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Research Article

Comparative Study Of In-Vitro Toxicity Of Pure Honey And Fermented Honey Using The BSLT (Brine Shrimp Lethality Test) Method

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ABSTRACT

Honey is an alternative product used in traditional medicine because of its many benefits, honey is a sweet liquid made by bees using flower nectar. The purposed of this study was to determine the phytochemical screening and the potential for acute toxicity of pure honey and fermented honey to Artemia salina larvae using the BSLT method (Brine Shrimp Lethality Test) as indicated by the LC50 value. This experimental study used 540 Artemia salina larvae which were divided into 1 negative control, 1 positive control and 7 groups of pure honey and fermented honey concentration series, each consisting of 10 larvae with replication 3 times for each treatment. The concentrations of pure honey and fermented honey were 1000, 750, 500, 100, 50, 10, 1 ppm, respectively. The LC50 value was determined using a probit analysis after the percentage of fatalities findings were obtained. Pure honey yielded a toxicity of 59.75 µg/mL. While the fermented honey obtained an LC50 value of 3.28 µg/mL, which means the LC50 value was 30 ppm, indicating that the fermented honey sample was included in the very toxic category. Toxicity test on pure honey showed the LC50 value was in the toxic range. In the toxicity test, the fermented honey showed an LC50 value of 30 ppm, which means that the fermented honey has a very toxic toxicity value. There are differences in LC50 values in the second sample. However, statistically, there is no significant difference between pure honey and fermented honey

Keywords: *BSLT, fermented honey, pure honey, toxicity*

INTRODUCTION

Free radicals are molecules with unpaired electrons that can exist on their own. Free radicals attempt to interact with other molecules in order to create pairs of electrons when there are unpaired electrons present. Important macromolecules are attacked by free radicals, resulting in cell death and homeostasis disturbance (Lobo et al., 2010). The body's defensive system, which takes the form of antioxidants, is required throughout to counteract the impacts of free radicals. By attaching to free radicals, antioxidants are substances that can stop oxidation events in the body. By giving one

of the electrons they possess to free radicals, antioxidants are necessary to prevent an imbalance in the quantity of free radicals in the body (Werdhasari, 2014).

Honey is a sweet liquid produced by bees utilizing floral nectar and is one of the alternative items used in traditional medicine in Indonesia. The monosaccharides glucose and fructose, which are present in honey, give it its sweet flavor. Due to the involvement of yeasts from the genus Saccharomyces, which are resistant to high sugar concentrations, pure honey has a low water content and a high sugar content. When the water content in honey is high, it will make honey easily ferment (Purnomo et al., 2018). Acidity in honey can be utilized as a sign of a fermentation process that can convert sugar into alcohol and carbon dioxide, and acetic acid produced by the oxidation of alcohol can be reduced sugar levels and increase acidity in honey (Biologi, 2017). Both enzymes and non-enzymatic substances are found in honey. Examples of enzymes found in honey include catalase, glucose oxidase, and peroxidase. Organic acids, amino acids, carotenoids, and hundreds of polyphenolic chemicals, including flavonoids and phenolic acids, make up honey's non-enzymatic composition. Due to its composition, honey has significant anti-oxidant qualities and may lower the risk of cancer, inflammation, and compromised physiological systems (Saputri DS & Putri YE, 2017).

Both fermented honey and pure honey were utilized as samples in this investigation. By doing screening tests for anticancer chemicals in the form of assessing toxicity tests using the Brine Shrimp Lethality Test (BSLT) technique, it is possible to identify phenolic compounds in pure honey and fermented honey that can function as anticancer candidates. In this test for toxicity, samples of fermented honey and pure honey were used to assess the harmful effects of the two samples and determine which sample had the greatest toxicity value. The researcher wishes to highlight the difference in toxicity between pure honey and fermented honey using the Brine Shrimp Lethality Test (BSLT) technique, despite the fact that research on the comparison between pure honey and fermented honey is still uncommon (Abubakar, 2012).

To determine the toxicity level, value of LC50 is counted through probit analysis. The potency of pure honey and fermented honey were determined by comparing the LC50 value which is less or equal to 1,000 ppm. This recent study aimed to observe whether the pure honey and fermented honey produced toxicity on larvae of Artemia salina by using method of BSLT.

MATERIAL AND METHODS

Tools and Materials

The equipment includes a test tube rack, vortex, bottle, fermentation container, set of egg incubators, volume pipette, dropper pipette, analytical balance, spatula, stirring rod, and a UV-VIS spectrophotometer. The ingredients used are honey, lanang garlic, 80% methanol, ascorbic acid, aquades, 1% DMSO, NaCl, Artemia salina larvae.

Preparation of Fermented Honey Samples

Put pure honey and garlic lanang in a pot. Put the honey in an airtight container. To peel the garlic. Crushed garlic is added to honey. Bottle must be properly closed. For 1-3 days, incubate or leave standing (Jacqueline, 2021).

Producing 500 PPM of both pure and fermented honey

Weigh the samples of fermented honey and pure honey separately by up to 25 mg. Combine 50 cc of methanol with honey to dissolve it, and then homogenize. Replication should be done on each sample.

Alkaloid test

On a porcelain plate, the 2 mL test extract solution was evaporated until a residue was formed. Following that, the residue was dissolved in 5 mL of 2 N HCl, and the resulting solution was added to three test tubes. The first was left empty and 2 N HCl was added to it. The second tube received three drops of Dragendorff's reagent, while the third tube received three drops of Mayer's reagent. In addition, the alkaloids were observed in the second and third tubes during the development of an orange precipitate and a white to yellowish precipitate, respectively (Harborne, 1987).

Steroid/terpenes Test

Aqueous extracts underwent testing using the Liebermann-Burchard reagent. The concentrated sulfuric acid is then squeezed out after first adding a chloroform solution that has been dissolved in acetic anhydride to the solution. A shift in hue from orange to purple denotes the presence of terpenoids. Last but not least, if it becomes blue, steroids are present (Harborne, 1987).

Saponin Test

All four techniques required putting 2 mL of the sample into a test tube, adding 10 mL of distilled water, shaking for 30 seconds, and then evaluating the findings of the saponin assay. When a thick foam forms in less than 30 seconds, saponins are present (Harborne, 1987).

Phenol/tannin Test

To identify phenol and tannin components, the test extract solution was reacted with a 10% solution of iron (III) chloride if the color was dark blue, blue-black, or greenish-black (Harborne, 1987).

Preparation of Artemia salina Larvae

Weigh the Artemia salina eggs to a maximum of 2 grams. 500 ml of seawater or NaCl should be ready. Place the eggs in a seawater-filled container. Set the Artemia salina oxygen machine's aerator to on. Wait until the eggs hatch and develop into larvae, which takes around 48 hours.

Preparation of 1% DMSO Solution

1 gram of 1% DMSO should be weighed and then dissolved in 100 cc of distilled water (Waghulde et al., 2019).

Preparation of Concentration of Pure Honey and Fermented Honey Samples

Weigh the samples of fermented honey and pure honey to a maximum of 25 mg each. Homogenize the honey after dissolving it in up to 25 ml of 1% DMSO to get a 1000 ppm concentration. Take 5 ml, and then pour it onto a petri dish. Pipette 18.75 ml from a dilution of 1000 ppm to a concentration of 750 ppm, transfer to a 25 ml volumetric flask, and then re-dilute at this concentration. Finally, add 1% DMSO to the limit mark. Take 5 ml, and then pour it onto a petri

dish. Pipette 16.66 ml from a 750 ppm dilution to a 25 ml volumetric flask at a concentration of 500 ppm. Add 1% DMSO. Pipette 16.66 ml from a dilution of 750 ppm to a 25 ml volumetric flask at a concentration of 500 ppm. Add 1% DMSO to the limit mark. Take 5 ml, and then pour it onto a petri dish. Pipette 5 ml from a 500 ppm dilution to a 100 ppm concentration, transfer it to a 25 ml volumetric flask, and then re-dilute at that concentration. Finally, add 1% DMSO to the limit mark. Take 5 ml, and then pour it onto a petri dish. Pipette 12.5 ml from a 100-fold dilution at a concentration of 50 ppm, transfer it to a 25 ml volumetric flask, and then add 1% DMSO to the limit mark. Transfer 5 ml to a petri plate after taking it. Pipette 5 ml from a dilution of 100 transferred to a 25 ml volumetric flask at a concentration of 10 ppm, and then add 1% DMSO to the mark. Transfer 5 ml to a petri plate after taking it. Pipette 2.5 ml of the 10 ppm dilution into a 25 ml volumetric flask to perform a 1 ppm dilution. Take 5 ml from the mark and transfer it to a petri plate along with 1% DMSO.

Preparation of A Control Solution

In the petri plate used as the negative control, add 5 ml of 1% DMSO. 5 cc of ethanol should be added to the positive control's petri plate.

Toxicity Testing

The Brine Shrimp Lethality Test, also known as shrimp larvae (A. salina Leach) used in toxicology testing on animals (BSLT). The number of shrimp larvae that die after being exposed to a test substance for 24 hours indicates how toxic it is. To determine the concentration at which up to 50% of the treated shrimp larvae perished, the extract solution was created in a variety of concentrations. If the extract's LC50 value is less than 1000 g/ml, it is considered to be active, toxic, or potentially harmful to A. salina. This experimental study used 540 Artemia salina larvae which were divided into 1 negative control, 1 positive control and 7 groups of pure honey and fermented honey concentration series, each consisting of 10 larvae with replication 3 times for each treatment. The concentrations of pure honey and fermented honey were 1000, 750, 500, 100, 50, 10, 1 ppm, respectively. Check each petri dish for the quantity of live and dead larvae (Waghulde et al., 2019). Artemia salina larval deaths after 24 hours are counted, the LC50 value is calculated using Microsoft Excel, and the difference between the two samples is tested using the SPSS 25 program.

DATA ANALYSIS

The proportion of Artemia salina larval mortality at each concentration of pure honey and fermented honey was determined by analysis of the toxicity test results and it can be computed using the following formula:

$$\textbf{\% Mortality} = \frac{\boldsymbol{Death\ Larvae\ Total}}{\boldsymbol{Larva\ Total}} \textbf{ x 100\%}$$

The LC50 value was determined using a probit analysis after the percentage of fatalities findings were obtained. Microsoft Excel was used to conduct this analysis (Septiana E and Simanjuntak P, 2017). The results of toxicity in fermented honey and pure honey were obtained. With the SPSS 25 program, a separate test will be used to compare the data collected. The potential of variations between samples or groups of honey that have not gone through the fermentation process and groups of honey that have been investigated using various assays. The independent t-test was the statistical analysis technique that was applied. The purpose of the study was to compare

toxicity of fermented and unfermented honey, and the test was used to assess these differences. The data utilized were unpaired data (Tyastirin E. & Hidayati I., 2017).

RESULTS AND DISCUSSION

In this study, samples of both fermented and pure forest honey underwent qualitative analysis using phytochemical screening. Fermented forest honey is honey that has been soaked in lanang garlic for three to twelve days. It is thought that a successful fermentation process is one that is characterized by gas bubbles on the surface of the honey. This is because gas bubbles on the surface of honey are a sign that the fermentation process using garlic is working because they appear during the honey fermentation process. A preliminary test to find secondary metabolites in pure forest honey and forest honey fermented with lanang garlic is the phytochemical screening. The assays used in phytochemical screening include those for alkaloids, flavonoids, tannins, steroids, and saponins. The result are shown in the table 1 below.

Table 1. Results of the phytochemical analysis of fermented forest honey and pure forest honey

No	Phytochemical Screening	Samples	
		Pure honey	Fermented Honey
1	Alkaloid	+	+
2	Flavonoids	+	+
3	Tannin	+	+
4	Steroids	+	+
5	Saponin	+	+

In Handayani's research from 2018, samples of forest honey that had undergone phytochemical testing yielded good results for alkaloids, flavonoids, tannins, saponins, and steroids, with an IC50 value of 2826,471 ug/mL. The findings of the phytochemical screening test on lanang garlic in the research by Poernomo and Ma'ruf (2020) reported positive results containing active components, namely flavonoids, alkaloids, phenolics, and tannins. The information found in lanang garlic is consistent with earlier studies.

In the toxicity test, samples of both fermented and pure honey were diluted with 1% DMSO solvent by varying concentrations numerous times before the BSLT (Brine Shrimp Lethality Test) test was conducted. The BSLT test involves a 24-hour incubation period, after which the sample's larvae were seen to die. The percentage of larval mortality is used to calculate the probit value, which is then displayed in the probit table. By computing the standard curve of the connection between the concentration log and the probit value, the LC50 value was determined. The standard curve for the correlation between concentration logs and probit is as follows:

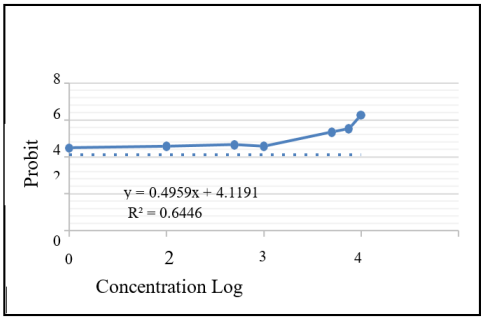

Figure 1. Concentration Log and Probit Relationship curve of Pure Honey

The standard curve in Figure 1 yields the equation y = ax + b, where y = 0.4959x + 4.1191. The LC50 value is calculated from this equation by entering the value 5 in the straight-line equation, the value of y is then calculated, and finally the value of x. The value of 5 here represents the probability of 50% of the experimental animal deaths. The curve above is obtained from data processing through the Microsoft Excel 16 application. The antilog X value was used to get the LC50 value. The pure honey sample's LC50 value was 59.75 µg/mL.

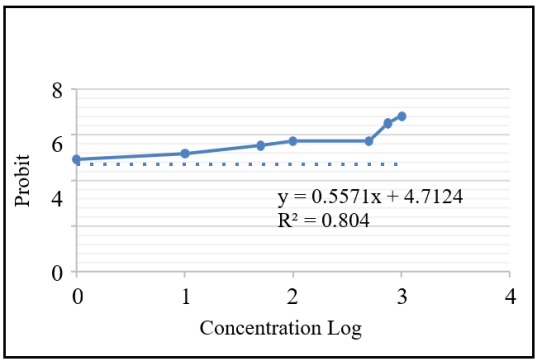

Figure 2. Concentration Log and Probit Relationship curve of Fermented Honey

Figure 2 shows the standard curve with the line equation y = ax + b where y = 0.5571x + 4.7124. The curve above is obtained from data processing through the Microsoft Excel 16 application, from this equation, the LC50 value is calculated by entering the value 5 in the y value and then calculating the x value. The LC50 value is obtained from the antilog x value. The LC50 value obtained from the fermented honey sample was 3.28 µg/mL.

Based on the LC50 value that has been obtained in each sample, it can be categorized as follows:

Table 2. LC50 Result of Pure Honey and Fermented Honey

No.	Sample	LC50	Category
1	Pure Honey	59,75	Toxic
2	Fermented Honey	3,28	Very Toxic

According to Martiningsih (2013), the pure honey sample had a toxicity result of 59.75 based on Table 2's findings. This implies that pure honey has an LC50 value between 30 and 1000 ppm, indicating that it falls within the poisonous category. While the LC50 result for the sample of fermented honey was 3.28, this indicates that the LC50 value was less than 30 ppm, placing the sample in the highly dangerous category. It may be concluded that there is no significant difference between the toxicity data for Pure Honey and Fermented Honey in the Mann-Whitney test, with Sig. 0.317 > 0.05.

A natural product called honey, which is high in flavonoids, phenolics, tocopherols, peroxides, oxidized glucose, and other phytochemical substances, offers various health advantages (Dinar SS, and Yolli EP, 2017). Forest honey (Apis Dorsata), the type of honey utilized in this study, includes several essential minerals, vitamins, and enzymes for the body (Wulandari, 2017). Minerals including salt, calcium, and phosphorus were present. Vitamins including thiamin (B1), riboflavin (B2), pyridoxine (B6), ascorbic acid (C), and folic acid were also present. The enzymes diastase, peroxidase, and glucose oxidase are all present in forest honey. Compared to regular honey, forest honey is deeper in hue asserts that black honey has more antioxidants than light honey (Wulansari, 2018). Polyphenols are one of the antioxidants found in forest honey. By combining wild honey with lanang garlic, samples of fermented honey were produced. Crushing or chopping the lanang

garlic is done before adding it to the honey. After that, seal the honey jar and let the fermentation take place for around 1-3 days. The appearance of bubbles that form in the honey jar, which can be observed immediately, indicates that fermentation has been successful. Lanang garlic is utilized as a component in fermentation because it is a form of onion that has long been popular among people as a source of therapeutic substances. Since lanang garlic only has one clove, it is also sometimes referred to as "single garlic." This onion variety's exceptionally pungent aroma, in contrast to other varieties, may indicate that it contains more nutrients than other varieties of onions (Dewi et al., 2021). Lanang garlic has several pharmacological properties, including anticancer, antioxidant, antibacterial, antifungal, hypoglycemic, and hypolipidemic, according to (Song, 2001) in (Dewi, et al., 2021). A toxicology test is one that is performed to determine if a drug or molecule is poisonous. The information gathered is used to assess the test preparation's degree of safety in the event that humans are exposed to it or consume it (Rumaseuw SE, et al., 2014). The Artemia salina shrimp larvae are employed as experimental animals in the BSLT (Brine Shrimp Lethality Test), which is the procedure used to test for toxicity. 24 hours were then spent incubating. The number of larval fatalities during incubation after 24 hours is the metric used in the BSLT test. Because Artemia salina larvae are extremely sensitive to their surroundings, this species is used (Ningdyah, 2015).

The goal of this toxicity research is to compare the toxicity values of fermented honey with pure honey using the LC50 value. The 10% DMSO (Dimethyl sulfoxide) solvent employed in this investigation was subsequently diluted to 1% DMSO (Dimethyl sulfoxide). A solvent or transparent liquid with the ability to effectively absorb water molecules is DMSO. The universally non-toxic qualities of DMSO 1% mean that it doesn't kill many larvae. This solvent is safe to use since it has a relatively low toxicity level. Therefore, one of the primary chemical solvents that may be utilized in the BSLT test, in addition to employing distilled water, is 1% DMSO (Andini A, et al., 2021). Samples of fermented honey and pure honey were each weighed up to 25 mg in 25 ml of 1% DMSO for the toxicity test. Then, 1000 ppm, 750 ppm, 500 ppm, 100 ppm, 50 ppm, 10 ppm, and 1 ppm of the original concentration were serially diluted. Then, 5 ml of each concentration was collected, poured to a petri dish together with 10 shrimp larvae and 5 ml of salt water, and incubated for 24 hours. Using the same Microsoft Excel program that was used to get the LC50 value, the results of larval mortality after 24 hours were then determined using the percent mortality and the probit value. based on the results of the BSLT tests that have been performed as follows:

The mortality findings of larval deaths in samples of pure honey are shown in Table 2. The measurement of LC50 was 59.75 μg/mL. As a result of the findings, pure honey had the potential to be an anticancer candidate because its toxicity value fell into the harmful range. According to (Sumarlin et al., 2014), the fructose and other organic acids found in glycoside chemicals, such as phenolic acid, are likely to be the source of honey's toxicity. While the sample of fermented honey had an LC50 of 3.28 μg/mL. These findings suggest that the fermented honey sample has an extremely poisonous toxicity value. Honey's LC50 value is defined as the compound's LC50 value, and LC50 levels below 30 ppm are considered active and may be a candidate for an anticancer drug (Sumarlin et al., 2014). This is consistent with (Albuntana, et al., 2011) in (Zulfiah et al., 2020), which claims that a substance is considered to be very active if the LC50 value is close to the standard value of American activity, according to the National Cancer Institute (NCI), the standard of effectiveness of bioactive components against cancer cells is 30 ppm (Zulfiah, et al., 2020).

These findings show that the LC50 of samples of pure honey and samples of fermented honey differ, with samples of fermented honey having greater hazardous qualities than samples of pure honey. There is no discernible difference in the toxicity values of pure honey and fermented

honey, according to data analysis using the Mann-Whitney test on the toxicity of pure honey and fermented honey samples. The results acquired a Sig value of 0.317, where the Sig value is > 0.05. Although the LC50 value from the probit test yielded different findings in the dangerous and extremely toxic categories, statistically speaking there was no significant difference between the two samples.

CONCLUSION

1. Positive results from phytochemical screening of pure forest honey and wild honey fermented with lanang garlic suggested the presence of secondary metabolites of alkaloids, flavonoids, tannins, steroids, and saponins in the samples.
2. A toxicology test on samples of pure honey revealed that the LC50 value was greater than 30 and less than 1000 ppm, indicating that pure honey was harmful; the LC50 value was 59.75 μg/mL. The toxicity test on the sample of fermented honey, however, revealed an LC50 value 30 ppm, indicating that it had an extremely toxic toxicity value, with an LC50 result of 3.28 μg/mL.

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