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ANTIDEPRESSANT-LIKE ACTIVITY OF METHANOLIC EXTRACT FROM OLD ARECA NUT USING FORCED SWIM TEST METHOD IN MICE (*Mus Musculus*) TESTED INDEPENDENTLY

Riryn Novianty[1]*,Yuharmen[1], Nery Sofyanti[2], Fitra Audhi Ya'la[1]

[1]Chemistry Study Program, Faculty of Mathematics and Natural Sciences, Universitas Riau Pekanbaru, Indonesia
[2]Biology Study Program, Faculty of Mathematics and Natural Sciences,Universitas Riau Pekanbaru, Indonesia

*Email: riryn novianty@lecturer.unri.ac.id

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Abstract: This study aims to investigate the potential of methanolic extract from aged areca nut as an antidepressant using acute and sub-chronic Forced Swim Test (FST) methods. In the acute FST, mice were injected with methanolic extract doses of 10, 50, and 100 mg/kg intraperitoneally as a single dose to determine the dose with the best activity. Meanwhile, in the sub-chronic FST, the best dose was administered for 7 consecutive days, and a re-test was conducted on the seventh day after acute FST. A significant reduction in immobility time after acute treatment was only observed with a dose of 100 mg/kg. Therefore, mice were injected with this dose for the next 7 days. However, this dose did not significantly decrease immobility time based on the results of sub-chronic FST. Thus, the methanolic extract at a dose of 100 mg/kg was considered not potentially effective for continuous use. Phenolic compounds, steroids, and saponins as the main constituents in the extract did not induce toxic effects after treatment for 7 days.

Keywords: Old areca nut, Depression, Methanolic extract, Forced swim test

INTRODUCTION

Depression, as one form of mental disorder, has become increasingly prevalent among communities lately, contributing to a decline in quality of life, disability, and even mortality[1]. In 2020, depression ranked second as the leading cause of disease burden worldwide. Approximately 3.8% of the global population, totaling 280 million people, were reported to be affected by depression based on data from the Institute of Health Metrics and Evaluation[2]. Data reported through the Basic Health Research[3], shows that 6.1% of the Indonesian population aged 15 years and older suffer from depression. Individuals with depression typically exhibit symptoms such as frequent feelings of hopelessness, low self-esteem, and sadness. This condition is often underestimated by the general public, starting from unresolved stress leading to depressive phases[4].

Factors contributing to the occurrence of depression include psychological factors (psychological pressure), socio-environmental factors (impact of daily life situations), and organobiological factors (imbalance in brain neurotransmitters)[4]–[6].Imbalance in neurotransmitters such as dopamine, serotonin, and noradrenaline can occur due to oxidative deamination by the enzyme Monoamine Oxidase-A (MAO-A)[7]. The activity of the MAO-A enzyme can be inhibited by a class of antidepressant drugs known as Monoamine Oxidase Inhibitors (MAOIs)[8].The inhibition process occurs when the substrate can react covalently or non-covalently with the target enzyme[9]. This leads to an increase in the three neurotransmitters mentioned above. Therefore, antidepressants are often used to restore the chemical balance in the brains of individuals with depression.

Various synthetic antidepressant drugs have indeed been widely marketed. However, patients report experiencing various side effects after consuming these drugs[10]. One of the standard antidepressant drugs is imipramine. The anticholinergic properties of imipramine can trigger constipation, dry mouth, tachycardia, blurred vision, and urinary retention[11]. Different from synthetic antidepressants, herbal antidepressant drugs are more preferred, such as St. John's Wort, which has been proven to be more potent with lower toxicity compared to synthetic antidepressants like paroxetine and fluoxetine[12], [13]. This has triggered an increasing demand for new antidepressant drugs with better efficacy.

One alternative that can be pursued is to utilize natural ingredients derived from local plants to be used as candidates for antidepressants. In the era of digitalization, experiments can be conducted using computer applications [14][15] and drug discovery research through molecular computational approaches (in silico) is often used to assess the efficacy and toxic risks of a drug compound. Previous researchers have conducted in-silico studies showing that several secondary metabolites derived from Indonesian local plants have the potential to be antidepressants[16][17][18][19], as well as drugs to alleviate symptoms of COVID-19[20]. The diverse inhabitants that nature has bestowed upon humans are useful in various fields, such as antimicrobial activity of fungi bioactive constituents [21] plants' secondary metabolites asantidiabetic agents[22], [23] alternative oil [24] [25] and immunomodulators[26] [27] also the abilities of native microbes to degrade the hazardous naphthalene compounds [28][29][30][31][32] and petroleum hydrocarbon components [33][33][35]35][36][37][38] that can contaminate the environment.This certainly serves as

evidence that researchers' interest in searching for drug candidates utilizing natural ingredients is increasing.

Areca nut (*Areca catechu L.*) is one of the local plants in Indonesia that is widely distributed in various provinces, including Riau Province. The most important part of the areca plant is its nut. In the past, people had rituals of chewing areca nuts and areca leaves associated with psychostimulant effects, satisfaction, well-being, and stress reduction[39].Studies on the potential of active compounds from areca nuts as antidepressants have been extensively conducted by researchers through in-silico approaches. Arecoline compounds have good bioavailability to be used as oral antidepressant drugs[40], guvacoline and homoarecoline are potential MAO inhibitors [41], and these compounds can work synergistically with standard antidepressant drugs like fluoxetine [42]. Other metabolites such as L-phenylalanine and L-tyrosine, through pharmacokinetic analysis, drug-likeness, and toxicity, have been confirmed as antidepressant candidates without toxic properties[43].

Several studies have conducted in-vivo tests using rodents to investigate the antidepressant activity of crude extracts from various plants[44]–[49]. Previous studies followed the standard Forced Swim Test (FST) protocol without modification. The test animals were only given acute treatment before the test. However, clinically, antidepressants often show therapeutic effects[50]. Therefore, sub-chronic FST is needed to assess the potential of the tested compounds after the animals have been given the drug samples for several days[51].

Based on the background, the researchers investigated the antidepressant activity of methanolic extract from oldareca nuts through acute and sub-chronic Forced Swim Tests. The main constituents potentially acting as antidepressants were identified by conducting phytochemical screening on the extract, and any potential toxic effects were also observed through organoleptic observations of key organs involved in drug metabolism.

RESEARCH METHOD

This research was conducted in several stages. In the initial stage, the crude drug was prepared by drying oldareca nuts and grinding them into fine granules. Then, the extraction stage was carried out by soaking the fine granules of the crude drug in methanol solvent at room temperature. The macerate was concentrated to obtain the concentrated methanolic extract of oldareca nuts.The Forced Swim Test (FST) method was conducted to investigate the antidepressant activity of the methanol extract. The test animals were given 7 days to adapt to the laboratory environment. Then, the pre-test stage was conducted one day before the acute FST took place. Both the pre-test and the test were conducted in the same room and container conditions. The acute FST was performed by injecting the drug sample in a single dose. Subsequently, for the next 7 days, the mice were injected with the optimal dose and subjected to a re-test in the sub-chronic FST series.

Materials

The materials required for this research include methanol as the solvent for the extraction process, Kalxetin® capsules (standard antidepressant) containing fluoxetine hydrochloride 20 mg/capsule, and 0.9% saline used as the drug carrier and control medium.

Test Animals

The test animals used in this study were male BALB/c mice aged eight to ten weeks with a weight of 20-25 grams. The mice were housed in groups of 5-6 individuals in one cage with unrestricted access to food and water. Acclimatization of the mice lasted for 7 days before the experiment, and they were given standard care with a 12-hour light/dark cycle at a temperature of 23-26°C. The research procedure began at least after the acclimatization period in the laboratory conditions and was conducted between 08:00-15:00. The mice used were healthy, showed no significant changes in body weight (maximum deviation of 20%), and showed normal behavior. The procedures carried out in this study were approved by the Research Ethics Committee of the Faculty of Medicine, University of Riau (Letter No.: B/004/UN19.5.1.1.8/UEPKK/2023).

Extraction of OldAreca Nuts

A total of 500 grams of oldareca nuts were obtained from local farmers (Pekanbaru, Riau Province). The areca nuts were separated from any adhering impurities and then ground into fine granules. Subsequently, they were macerated in 2.5 liters of methanol for 24 hours with occasional stirring. Afterward, the extract solution was filtered, yielding a macerate. The residue was macerated again for up to four repetitions until the solution became clear[52]. The clear solution indicates that all metabolites have been thoroughly extracted[53].Next, the macerate was collected, and the solvent residue was evaporated using a vacuum rotary evaporator.

The secondary metabolites present in the samples of oldareca nuts and methanol extract will be analyzed through phytochemical screening, which allows for the estimation of the main constituents acting as antidepressants. The phytochemical screening protocol refers to previous studies[54]–[56].The presence of alkaloid compounds is determined by adding Mayer and Dragendorff reagents, FeCl3 reagent for phenolic testing, alkali for identifying flavonoids, and Libermann-Burchard reagent for steroid/terpenoid testing. The presence of saponins is identified through the formation of stable foam in the test solution after shaking.

The Forced Swim Test (FST)

The Forced Swim Test (FST) was conducted following the modified Porsolt method. Based on the duration of treatment, mice underwent acute and sub-chronic FST[57]. The forced swimming method in

the pre-test was conducted for 15 minutes a day before observation. Mice were placed in FST tanks (size 11 cm x 20 cm) filled with water to a depth of 10 cm. The water temperature was adjusted to exactly 25°C. Animals that were injured or showed bleeding from the nose were eliminated from the experiment[58].

The next day, the testing session lasted for 10 minutes. However, the behavior of the mice was only observed in the last 8 minutes because almost all rodents would continuously swim while attempting to escape during the first 2 minutes[59].

a. Acute FST

Observations were conducted on the day after the pre-test. Mice were injected with 0.9% saline (negative control 0.1 mL/20 grams); fluoxetine (positive control 20 mg/kg); and methanol extract at doses of 10, 50, and 100 mg/kg. Drug samples were administered intraperitoneally 1 hour before the test. The selection of dose variations and drug administration routes was based on several literature sources[60]–[62]

b. Sub-chronic FST

Test animals were injected with an extract dose of 100 mg/kg, fluoxetine 20 mg/kg, and saline 0.1 mL/20 grams for 7 consecutive days. Re-testing was conducted on the seventh day after the acute FST.

Toxicity Testing

Toxicity testing is aimed at detecting toxic effects that arise after repeated administration of antidepressants (sub-chronic treatment) in test animals. The initial stage in toxicity testing involves sacrificing the mice (euthanasia) by cervical dislocation. The drug administration site, namely the peritoneal area, is observed to identify any nodules indicating tumor growth. The mice were skinned in that area to ensure tumor growth and then dissected to examine any damage that may have occurred to the intestines and liver. Toxic effects caused by plant extracts have been reported by previous researchers[49]including tumor growth in the peritoneal area, accumulation of yellow fluid, and lesions in the liver and intestines.

Statistical Analysis

The immobility time of each group was expressed as the mean ± SEM in seconds. Data analysis was performed using a one-way ANOVA followed by Tukey's test for both acute and sub-chronic FST. A p-value < 0.05 indicates a significant difference between the negative control and experimental groups.

RESULTS AND DISCUSSION

1. Extraction and Phytochemical Screening

2. The crude methanol extract of oldareca nuts obtained was dark red in color with a weight of 35.76 grams. Secondary metabolites present in the oldareca nut sample were identified through initial screening and further confirmed by conducting phytochemical screening on the methanol extract. Both the oldareca nut sample and the methanol extract showed positive results in phenolic, saponin, and steroid testing[57], [63], [64].

3. Acute FST

Forced Swim Test, or forced swimming test, is one of the procedures utilizing test animals validated with depression-like behavior to determine the efficacy of antidepressant drugs. This method is based on the finding that rodents placed in a cylinder filled with water will attempt to escape, such as swimming and climbing, but show increased periods of inertia after initial efforts when escape is considered impossible[65]. The typical immobility posture, reflecting despair-like behavior akin to depression in humans, is characterized by floating near the water surface with minimal movements to keep the nose above the surface[66].

Mice were grouped based on the treatment administered, including negative control, positive control, and oldareca nut methanol extract. In this study, 0.9% saline was injected into the control group. This decision was made considering that saline has been widely used as a drug vehicle and vehicle control[67]. The ability of saline to dissolve drugs facilitates the body's absorption of drug samples.Fluoxetine, one of the Selective Serotonin Reuptake Inhibitor (SSRI) antidepressants, was used as the positive control due to its ability to treat depression by increasing serotonin neurotransmitter levels through inhibition of the serotonin transporter (SERT) in the brain[68]. This serves as a determinant to validate whether the procedures performed are correct and whether the tested extract has the potential as an antidepressant.

The effect of a single dose of oldareca nut methanol extract and fluoxetine on reducing immobility time in mice can be seen in **Table 1.** Mice injected with a dose of 100 mg/kg of methanol extract showed the lowest immobility time. Based on statistical analysis using one-way ANOVA followed by Tukey's test, this dose exhibited a similar action to the standard antidepressant, fluoxetine (20 mg/kg), in reducing the immobility time of mice. The highest percentage reduction in immobility time was also caused by this dose, which was 12.41%.

Table 1.Effect of oldareca nut methanol extract on mice subjected to acute FST

Treatment	Immobility Time (seconds)	Reduction in Immobility Time (%)
Control (Saline 0.1 mL/20 g)	408.33 ± 9.82^{b}	-
Fluoxetine (20 mg/kg)	324 ± 6.35^{a}	20.65
Methanol extract (10 mg/kg)	429 ± 4.72^{b}	-
Methanol extract (50 mg/kg)	405 ± 14.57^{b}	0.82
Methanol extract (100 mg/kg)	**357.67 ± 9.02^{a}**	**12.41**

Note:
Different letters (a and b) in the same column indicate significantly different immobility times of mice ($p < 0.05$) based on Tukey's test.

The ability of methanol extract and positive control in reducing immobility time during acute FST compared to the control group is shown in **Figure 1.** As previously described, the methanol extract at 100 mg/kg dose exhibits the best activity compared to the other three doses. This dose can significantly reduce immobility time compared to the control group. Meanwhile, fluoxetine can induce a significant reduction in immobility time. Unsatisfactory results were obtained from the groups of mice injected with extract doses of 10 and 50 mg/kg. Both doses have immobility times that are not significantly different from the control group. This indicates that these doses cannot alleviate depression in the test animals. Therefore, the methanol extract at a dose of 100 mg/kg was selected as the dose to be administered to the mice during sub-chronic treatment.

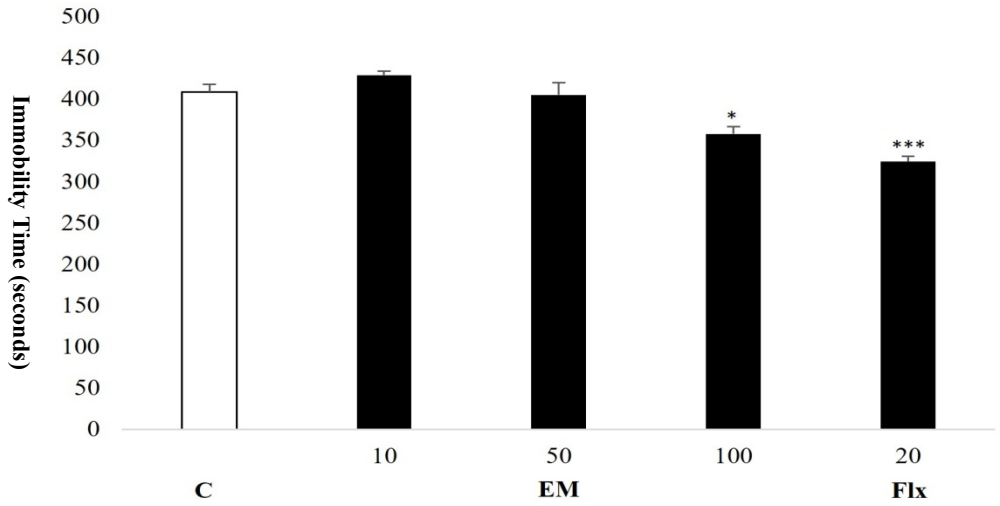

Figure 1. The effect of methanol extract of old areca nut (EM) and fluoxetine (Flu) on immobility time of mice in acute FST. *$p < 0.05$-significant, ***$p < 0.001$-very significant compared to the control group (K)

The results obtained from this study show that acute treatment with methanol extract from old areca nuts induces a dose-dependent response trend as depicted in **Figure 1.** This pattern has also been reported in previous studies examining the potential of natural substances as antidepressants[69]–[71]. Previous researchers have reported the potential of areca nut extract; administration of aqueous ethanol extract and hexane fraction at doses of 20 and 13 mg/kg, respectively, to Wistar rats resulted in decreased immobility time during FST[72].

The best response was shown by the group of mice injected with the 100 mg/kg dose of extract, characterized by the lowest immobility time and the highest percentage reduction in immobility time. Meanwhile, the 10 and 50 mg/kg doses were unable to reduce the immobility time of mice because they were considered to have deficient metabolite concentrations, thus binding to only a few allosteric sites of the target protein. When the dose was increased to 100 mg/kg, more allosteric protein sites could bind to the metabolite, thereby reducing the substrate (neurotransmitter) binding affinity to the protein. This causes the neurotransmitter levels to increase, and depression to be treated. Referring to the organobiological factors causing depression, among them are the catalytic activity of MAO-A, which oxidizes neurotransmitters, and the activity of transport proteins (such as Serotonin Transporter or SERT), which reuptake serotonin, resulting in decreased levels in the brain [54]. We suspect that the mechanism of action of the methanol extract of areca nut is similar to the positive control used, which is Fluoxetine (a Selective Serotonin Reuptake Inhibitor or SSRI), as explained in the next paragraph. Thus, the inhibition through the allosteric sites of SERT

reduces the affinity of serotonin binding to its active site. This prevents the reuptake process, leading to an increase in serotonin levels in the brain.

Secondary metabolites such as saponins, steroids, and phenolics are believed to work synergistically as the main constituents responsible for the antidepressant activity in the methanol extract of areca nut. This is supported by previous research indicating that phenolic compounds[73]and saponins[57]have potential antidepressant effects and exhibit monoaminergic actions similar to the mechanism of SSRIs[74].Steroid compounds have also been reported to have antidepressant activity. β-sitosterol isolated from Sargassum Horneri belongs to the group of steroid metabolites that can increase serotonin concentration in the hippocampus of rats similar to fluoxetine[70].Thus, it can be speculated that the methanol extract of areca nut may have a mechanism acting as a serotonin reuptake inhibitor, namely Selective Serotonin Reuptake Inhibitor (SSRI).

4. Sub-chronic FST

Administration of methanol extract at a dose of 100 mg/kg for 7 consecutive days in the test animals resulted in a significant difference in immobility time between the extract group and the positive control. Fluoxetine (20 mg/kg) showed better activity, as seen in **Table 2.** The percentage reduction in immobility time in the positive control group increased to 43.53%. Meanwhile, the increase in the extract group was only 13.87%.

Table 2. Effect of methanol extract of old areca nut on mice subjected to sub-chronic FST

Treatment	Immobility Time (seconds)	Reduction in Immobility Time (%)
Control (Saline 0.1 mL/20 g)	420.33 ± 17.14^{b}	-
Fluoxetine (20 mg/kg)	237.33 ± 4.63^{a}	43,53
Methanol Extract (100 mg/kg)	362 ± 16.01^{b}	13,87

Note:
Different letters (a and b) in the same column indicate significantly different immobility times in mice ($p < 0.05$) based on Tukey's test.

The optimal dose of methanol extract is less potent as an antidepressant. This is evidenced by its ability to reduce immobility time not significantly different from 0.9% saline. Different results were obtained in the positive control group, which significantly reduced immobility time as shown in **Figure 2.**

Based on the results of the sub-chronic FST, the methanol extract is not potent as an antidepressant. This is evidenced by mice showing more immobility posture compared to the acute FST with immobility time not significantly different from the control group. These results are contrary to previous studies that antidepressants would be more potent after sub-chronic or chronic treatment[75]. The behavior of test animals during FST can be affected by environmental conditions.[51]. The administration of drugs to mice during the sub-chronic FST lasts for 7 days, requiring the mice to be housed for a longer period. During this period, the room conditions where the mice are housed are quite odorous, thus disturbing the mice's sense of smell. Olfactory deficits can affect the function of neural circuits involved in mood regulation[76]. Therefore, the less conducive cage conditions and the noise around the cage and laboratory due to construction activities are suspected to cause the mice to become more depressed, as indicated by increased immobility time.

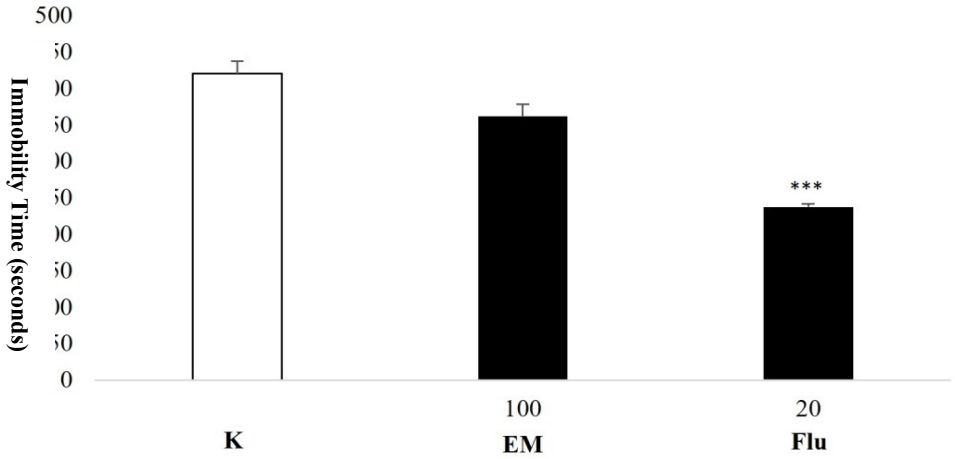

Figure 2. The effect of methanol extract of areca nut (EM) and fluoxetine (Flu) on the immobility time of mice in the sub-chronic FST. *p<0.05-significant, ***p<0.001-very significant compared to the control group (K)

5. Toxicity Test

Mice injected with methanol extract and fluoxetine for 7 consecutive days showed no signs of toxic effects from the drug samples. This is evidenced by the absence of tumor growth. Internal organs such as the liver and intestines showed no signs of inflammation similar to the control group **(Figure 3).**

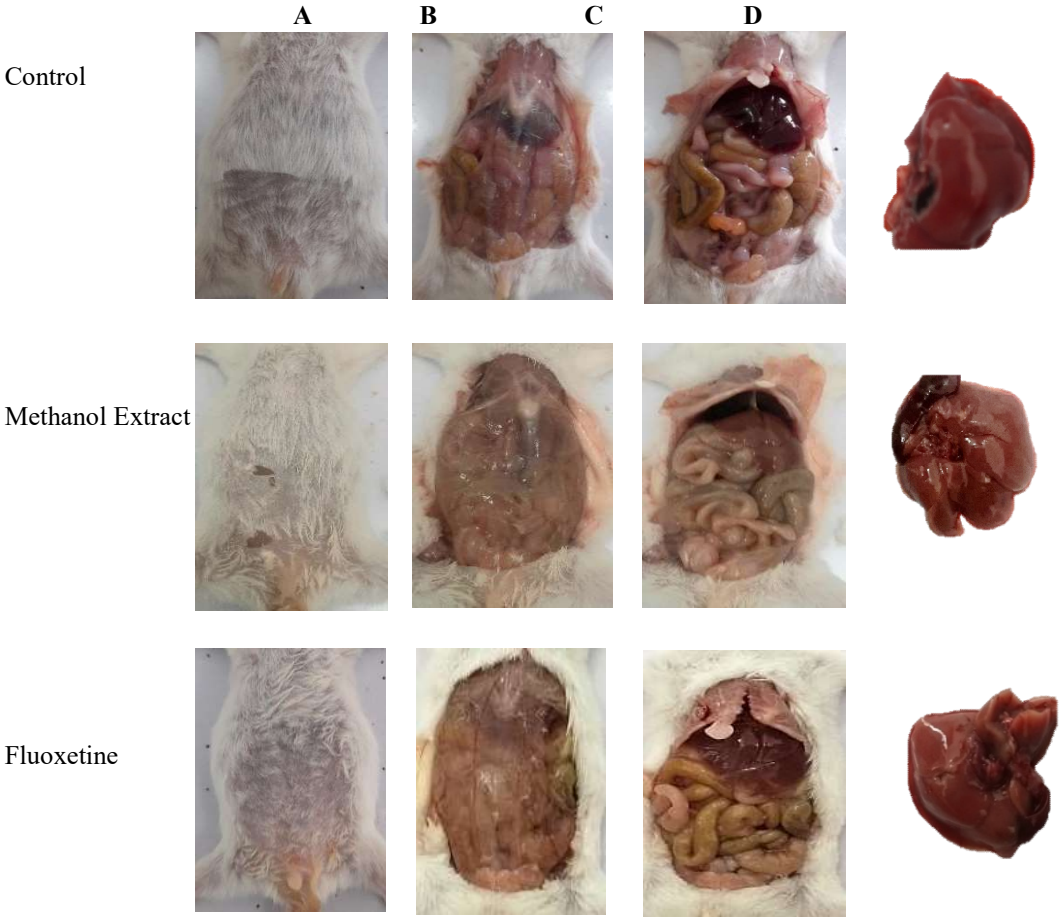

Figure 3. Effect of saline, methanol extract, and fluoxetine on toxicity in mice. (A) Cross-section of the abdomen. (B) Cross-section of the abdomen after skin removal. (C) Intestinal organ view. (D) Liver.

The toxicity test aims to obtain information on the toxic properties after repeated administration of the test substance. The results of toxicity tests using animal models can serve as an indication of relative toxicity if exposure to humans is also considered[77]. Methanol extract of areca nut contains primary constituents such as phenolics, saponins, and steroids. These three compounds are unlikely to induce toxic effects in animal models after sub-chronic treatment. This finding confirms the results of in-silico tests previously conducted, where the ADMET approach on several phenolic compounds found in areca nuts showed non-toxic characteristics.

The intestines of mice were observed macroscopically in this study to assess any potential toxic effects. Within the intestines, there are mesenteric capillaries that absorb the drug after intraperitoneal injection. Subsequently, the drug flows into the portal vein to pass through the liver[78]. If there is exposure to a toxic substance, it can induce inflammation in the intestines as reported by Abbas et al.[57]. However, the results we obtained did not show any inflammation in the intestines, indicating that the intestines of mice were in a healthy condition, similar to the control group that was only injected with 0.9% saline.

The livers of mice were also observed as they serve as the center for drug metabolism and detoxification[79].Drugs injected intraperitoneally are metabolized in the liver first, so if a drug has toxic effects on the body of the test animal, liver damage will be evident. A healthy mouse liver typically appears reddish-brown, while a pale and spotted liver indicates abnormalities[80]. A change in liver color to reddish-yellow indicates liver damage due to exposure to toxic substances[81].Macroscopic observations of the

liver organs revealed that each group of mice exhibited characteristics of healthy livers.

CONCLUSION

Based on the conducted research, it can be concluded that the methanol extract of areca nut at a dose of 100 mg/kg shows potential as an antidepressant after acute treatment. Its action in reducing the immobility time of mice did not significantly differ from fluoxetine. This activity is presumed to be due to the synergistic effects of phenolic compounds, steroids, and saponins present in the extract in addressing depression in the test animals. However, the immobility time of mice injected with the extract during sub-chronic treatment did not differ significantly from the control group. This indicates that the dose is not effective when used continuously. Nevertheless, the secondary metabolites present in the extract did not induce toxic effects after 7 days of treatment.

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