HYPNOTIC-SEDATIVE ACTIVITY TEST OF 70% ETHANOL EXTRACT OF LETTUCE (*Lactuca sativa* L.) IN MALE WHITE MICE SWISS WEBSTER STRAIN

Asman Sadino[1*], Hesti Renggana[1], Suwendar[2], Riza Apriani[3], Yuni Nurhandayani[1]

[1]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Garut, Jl. Jati No.42B, Tarogong Kaler, West Java, 44151, Indonesia
[2]Pharmacy Study Program, Faculty of Mathematics and Natural Sciences, Bandung Islamic University, Jl. Ranggagading No.8, Tamansari, Bandung Wetan, West Java, 40116, Indonesia
[3]Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Garut, Jl. Jati No.42B, Tarogong Kaler, West Java, 44151, Indonesia

*Corresponding author: Asman Sadino (asman@uniga.ac.id)

Abstract

Humans who use medicines for insomnia may experience a variety of adverse effects, including dependence. Traditional medicine frequently uses lettuce (Lactuca sativa L.) to treat various conditions, including sleep difficulties. Terpenoids, which are secondary metabolites found in lettuce plants, are to blame for this. This study aimed to see if an ethanolic extract of lettuce leaf had any sedative-hypnotic activity in Swiss Webster strain male white mice induced by phenobarbital at a dose of 90 mg/KgBB intraperitoneally. In this study, the post-test-only control strategy was employed. The animals were divided into six groups (n = 4): negative (aqua dest), positive (Na-CMC 0.5%), comparison (diazepam 1.3 mg/KgBB), and ethanolic extract of lettuce leaf at doses of 300 mg/KgBB, 400 mg/KgBB, and 500 mg/KgBB. The parameters observed in this study were a combination of parameters from previous studies, namely the number of falls, sleep onset, and sleep duration in mice. The data obtained were then processed statistically using the one-way ANOVA (analysis of variance) and post-hoc test follow-up analysis with the LSD (least significant difference) test. The results showed that the ethanolic extract of lettuce leaves at 300, 400, and 500 mg/KgBB had a sedative-hypnotic effect. Doses of 300 mg/KgBB only increased sleep duration. In contrast, 400 and 500 mg/KgBB increased the number of falls, rapid sleep onset, and increased sleep duration, significantly different from positive controls (p<0.05). The effective dose of ethanolic extract of lettuce leaf as a sedative-hypnotic was 400 mg/KgBB.

Keywords: insomnia, lettuce, hypnotic-sedative, traditional medicine

Introduction

Sleep is a need for everyone. Time spent sleeping allows the brain to recover from biochemical or physiological processes that gradually slow down while a person is

awake.[1] Certain individuals usually need less than six hours of sleep (short sleepers) and less than nine hours (long sleepers) per night to function properly.[2] Various conditions, including sleep time, greatly influence the health and performance of the human body. However, not a few individuals experience sleep deprivation or sleep disturbances (insomnia). This condition can cause decreased concentration, a decrease in the body's immune system, poor memory, and what most often occurs are emotional disorders.[3,4] Apart from that, insomnia can affect work safety, which is quite a considerable risk because it can cause accidents and workplace injuries.[5] Many variables, including stress, excessive anxiety, intake of alcoholic and caffeinated beverages, advanced age, medical conditions, and use of certain medications, cause individuals to experience insomnia. This insomnia generally occurs in older adults, women, and people who experience poor mental health.[6] Therefore, efforts are needed to overcome the problem of insomnia.

Many efforts have been made as a form of treatment for insomnia, one of which is using hypnotic-sedative drugs. However, continuous and irrational use of these drugs in high doses can cause dependence, tolerance effects, and withdrawal symptoms. The development of drugs derived from natural ingredients is directed at treating insomnia. A plant reported to have hypnotic-sedative activity is lettuce. A literature search found that the content of terpenoids such as lactucin, lacton, and lactucopicrin in lettuce leaves plays a role in hypnotic-sedative activity. This is supported by ethnomedicinal studies in Iran and Persia that prove the efficacy of lettuce (Lactuca sativa var. lettuce) and longifolia (Lactuca sativa var. Longifolia) as having significant activity as a sleep inducer. Based on the theory of plant relationships, it is stated that plant species in the same genus and family will contain the same chemical compounds. Still, the only difference is the intensity of the compounds in each species, so this is the basis for selecting lettuce plants in this research.[6]

Therefore, this research was carried out to analyze the hypnotic-sedative activity of the ethanol extract of lettuce leaves and identify the effective dose of lettuce leaves as a hypnotic-sedative in male white mice of the Swiss Webster strain.

Method

Equipment
The equipment used in this study were a stopwatch, rotary cage, oral probe, 1 mL syringe, analytical balance, mice storage container, glass funnel, dropping pipette, measuring cup, beaker, mortar, stamper, blender, macerator, evaporator, steam cup, gauze tripod, spirit burner, and test tube.

Material
The materials used in this research were lettuce leaves, diazepam, phenobarbital, aqua pro-injection, Na-CMC, distilled water, 70% ethanol, sulfuric acid, dragendorf's reagent, Mayer's reagent, magnesium powder, $FeCl_3$, steasny's reagent, ammonia 25%, chloroform, HCl 10%, amyl alcohol, gelatin solution, Na-acetate, 1N NaOH solution, benzene, ether, Na_2SO_4 and Lieberman Burchard reagent.

Procedure

Experimental Animals
The type of animal used in this study was Swiss Webster white mice with a body weight of 20-30 grams and 6-8 weeks old obtained from the Animal Laboratory, School of Pharmacy, Bandung Institute of Technology (ITB).

Material Preparation and Plant Determination

The plant samples in this study were lettuce obtained from the Cikajang area, Garut Regency, West Java. Furthermore, plant determination was carried out at the Bandungense Herbarium, School of Life Sciences and Technology, ITB, to ensure plant identity. After the determination, the lettuce leaves are processed to become simplicia through several processes, including wet sorting, washing, chopping, drying, dry sorting, storage, and grinding into powder.

Preparation of Lettuce Leaf Ethanol Extract

The ethanol extract of lettuce leaves was made by maceration, and the amount of simplicia used was 200 g. Then, the simplicia was macerated by adding 3 litres of 70% ethanol solvent three times a day, 24 hours, with occasional stirring. The first filtrate obtained was stored in a container, and the residue was macerated again with 1000 mL 70% ethanol two times for three times 24 hours, with occasional stirring, and then filtered using a cloth. Then, when all the filtrate has been collected, it is then concentrated using an evaporator to obtain a thick extract. Then, the thick extract obtained was dried over a water bath using a steam cup until a constant weight was obtained.[7]

Phytochemical Screening of Simplicia and Extracts

Phytochemical screening is carried out to detect secondary metabolite compounds in the extract, including alkaloids, flavonoids, saponins, quinones, tannins, and steroids/triterpenoids. Phytochemical screening is carried out using the Phytochemical Screening of Plants method.[8]

Preparation of Test Animals

The test animals used in this study were male white mice of the Swiss Wester strain. Before testing, mice were acclimatized for one week and given the same food and drink in each cage. According to visual observations, animals can be used for experiments if they are in good health after being cared for. During acclimatization, the test animals were weighed to determine their body weight, and their behaviour was observed to adapt to the environment to be used as an experiment. This study used test animals with a weight of 20-30 grams.

Testing the Sedative Hypnotic Effect of Ethanol Extract of Lettuce Leaves

There were 24 mice used in this study and then divided into six groups, each consisting of four mice. Before testing, test animals are not given food for 18-24 hours but are still given water. At the time of testing, all mice were weighed first to determine the body weight of the mice so that the volume of the preparation to be given could be determined. This test aims to assess the ability of the ethanol extract of lettuce leaves to extend the sleep duration of mice induced using phenobarbital and decrease the motor coordination ability in mice.[9]

The first group was the negative control group, which was given water. The second group was the positive control group, which was given 0.5% Na-CMC. The third group was the test group, which was given ethanol extract from lettuce leaves at 300 mg/KgBB. The fourth group was the test group, which was given 400 mg/KgBB of lettuce leaf ethanol extract. The fifth group was the test group, which was given 500 mg/KgBB of lettuce leaf ethanol extract. The sixth group, namely the comparison group, was given diazepam at a dose of 1.3 mg/KgBB. All preparations are given orally with an administration volume of 0.5 mL/KgBB. After being given the test solution and then allowed to stand for 30 minutes, after 30 minutes, all groups were given phenobarbital inducers at a dose of 90 mg/KgBB, which was injected intraperitoneally. Then, the mice were placed on the rotarod to observe the number of falls within 3 minutes at 1-minute

intervals. After that, the latency and duration of sleep in the mice were observed. Sleep latency was calculated when phenobarbital was administered until the righting reflex disappeared in mice. Meanwhile, sleep duration was calculated starting from the disappearance of the righting reflex until the righting reflex reappeared. In other words, the mice were in normal conditions again and then recorded as a test parameter.[9]

Data Analysis

The research data obtained are then processed statistically using SPSS software. The data processing was carried out using parametric statistics consisting of a normality test using the Shapiro Wilk and a homogeneity test with the Leven test. If the results of the two tests are typically distributed, then the ANOVA test is performed and followed by the LSD test to see further the differences between the control and test groups.

Result

The secondary metabolite screening results, number of falls, sleep onset, and sleep duration can be seen below:

Table 1. Results of Phytochemical Screening Examination of Simplicia Lettuce Leaves

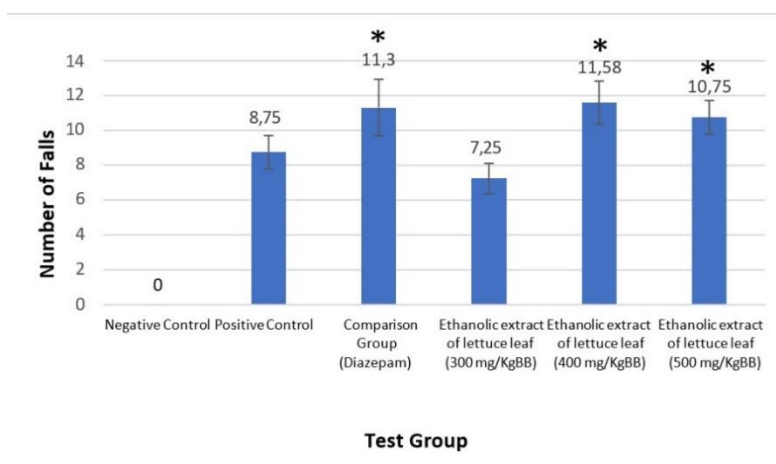| No.	Examination	Observation Result	
		Simplicia	Extract
1	Alkaloid	+	+
2	Flavonoids	+	+
3	Saponins	-	-
4	Tannin	+/Galat	+/Galat
5	Quinones	-	-
6	Phenol	+	+
7	Steroids/Triterpenoids	+	+

Description: (+) Detected; (-) Not detected

Figure 1. Bar chart of the average number of mice falling for 3 minutes
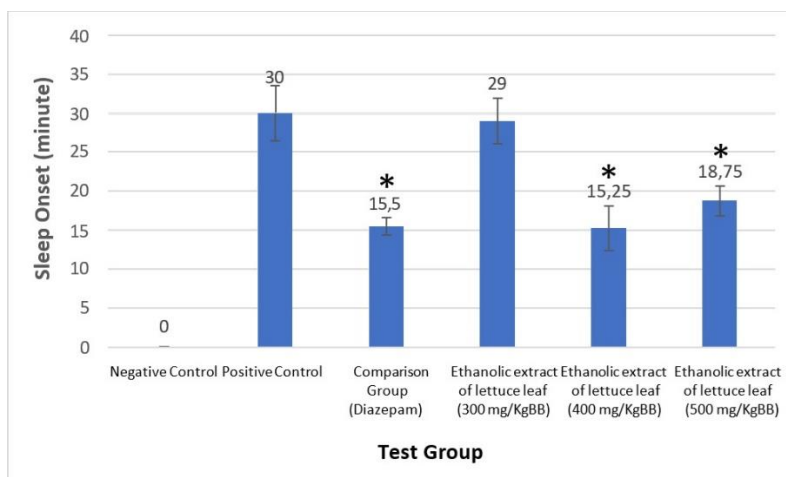Description: ($*$) = Significantly different from the positive control ($p<0.05$)

Figure 2. Bar chart of average sleep onset of mice
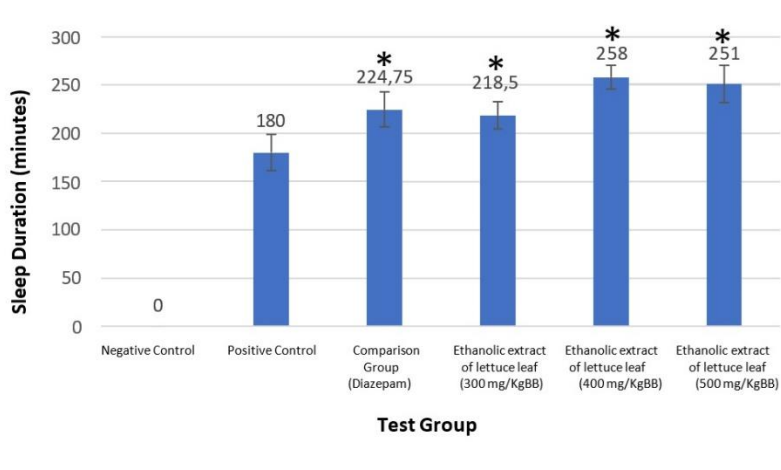Description: (*) = Significantly different from the positive control (p<0.05)

Figure 3. Bar chart of the average sleep duration of mice
Description: (*) = Significantly different from the positive control (p<0.05)

Discussion

The determination showed that the plant was lettuce with letter 2665/IT1.C11.2/TA.00/2022. Test plants are processed into dry simplicia through various processes, including collecting raw materials, wet sorting, washing, chopping, drying, dry sorting, storage, and grinding into powder.

The simplicia that was obtained was then macerated in 200 grams using 4 L of 70% ethanol solvent for 3×24 hours with repetition and filtration three times, producing a liquid extract. After obtaining the liquid extract, the liquid extract is then evaporated using a rotary vacuum evaporator and heated in a bath until a thick extract is obtained. From the extraction of 200 grams of simplicia from lettuce leaves, a viscous extract of 61.11 grams was obtained with a yield of 30.51%.

Phytochemical screening aims to analyze the secondary metabolite content found in lettuce leaves qualitatively. This phytochemical screening was carried out on simplicia powder and thick lettuce leaf extract. The phytochemical screening results in Table 1 showed that simplicia and ethanol extract of lettuce leaves contained secondary metabolite compounds in the form of alkaloids, flavonoids, tannins, phenols, and

steroids/triterpenoids. Phytochemical screening of simplicia powder and thick extract aims to determine whether the content of secondary metabolite compounds in simplicia powder and extract is the same or different. Apart from that, phytochemical screening of the thick extract aims to ensure that the extraction and concentration process does not damage the compounds in the simplicia.

Before the hypnotic-sedative activity testing process, the test animals were controlled first to minimize biological variations, including the weight of mice in the range of 20-30 grams, the same strain, namely Swiss Webster, gender, and the same treatment, drum conditions and feeding. The choice of male sex is because male mice do not have the hormone estrogen or if there is a relatively small amount. The hormonal condition of male mice is also more stable than that of female mice, which can experience hormonal changes at certain times and affect the psychological condition of the test animals.[10]

One way to test the hypnotic-sedative effect is by using the rotarod method. In the rotarod method, the parameters observed were the number of falls the mice fell for 3 minutes with a time interval of 1 minute. The rotarod is a tool used to view or assess the motor coordination of test animals.[11] In this study, phenobarbital at a dose of 90 mg/KgBW as an inducer was given 30 minutes after administration of the test preparation. After being induced, the mice were kept quiet for 5 minutes because the onset of action of phenobarbital given intraperitoneally was 5 minutes. After that, it was placed on the rotarod to count the number of falls. The effect of administering the test preparation can be seen from the motor coordination of mice with a decrease in their grip on the rotarod, which is indicated by an increase in the number of falls because the sedative-hypnotic effect can cause a reduction in the mice's gripping power as a sign of the relaxing effect of the test preparation so that the mice can fall off the rotarod.[11]

Diazepam can increase the number of falls because the mechanism of action of diazepam itself is that it works by binding to the asteric site on the $GABA_A$ receptor, which causes an increase in the frequency at which the chloride channel opens, which leads to an increase in the conductance of chloride ions thereby causing an anti-anxiolytic effect.[12] Meanwhile, phenobarbital works by increasing the number of drops. Time for the chloride channels to open, resulting in suppression of the central nervous system. This occurs because of $GABA_A$ receptor subunits, so when phenobarbital binds to this receptor, the chloride ion gate will remain open and allow ion flow into neuronal cells. This action causes hyperpolarization of the cell membrane, thereby increasing the action potential threshold and causing a relaxing effect on muscles, causing drowsiness and being anti-anxiolytic.[13,14] In Figure 1, the test group of ethanol extract of lettuce leaves doses of 400 and 500 mg/KgBB increased the number of falls in the test animals. When compared with the positive control, there was a statistically significant difference ($p < 0.05$) with a value of $p=0.005$ at a dose of 400 mg/KgBB and $p=0.034$ at a dose of 500 mg/KgBW. The statistical analysis results at a test dose of 300 mg/KgBB showed the average number of falls was not significantly different from the positive control ($p>0.05$) with a value of $p=0.120$. This could be caused by dose ranges that are too close together due to biological and physiological variations. Different mice, when given treatment, can affect the test results.

In testing the sedative-hypnotic activity using the sleep onset method, the mice were induced using phenobarbital to assess the onset of the mice after being given the test preparation. The onset of sleep in mice can be seen after administration of phenobarbital until the righting reflex disappears in mice. The righting reflex is the ability of the test animal to return to its original position. At the onset of sleep, the loss of the righting reflex is characterized by the inability of the mice to return to their normal position (standing on all fours) from a passive state when touched.[9]

The results in Figure 2 show that the negative control, which was only given aquadest, did not show any sedative-hypnotic effect in the form of sleep onset (time needed to start sleeping), meaning that the mice in the negative group remained active so that sleep onset was counted as 0 minutes. Then, in the comparison group given diazepam and phenobarbital, the onset required for the mice to fall asleep was 15.5 minutes. At the same time, in the positive control group given 0.5% Na-CMC and phenobarbital, the onset of sleep was 30 minutes. In the test group given ethanol extract of lettuce leaves at a dose of 300 mg/KgBB, the onset of sleep was 29 minutes, while the test dose of 400 mg/KgBB had a sleep onset of 15.25 minutes and was the fastest onset as was the comparison group. At the test dose of 500 mg/KgBB, the sleep onset of mice was 18.75 minutes, slightly slower than 400 mg/KgBB. In the test group, the ethanol extract of lettuce leaves at doses of 400 and 500 mg/KgBB was able to accelerate the onset of sleep. When compared with positive controls, there was a statistically significant difference ($p < 0.05$) with a p-value = 0.000 at 400 and 500 mg/KgBB doses. The 300 mg/KgBB test dose was not statistically significantly different from the positive control ($p > 0.05$) with p=0.614. A dose of 300 mg/KgBB could not accelerate the onset of sleep due to the dose range being too close, and the biological and physiological variations in different mice, when given treatment, could affect the test results.

Another parameter in testing hypnotic-sedative activity is sleep duration. Sleep duration is the time interval observed after the mouse loses the righting reflex until the righting reflex appears again in the mouse. The appearance of the righting reflex was indicated by the ability of the mice to return to their normal position (standing on all four legs).[15]

The results in Figure 3 show that the negative control group that was only given aquadest did not fall asleep or keep moving, so the duration of sleep in this group was calculated as 0 minutes. In the comparison group given diazepam and phenobarbital, the sleep duration of the mice was 224.75 minutes. In contrast, in the positive control given 0.5% Na-CMC and phenobarbital, the sleep duration was 180 minutes. Then, in the group given the ethanol extract of lettuce leaves at a dose of 300 mg/KgBB, the sleep duration was 218.5 minutes, the test dose was 400 mg/KgBB 258 minutes, and the test dose was 500 mg/KgBB 251 minutes. From the statistical analysis results, the positive control group was significantly different from the comparison group ($p<0.05$) with a value of p=0.002. The lettuce leaf ethanol extract test group at doses of 300, 400, and 500 mg/KgBB was able to extend the duration of sleep in mice because it was significantly different from the positive control ($p<0.05$) with a value of p=0.006 for the test dose of 300 mg/KgBW, p =0.000 for test doses of 400 and 500 mg/KgBB.

Conclusion

Based on the results of the sedative-hypnotic activity test that has been carried out, it can be concluded that the ethanol extract of lettuce leaves at doses of 400 and 500 mg/KgBB has sedative-hypnotic activity, which is characterized by a large number of mice falling on the rotarod, a short onset of sleep in mice and an extended sleep duration. Sleep duration in different mice was significant to the positive control ($p<0.05$). The effective dose of ethanol extract from lettuce leaves, which can have a sedative-hypnotic effect, is 400 mg/KgBB.

Acknowledgement

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