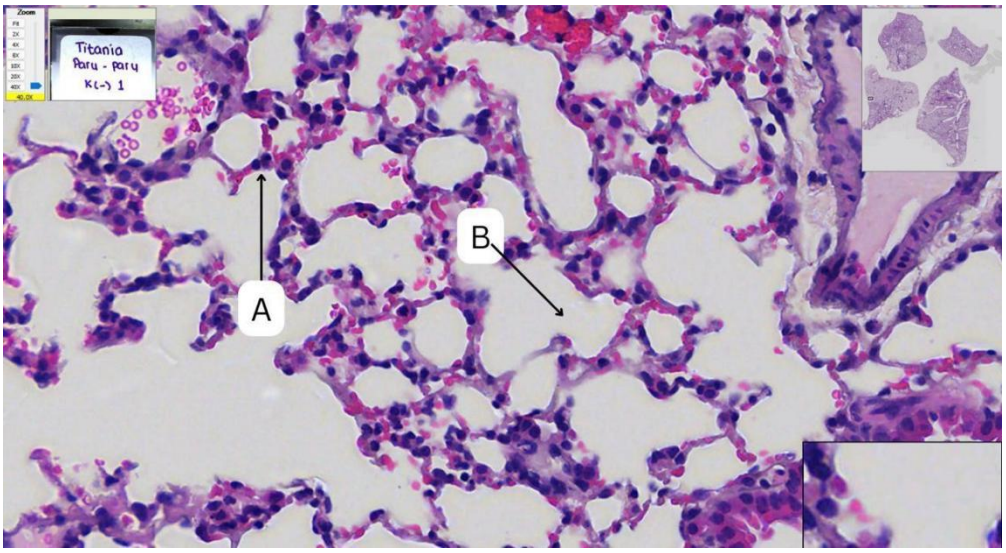

Figure 1 Inflammatory cell infiltration part of the alveolar septum (A) and alveolar septal destruction (B) in the control group.

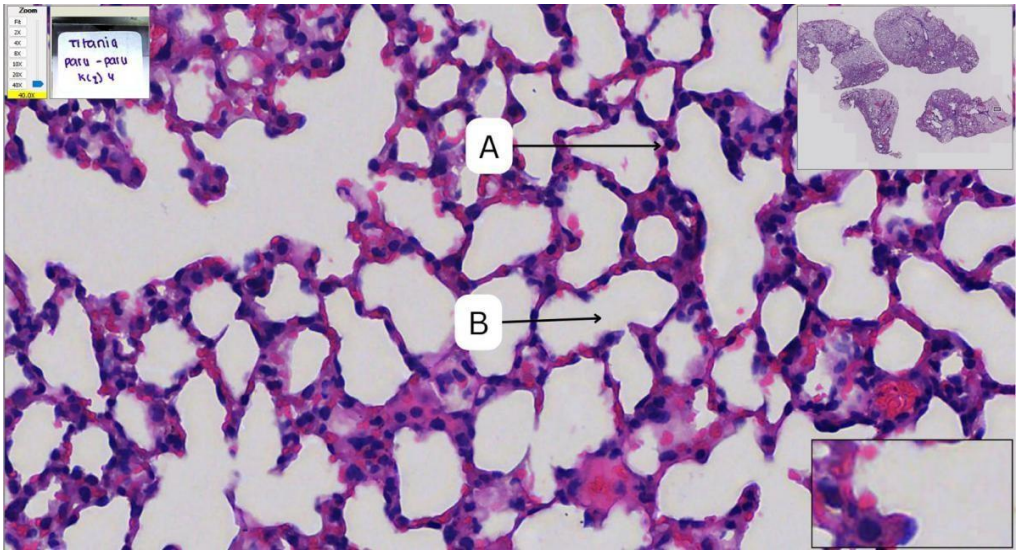

Figure 2 Inflammatory cell infiltration part of the alveolar septum (A) and alveolar septal destruction (B) in the treatment group receiving 250 mg/kg BW (P1).

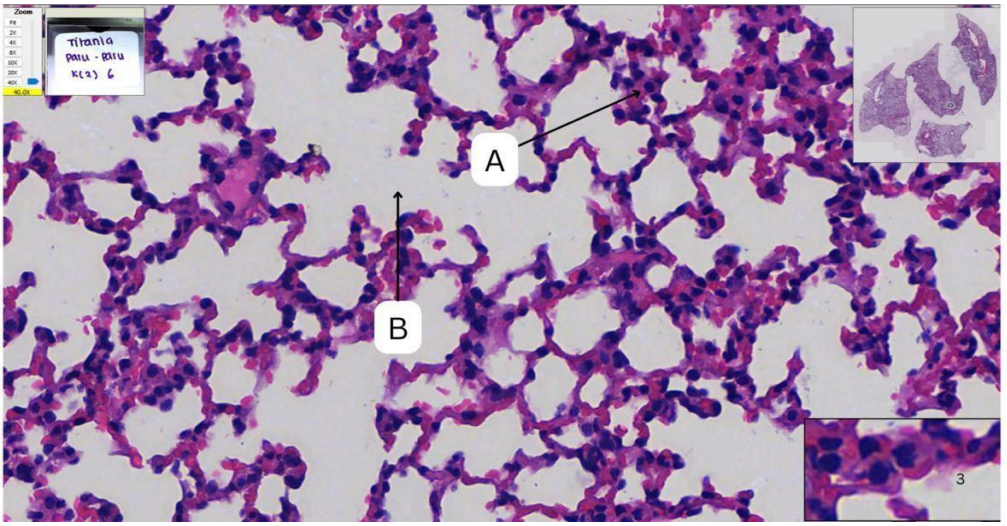

Figure 3 Inflammatory cell infiltration part of the alveolar septum (A) and alveolar septal destruction (B) in the treatment group receiving 500 mg/kg BW (P2).

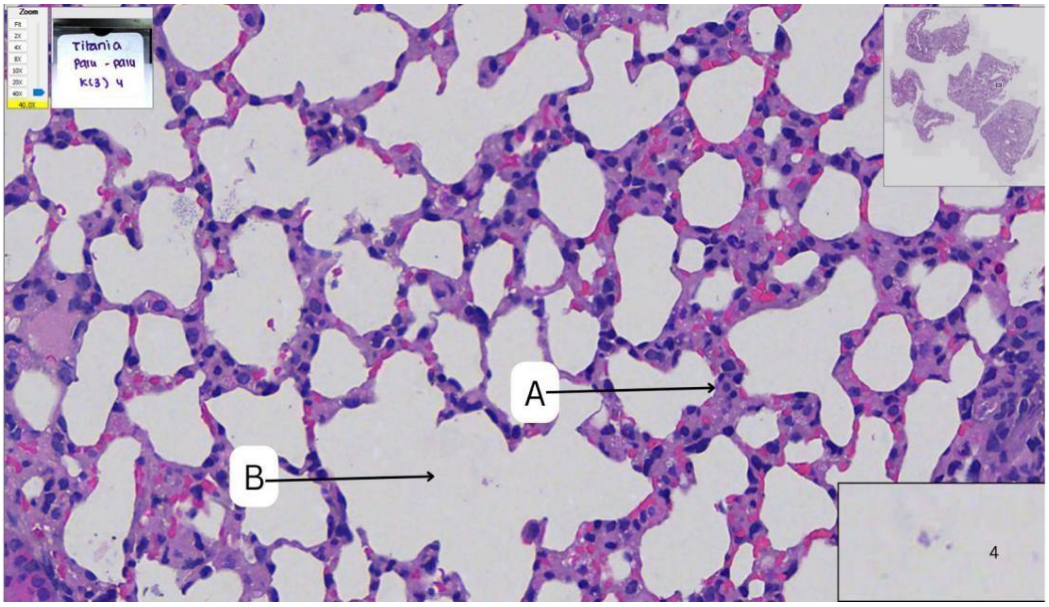

Figure 4 Inflammatory cell infiltration part of the alveolar septum (A) and alveolar septal destruction (B) in the treatment group receiving 1000 mg/kg BW (P3).

CONCLUSION

According to the findings of this study, the administration of ethanolic bay leaves extract (*Syzygium polyanthum*) at doses of250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW for 28 days did not cause significant histopathological changes in the lungs of female DDY mice. All groups, including the control group, exhibited mild lymphocytic infiltration and

mild alveolar septal destruction with a percentage change of less than 33% (score 1), with no evidence of dose-response relationship. These findings indicate that, under the conditions of this study, ethanolic bay leaves extract demonstrated a good safety profile for the lungs following 28 days of subchronical administration. However, further research involving longer exposure periods, higher doses, and the evaluations of biochemical and molecular parameters are still required to establish a more comprehensive safety profile.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this study.

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