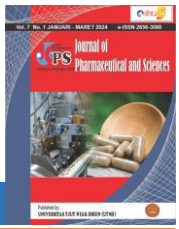
Journal of Pharmaceutical and Sciences
Electronic ISSN: 2656-3088DOI:https://doi.org/10.36490/journal-jps.com
Homepage: https://journal-jps.com
ORIGINAL ARTICLE JPS. 2026, 9(1), 98-106



# Protective effect ethanol extract of B*idens pilosa* L. on the immune system based on histological spleen of *Rattus norvegicus* alcohol-induced

# Efek protektif ekstrak etanol *Bidens pilosa* L. terhadap sistem imun tubuh berdasarkan histologis limpa *Rattus norvegicus* yang diinduksi alkohol

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**Abstract**

This study aims to determine the protective activity of *Bidens pilosa* L. leaf ethanol extract (EEDK) on the immune system based on the white pulp diameter, and histopathology of the spleen of white rats given alcohol. The study was conducted experimentally using a completely randomized design (CRD), with treatments divided into 5 groups: negative control (K-) without EEDK or alcohol administration, positive control (K+) administered 10 ml/kg body weight of alcohol, treatment 1 (P1) administered 250 mg/kg body weight of EEDK and 10 ml/kg body weight of alcohol, treatment 2 (P2) given 500 mg/kgbb EEDK and 10 ml/kgbb alcohol, and treatment 3 (P3) given 750 mg/kgbb EEDK and 10 ml/kgbb alcohol. EEDK was administered 1 hour after alcohol administration and was given orally every day for 43 days. The parameters observed in this study were diameter of the white pulp, and histopathology of the white rat spleen. The observation data were analyzed using one-way ANOVA and followed by a post hoc test with the DMRT test to see the differences between treatments. The results showed that the ethanol extract *Bidens pilosa* L. potential as an immunomodulator by increasing the diameter of the white pulp of the spleen, and had a significant effect on spleen histology by showing protective activity with a decrease in the level of spleen tissue damage due to alcohol, particularly tissue fibrosis, lymphocyte apoptosis, and necrosis ($p \leq 0.05$).

***Keywords*: Bidens pilosa L., immune syatem, white pulp diameter, spleen histopathology, alcohol.**

**Abstrak**

Penelitian ini bertujuan untuk menentukan aktivitas protektif ekstrak etanol daun *Bidens pilosa* L. (EEDK) terhadap sistem kekebalan tubuh berdasarkan diameter pulp putih, dan histopatologi limpa tikus putih yang diberi alkohol. Penelitian ini dilakukan secara eksperimental dengan desain acak lengkap (CRD), dengan perlakuan dibagi menjadi 5 kelompok: kelompok kontrol negatif (K-) tanpa pemberian EEDK atau alkohol, kelompok kontrol positif (K+) yang diberikan 10 ml/kg berat badan alkohol, perlakuan 1 (P1) diberikan 250 mg/kg berat badan EEDK dan 10 ml/kg berat badan alkohol, perlakuan 2 (P2) diberikan 500 mg/kg berat badan EEDK dan 10 ml/kg berat badan alkohol, dan perlakuan 3 (P3) diberikan 750 mg/kg berat badan EEDK dan 10 ml/kg berat badan alkohol. EEDK diberikan 1 jam setelah pemberian alkohol dan diberikan secara oral setiap hari selama 43 hari. Parameter yang diamati dalam studi ini adalah diameter pulp putih dan histopatologi limpa tikus putih. Data pengamatan dianalisis menggunakan uji ANOVA satu arah dan dilanjutkan dengan uji post hoc menggunakan uji DMRT untuk melihat perbedaan antara perlakuan. Hasil menunjukkan bahwa ekstrak etanol daun ketul (*Bidens pilosa* L.) berpotensi sebagai imunomodulator dengan meningkatkan diameter pulp putih limpa, dan memiliki efek signifikan pada histologi limpa dengan menunjukkan aktivitas protektif melalui penurunan tingkat kerusakan jaringan limpa akibat alkohol, terutama fibrosis jaringan, apoptosis limfosit, dan nekrosis ($p \leq 0,05$).

***Kata Kunci*: Bidens pilosa L., sistem imun, diameter pulpa putih, histopatologi limpa, alkohol.**

https://doi.org/10.36490/journal-jps.com

**Article History:**

Received: 13/11/2025,
Revised: 13/01/2026
Accepted: 13/01/2026,
Available Online : 20/01/2026.

**QR access this Article**

## Introduction

Alcohol is addictive and is a psychoactive substance that causes acute and chronic changes to the body's systems. Alcohol abuse can cause various functional disorders in human organs. Alcohol abuse is a growing problem worldwide. The number of deaths due to alcohol consumption worldwide reaches 3 million cases per year, or 5.3% of deaths caused by tuberculosis, HIV/AIDS, and diabetes [1]. Based on data from the Central Statistics Agency (BPS) in 2023, alcohol consumption among people aged 15 years and above in the last year in urban and rural areas reached 0.33 liters per capita in 2022. Indonesia itself is still at the lowest point of alcohol abuse, but it must be understood that the alcohol problem in Indonesia is very complex.

Alcohol is toxic to humans and has a negative impact on the spleen. Alcohol affects the immune system by altering the production of molecules that function as signals (i.e., cytokines) for coordinating the body's defenses. The result of this effect is a weakened immune system that is vulnerable to bacterial infections, such as tuberculosis or pneumonia. Acute chronic alcohol consumption increases cytokine production, causing excessive inflammation in the body.

The immune system is the body's mechanism for protecting it self from various harmful foreign substances [2]. An imbalanced immune system response can lead to autoimmune conditions, chronic inflammation, cancer, chronic kidney disease, cirrhosis, and neurodegenerative disorders. These diseases have a high prevalence, cause disability, and have high mortality rates worldwide [3].

The spleen is a lymphoid organ that plays an important role in the immune system and is the largest lymphoid organ [2]. The spleen contains white pulp, which functions as a site for antibody production and the maturation of T and B lymphocytes and macrophages. T cells and B cells are the main effectors in the adaptive immune system and are found throughout the spleen, with B cells being the producers of antibodies [4]. The response to alcohol is associated with abnormal white and red pulp morphology of the spleen, abnormal spleen cell ratio, a decrease in the count of splenocytes, abnormal physiological function of the spleen, increased apoptosis of splenocytes, and reduced proliferation of splenocytes. Alcohol also causes the loss of immune cells, alters the normal ratio of different immune cells and the distribution of systemic immune cells. Alcohol and its metabolite acetaldehyde also affect antigen presentation, T and B cell-mediated immune responses, cytolytic activity, NK, and the production of various cytokines and chemokines, resulting in local and systemic dysfunction of immune function. Das et al,. reported that ethanol causes oxidative stress in the spleen due to the formation of superoxide radicals and can result in organ damage [5].

Immune system activity is associated with the presence of bioactive compounds in herbal plants in the management of symptoms or regulation of immune responses and fewer side effects. As one of the plants used in traditional medicine, *Bidens pilosa* L. has been used to treat various symptoms and diseases. *Bidens pilosa* L., also known as Ketul or Ajeran (Central Java), has many local names, one of which is Halosi (Samosir, North Sumatra). The ketul plant (*Bidens pilosa* L.) belongs to the Asteraceae family, which is widespread in Indonesia and has been utilized in ethnobotany. For example, the people of Samosir, North Sumatra, use the leaves of the halosi/ketul plant as traditional medicine [6].

Based on Bartolome *et al.*, polyenes, flavonoids, phenylpropanoids, fatty acids, and phenolics are the main bioactive compounds of B. pilosa, and are reported to be effective in the treatment of tumors, inflammation/immune modulation, diabetes, viruses, microbes, protozoa, gastrointestinal diseases, hypertension, and cardiovascular diseases [7]. The most abundant compound found in the Ketul plant is flavonoids. Mtenga and Ripanda, reported the flavonoid content of *Bidens pilosa* L. showed great potential as an immunomodulator. *Bidens pilosa* L. contains chemical compounds such as glycosides, alkaloids, tannins,

terpenoids, coumarins, flavonoids, polysaccharides, and lignans, which are known to have immunomodulatory activity for HIV patients [8].

In a toxicity study of *Bidens pilosa* L. administered to test animals at daily doses of 0%, 0.5%, 2.5%, 5%, and 10% for 24 weeks, the test animals were found to be healthy, and an increase in body weight was observed over time. Histological examination of the vital organs of the test animals, namely the heart, liver, lungs, kidneys, brain, and reproductive organs, revealed that administration of different doses of *Bidens pilosa* L. had no toxic effects on any of these vital organs. Furthermore, in terms of survival rate and genotoxicity studies, all test animals in each group that received daily doses of 0%–10% *Bidens pilosa* L. had a 100% survival rate during the 24-week treatment period [9]. According to Ezeonwumelu et al. [10], the administration of *Bidens pilosa* L. ethanol extract at doses of 200 and 400 mg/kgBW was more effective than the 800 mg/kgBW dose, but the analgesic activity of *Bidens pilosa* L. extract was statistically confirmed to be the same, where the analgesic activity was associated with the presence of flavonoid compounds from *Bidens pilosa* L. that were able to reduce pain due to inflammation. *Bidens pilosa* is a plant with no side effects [9].

Therefore, this study is important to test the efficacy of ethanol extract of ketul leaves (*Bidens pilosa* L.) on the immune system by observing the histology of the spleen, namely the diameter of the white pulp, tissue fibrosis, lymphocyte apoptosis, and lymphocyte necrosis, by observing changes in the histopathology of the spleen of white rats (*Rattus norvegicus*) induced by alcohol.

## Experimental Section

### Plant identification and extract preparation

Ketul leaves (*Bidens pilosa* L.) collected from Sabulan Village, Sitiotio District, Samosir Regency, North Sumatra. A total of 7 kg of ketul leaves were used, consisting of dark green and light green leaves. Next, the drying process was carried out indoors, away from sunlight, to prevent damage to the chemical content of the dried material [11]. After the ketul leaves were dry, they were blended and filtered. The powdered simplisia was then extracted using the maceration method with 96% ethanol solvent, where the simplisia is placed in a glass jar with a weight of 250 grams each. Then ethanol is added with a ratio of 1:10 between the simplisia and the solvent (for example, 500 grams of simplisia with 5 liters of ethanol). The maceration process was carried out for 4 days, with stirring performed every day. The maceration solution was then filtered using filter paper. The liquid extract obtained was then concentrated (extracted) using a rotary vacuum evaporator at a temperature of 30-40°C to produce a thick extract.

**Figure 1.** *Bidens pilosa L.* was found by author on Sabulan villages.

### Animal and experimental design

**Tabel 1.** Experimental treatments

| Group | Treatments |
|---|---|
| K- | Rats given 0,5% CMC, not given EEDK and alcohol |
| K+ | Rats given 30% alcohol at a dose of 10 ml/kgBW |
| P1 | Rats given EEDK 250 mg/kgBW 1 hour after administration of 30 % alcohol |
| P2 | Rats given EEDK 500 mg/kgBW 1 hour after administration of 30 % alcohol |
| P3 | Rats given EEDK 750 mg/kgBW 1 hour after administration of 30 % alcohol |



Electronic ISSN :2656-3088
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The experimental animals used in this study were 25 male Wistar rats (*Rattus norvegicus*) obtained from the Pharmacy Laboratory of the University of North Sumatra, aged 2-3 months and weighing 150-200 grams. This study was an experimental study using a completely randomized design (CRD), consisting of 5 groups, with 5 rats in each group (replication). The alcohol ($C_2H_5OH$) used in this study had a concentration of 30%. To determine the alcohol dosage, a dose of 10 ml/kgbw was converted to the body weight of the rats. Alcohol and ethanol extract of ketul leaves were administered orally every day for 43 days.

**Measurement of white pulp diameter**

The diameter of the white pulp of the spleen was observed using hematoxylin and eosin (HE) staining and measurement of white pulp diameter using a light microscope with 100x magnification. One spleen tissue slide was prepared for each rat, so that each treatment group had five spleen tissue slides. The preparations were observed in five (5) fields of view per spleen tissue slide, then the white pulp was measured by adding the maximum diameter of the horizontal axis to the maximum diameter of the vertical axis and then dividing by two (2). The diameter of the white pulp of the preparation was measured and expressed in μm [12,13].

**Spleen damage score**

Histological slides stained with hematoxylin eosin (HE). Observations were made on 5 fields of view around the red pulp and white pulp of the spleen using a light microscope with 400x magnification. The observations included three indicators, namely tissue fibrosis, lymphocyte apoptosis, and necrosis. The assessment was performed using a semi-quantitative scoring system based on the distribution pattern of the lesions, namely focal, multifocal, and diffuse. A score of 0 indicated no lesions or a normal category in the spleen tissue, a score of 1 (focal) indicated mild lesions, limited to one or several small areas, a score of 2 (multifocal) indicated moderate lesions, found in several separate areas, and a score of 3 (diffuse) indicated severe lesions, spreading throughout almost the entire spleen tissue. Observations were made using 5 fields of view per spleen tissue slide, and the final score used was the total average score of spleen tissue lesions from the five fields of view observed [14].

**Data Analysis**

The data obtained from the research results, namely the diameter of the white pulp of the spleen and the level of histological damage to the spleen, were tabulated and subjected to normality tests and parametric homogeneity tests (one-way ANOVA). The ANOVA results showed significant differences ($p \leq 0.05$), so the data analysis was continued with the DMRT (Duncan Multiple Range Test). If the data were not normally distributed or homogeneous, the analysis was continued with a nonparametric statistical test, namely the Kruskal Wallis test with a 95% confidence level, followed by the Mann-Whitney test. Data analysis was processed using SPSS 26 (Statistical Product and Service Solution) software.

## Results and Discussion

**Table 2.** Mean diameter white pulp of spleen

| Treatments Group | Diameter of white pulp (μm) ± SD |
|---|---|
| K- | 709,40 ± 169,50$^{ab}$ |
| K+ | 618,60 ± 110,73$^{a}$ |
| P1 | 977,40 ± 183,11$^{bc}$ |
| P2 | 1090,60 ± 92,52$^{c}$ |
| P3 | 817,40 ± 378,73$^{ab}$ |

Description : Each value represents the mean ± standard deviation (n=5). Different superscripts in the same column indicate significant differences between treatments ($p \leq 0.05$)

Table 2 shows that the mean diameter of white pulp in the spleen in all treatment groups showed a significant difference ($p \leq 0.05$). Based on the results of spleen histology observations, there were differences in the diameter of the white pulp of the spleen in all treatment groups. Based on statistical results, the highest average enlargement of the diameter of the white pulp of the spleen was in group P2, which was significantly different from groups K-, K+, P1, P2, and P3. However, K+ was the group with the lowest average diameter of


Electronic ISSN :2656-3088
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white pulp of the spleen among all groups. This indicates that exposure to 30% alcohol in the positive control group (K+) significantly affected the diameter of the white pulp of the spleen.

Groups P1 and P2 showed an increase in the diameter of the white pulp of the spleen, while group P3 showed a decrease in the diameter of the white pulp of the spleen that was not much different from K-, and this value showed a significant difference. This indicates that administration of EEDK to white rats (Rattus norvegicus) at a dose of 250 mg/kgBW (P1) and a dose of 500 mg/kgBW (P2) was able to increase the enlargement of the white pulp diameter of the spleen, and a dose of 750 mg/kgBW (P3) was able to decrease the size of the white pulp diameter of the spleen.

### Spleen Histopathology

**Table 3.** Mean histological damage score of the spleen

| Perlakuan | Skor fibrosis ± SD | Skor lymphocyte apoptosis ± SD | Skor necrosis ± SD |
|---|---|---|---|
| K- | 4,00 ± 1,58$^{a}$ | 7,00 ± 2,54$^{a}$ | 2,20 ± 0,83$^{a}$ |
| K+ | 13,20 ± 0,83$^{c}$ | 13,80 ± 1,09$^{d}$ | 13,00 ± 1,58$^{d}$ |
| P1 | 11, 40 ± 1,14$^{bc}$ | 12,60 ± 1,34$^{cd}$ | 11,00 ± 2,34$^{c}$ |
| P2 | 7,40 ± 1,14$^{ab}$ | 10,40 ± 1,14$^{b}$ | 10,40 ± 1,67$^{c}$ |
| P3 | 5,60 ± 2,40$^{a}$ | 9,20 ± 1,92$^{ab}$ | 7,20 ± 1,30$^{b}$ |

Description : Each value represents the mean ± standard deviation (n=5). Different superscripts in the same column indicate significant differences between treatments ($p \leq 0.05$).

The results of the study showed that the level of splenocyte damage in all treatment groups showed significant differences ($p \leq 0.05$). The evaluation of the protective activity of ketul leaf ethanol extract (EEDK) was based on histopathological observations of the spleen tissue of white rats (*Rattus norvegicus*). There were three main parameters, namely fibrosis, lymphocyte apoptosis, and necrosis. These three parameters showed the level of damage that occurred in spleen tissue treated with 30% alcohol. Based on Table 3, the highest splenocyte damage score was found in the positive control group (K+), which was only given 30% alcohol at 10 ml/kgBW. This indicates that alcohol administration in the K+ group can cause splenocyte damage and tissue changes such as necrosis, lymphocyte apoptosis, and tissue fibrosis. The group with the lowest average value was group P3, which was given 10 ml/kgBW of 30% alcohol and 750 mg/kgBW of EEDK. This value indicates that the administration of a high dose of EEDK was able to reduce histological damage to the spleen tissue to a level close to the normal value in group K-.

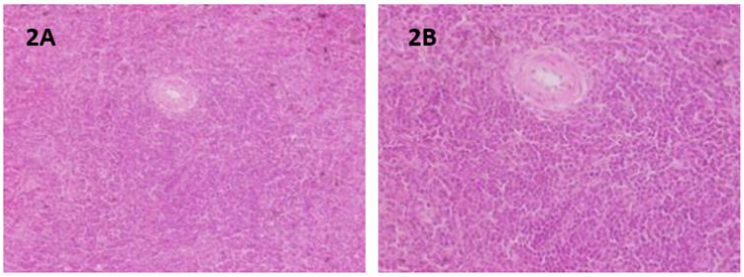


**Figure 2.** Normal splenic tissue on K- groups. Figure 2a are low magnification (200x, HE), figure 2b is a higher magnification (400x, HE).

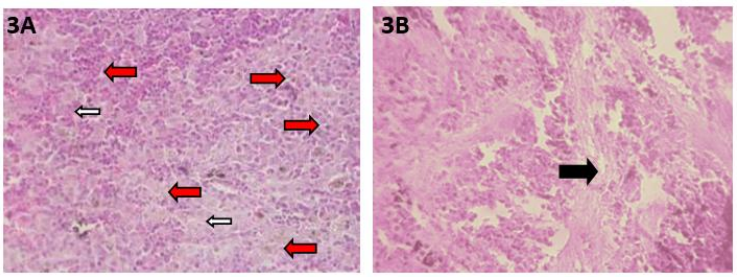


**Figure 3**. Splenic tissue K+ (alcohol groups). (a) diffuse and severe of necrosis lyphocytes (red arrows), and multifocal of apoptotic cells (white arrows). (b) there is diffuse and savere of fibrosis splenic tissue (black arrows). In higher magnification (400x, HE).


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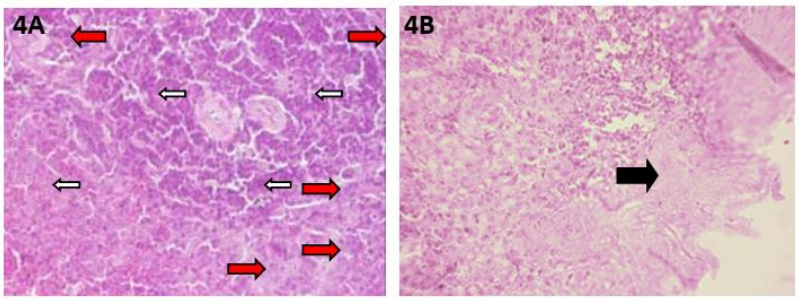


**Figure 4.** Spleen histology P1 gruops. (a) multifocal and savere of necrosis lymphocytes cells (red arrows), and multifocal of apoptotic cells (white arrows). (b) diffuse and savere of fibrosis splenic tissue (black arrows). In higher magnification (400x, HE).

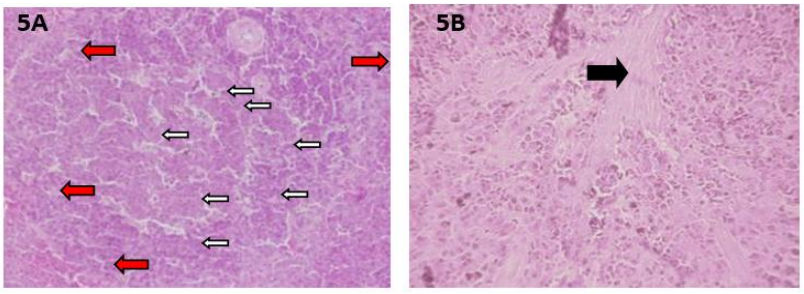


**Figure 5.** Spleen histology P2 gruops. (a) multifocal and moderate of necrosis lymphocytes cells (red arrows), and multifocal of apoptotic cells (white arrows). (b) focal and mild of fibrosis splenic tissue (black arrows). In higher magnification (400x, HE).

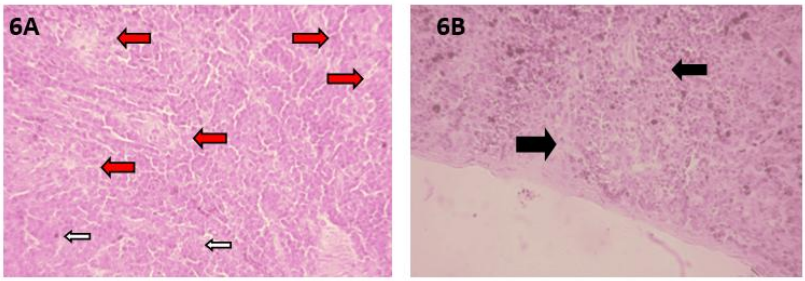


**Figure 6**. Spleen histology P3 gruops. (a) multifocal and decreased of necrosis lymphocytes cells (red arrows), and multifocal of apoptotic cells (white arrows). (b) focal and mild of fibrosis splenic tissue (black arrows). In higher magnification (400x, HE).

## Discussion

### Diameter white pulp spleen

The treatment with EEDK at a dose of 250 mg/kgBW (P1) and 500 mg/kgBW (P2) resulted in an increase in the diameter of the white pulp of the spleen compared to the K- and K+ groups, indicating that EEDK can enhance immune system activity. This finding is in line with the research by Makiyah and Zahra [15], which states that mice induced with a 50% dose of alcohol showed a decrease in white pulp diameter compared to the treatment group. The increase in white pulp diameter of the spleen indicates the presence of compounds that have the potential to act as immunomodulators.

Immunomodulatory compounds are compounds that can boost immunity or the body's defense system [16]. According to Rodríguez-Mesa *et al*., *Bidens pilosa* L. contains various bioactive compounds, in the flavonoid and polyenes groups, such as centaureidin, centaurein, and luteolin [3]. These compounds are known to have anti-allergic, antioxidant, antiproliferative, immunosuppressive, and anti-inflammatory properties. The increase in white pulp is due to the presence of flavonoids containing EGCG (quercetin and epigallocatechin 3-gallate), which can boost the immune system through the mechanism of increasing IL-6 and IL-10, thereby affecting the proliferation of T cells and B cells [17]. Flavonoids as immunomodulators can increase the proliferation index of lymphocytes and splenocytes, thereby increasing the diameter of white pulp, which is composed of lymphocytes [18].


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The treatment group administered EEDK at a dose of 750 mg/kgBW (P3) had the lowest mean white pulp diameter among the two EEDK doses, indicating that high-dose EEDK has been proven to reduce the white pulp diameter of the spleen. Statistical test results showed that the P3 treatment group had an average diameter that was not significantly different from the normal control group (K-). This effect is very likely due to the flavonoid content in ketul, which has an optimal level to suppress lymphocyte proliferation in the white pulp of the spleen, thereby acting as an optimal immunosuppressive agent. This finding is in line with Makiyah and Wardhani [15], who stated that flavonoids can reduce the diameter of the spleen pulp in mice. Flavonoids, as natural antioxidants, can act as immunosuppressants by capturing free radicals and suppressing the production of proinflammatory cytokines. The mechanism of action includes inhibition of enzymes that regulate the inflammatory response, reduction of arachidonic acid, and suppression of cytokine transcription, especially IL-12, which results in decreased immune cell activity and antibody production. These findings indicate that a dose of 750 mg/kg bw EEDK can reduce immune cell activity, as indicated by a decrease in the diameter of the white pulp of the spleen. The presence of various compounds contained in EEDK, such as flavonoids, allows EEDK to act as an immunomodulator. EEDK can not only enhance the immune system but also suppress it when its activity is excessive.

**Spleen Histopathology**

Alcohol is associated with sulfur amino acid metabolism disorders that cause hyperhomocysteinemia due to ethanol. High doses of alcohol cause DNA damage and disrupt the DNA repair system. This induces apoptosis [5]. The metabolic effects of alcohol are caused by acetaldehyde and may also be associated with changes in redox status. Ethanol increases the rate of free radical formation, decreases antioxidant levels, and potentiates oxidative stress.

An imbalance between the production of Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), and antioxidant defenses causes oxidative stress, resulting in cellular dysfunction and tissue damage. In the pathogenesis of fibrosis, oxidative stress plays a crucial role by triggering inflammation through increased cytokine production, enhancing myofibroblast differentiation and fibrogenesis, and as a result of DNA damage and p53 activation, ROS promotes apoptosis and these changes cause the development of fibrosis.

Ketul (*Bidens pilosa* L.) produces various bioactive compounds, some of which have been identified as porphyrin, tannin, aliphatic, terpenoid, alkaloid, phenylpropanoid, cardiac glycoside, flavonoid, and aromatic [19]. Ketul (*Bidens pilosa* L.) can offer a solution because it has a high polyphenol (antioxidant) content. Antioxidants are agents that reduce Reactive Oxygen Species (ROS) levels and reduce oxidative stress and restore redox in biological systems [20].

Phenolic compounds are known to play an important role in inhibiting the inflammatory process. Phenols work by reducing the production of free radicals and inhibiting inflammatory signaling pathways, such as the Mitogen Activated Protein Kinase (MAPK) pathway. This pathway regulates various cellular responses, including proliferation, differentiation, and cell death. By inhibiting MAPK pathway activity, phenolic compounds can reduce the expression of pro-inflammatory cytokines and increase the production of anti-inflammatory cytokines [21]. *Bidens pilosa* stimulates IFN expression by increasing IFN-γ promoter activity. Centaurein regulates IFN-γ transcription through NFAT and NFκB located within the IFN-γ promoter in T cells [22].

In vivo studies have demonstrated the effectiveness of the antioxidant drug N-acetylcysteine (NAC) in maintaining vital lung function in patients with idiopathic pulmonary fibrosis (IPF) (Estornut *et al.*, 2022). In Antar *et al.*, [23] stated that Halofuginone, which is one of the antifibrotics found in bioactive plant compounds, namely alkaloids, is known that alkaloids from the Dichroa febrifuga plant have been used as a drug in patients with cutaneous GvHD, a condition characterized by fibrosis and skin contractures. The drug causes a decrease in skin integrity and a decrease in skin collagen. Based on this, the antioxidant compounds contained in Ketul (*Bidens pilosa* L.) have the potential as antifibrotics.

Flavonoids and polyphenols act as antioxidants that reduce oxidative stress. Excessive oxidative stress can trigger apoptosis through increased production of free radicals that damage cells. By reducing cellular oxidation, these compounds can prevent the activation of cell death pathways. Antioxidants are able to convert oxidants into harmless compounds and can prevent the formation of free radicals and repair damage caused by these free radicals.

Flavonoids as antioxidants (suppressing the number of free radicals) can capture free radicals by releasing hydrogen atoms from their hydroxyl groups, thereby stabilizing the free radicals. Stabilized free


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radicals do not damage lipids, proteins, and DNA, which are the targets of cell damage [24,25]. The anti-inflammatory function of flavonoids works by inhibiting inflammation. At high concentrations, flavonoids can inhibit the release of arachidonic acid and the secretion of lysosomal enzymes from membranes by blocking the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and phospholipase A2 (PLA2). while low concentrations only block the lipoxygenase pathway. Flavonoids also inhibit neutrophil degranulation, thereby inhibiting the release of cytokines, free radicals, and enzymes that play a role in inflammation [26].

In addition, a study by Kviecinski *et al.*, [27] stated that several compounds contained in *Bidens pilosa* have free radical scavenging activity, which provides benefits in preventing liver damage in rats induced by CCI4, by protecting the liver from injury by blocking CCI4-induced lipid peroxidation and reducing protein carbonylation and DNA fragmentation. This antioxidant and hepatoprotective activity is due to the presence of polyphenolic compounds, namely flavonoids and quercetin, contained in *Bidens pilosa*.

The results of this study indicate that ketul leaf ethanol extract (EEDK) shows potential as an immunomodulator, with immunosuppressive effects at a dose of 750 mg/kg body weight and immunostimulatory effects at doses of 250 mg/kg body weight and 500 mg/kg body weight based on the diameter of the white pulp of the spleen. Furthermore, EEDK has potential as a protective agent at a safe and effective dose of 750 mg/kg body weight. Among the three tested doses, the 750 mg/kg body weight dose of EEDK showed the most consistent protective effect against histopathological damage to the spleen, with results approaching normal conditions without causing permanent tissue damage.

## Conclusions

Based on the results of the study, the following conclusions can be drawn: Ketul leaf ethanol extract (EEDK) has significant immunomodulatory properties by increasing the diameter of the white pulp of the spleen due to alcohol at a dose of 500 mg/kgBW. It can also reduce the diameter of the white pulp of the spleen and show significant protective effects by reducing the level of histological damage to the spleen due to alcohol, both in terms of tissue fibrosis, lymphocyte apoptosis, and lymphocyte necrosis at a dose of 750 mg/kgBW.

## Conflict of Interest

The authors shall declare that there is no conflict of interest if it is true.

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