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Potential antidepressant activity of n-hexane extract from old *Areca catechu* Nut by reducing depressive-like state in Swiss albino male mice

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ABSTRACT

Depression is a psychological disorder caused by an imbalance of neurotransmitters that can be managed with antidepressants. One of the local plants with the potential as an antidepressant is areca nut. However, its' antidepressant effects in nonpolar solvents have not been studied recently. Equipping the extract to animals sub-chronically could mimic the clinical antidepressant treatment. Thus, the current investigation studied the antidepressant action of the hexane extract of old areca nut by utilizing acute and sub-chronic FST. This research started with extracted areca nut using n-hexane and continued with phytochemical tests. During FST, the animals were treated with n-hexane extract (50 and 100 mg/kg), fluoxetine (20 mg/kg), and saline (0.1 mL/20 g). The phytochemical test of the extract showed positive results from the content of secondary metabolites, namely saponins and steroids. *n*-Hexane extract at a dose of 50 mg/kg gave the best action in decreasing the immobility period. After sub-chronic medication, the secondary metabolites of the extract did not induce any toxic effects. This study's findings imply that depression may be treated with conventional medicine, such as old areca nut extract.

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1. Introduction

Depression is a mental disorder characterized by several clinical symptoms that can appear as mood complaints, such as sadness, depression, and hopelessness (Hammen and Watkins, 2018) . When stress is not managed, a person can fall into a depression phase (Psychiatric, 2018). An article by the World Health Organization (2021) states that mental disorders such as depression are major health problems that must be taken seriously. There are 280 million people with mental disorders. Depression rates have increased by more than 18% since 2005 (WHO, 2021). Mental disorders are projected to be the top cause of the disease burden by 2030. This disease is frequently disregarded due to the misconception that it can heal independently. Depression can cause a decrease in a person's psychosocial functioning, leading to suicide. Suicide is the leading cause of death globally, contributing to 11% of years living with a disability (Ferrari et al., 2013).

Depression is thought to be brought on by a variety of reasons, such as a brain imbalance in neurotransmitters, socioenvironmental stress, and psychological strain (Novianty, Elvira, et al., 2023, 2024; Pradiningsih et al., 2017). The activity of the monoamine oxidase enzyme (MAO) is the reason for the low concentration of neurotransmitters, such as serotonin, dopamine, and norepinephrine (Stahl, 2013). Taking an antidepressant like MAOI, or monoamine oxidase inhibitor, that inhibits MAO is the best way to increase the amount of that neurotransmitter (Gerlach et al., 2014). The targeted enzyme can form a covalent or non-covalent bond with the ligand or active chemicals, which results in the inhibitory activity (Novianty et al., 2014).

There are many synthetic antidepressant drugs, one of which is imipramine. The anticholinergic properties of imipramine can cause unwanted side effects, such as blurred vision, constipation, tachycardia, dry mouth, and urinary retention (Tai et al., 2019). Treatment of depression using medicinal plants does not have side effects compared to synthetic drugs (Dhingra and Sharma, 2006). Antidepressant herbal medicines have also been widely used in the world because they can suppress the side effects of synthetic drugs. One is St. John's Wort which is considered to have a lower toxicity level compared to synthetic antidepressants, such as paroxetine and fluoxetine (Kumar et al., 2015; Sarris, 2013).

One nation with a wealth of biodiversity is Indonesia. Studying the possibilities of natural substituents is becoming more popular. Our previous studies have been investigated the antimicrobial activities from fungi metabolites (Octarya et al., 2021), the antidiabetic agent (Sy et al., 2017, 2019), alternative oils (Novianty, 2023d; Novianty, Jasril, et al., 2023), and immunomodulators (Novianty, 2023c; Novianty and Devy, 2023) from utilizing plants constituents. Native microbes are also able to degrade the potentially harmful compounds like petroleum hydrocarbons (Antika and Novianty, 2019; Novianty, Awaluddin, et

al., 2020, 2021; Novianty, Dahliaty, et al., 2020; Novianty and Yuharmen, 2023; Sari et al., 2019; Saryono et al., 2022) and the naphthalene (Fitrida et al., 2019, 2020; Novianty et al., 2022; Novianty et al., 2020) that can contaminant environment. In the modern era of digitalization besides wet lab, experiments can be carried out through computer programs (Ningsih et al., 2019a, 2019b), and molecular computational techniques (*in silico*) are widely employed in drug discovery research to evaluate the efficacy and potential side effects of a therapeutic compound. Some plant metabolites have been confirmed potent as antidepressants by using *in silico* approach (Ananta and Novianty, 2022; Maylinda and Novianty, 2022; Novianty, 2020; Sirait and Novianty, 2022) and drugs for COVID-19 (Novianty, Ananta, et al., 2021). An indigenous plant widely distributed in Indonesia is *Areca catechu* L. Its nut is generally used in traditional medicine. The active compounds of areca nut have been reported to have good bioavailability, making them suitable for use as oral antidepressants (Ningsih and Novianty, 2021; Novianty, 2023b; Novianty, Wardana et al., 2023; Novianty, Yuharmen, and Sofiyanti., 2024; Peng, Liu, Zhao, et al., 2015) and less toxicity (Novianty, 2023a) by in silico approach.

Earlier reports used *in vivo* method confirmed the effectiveness of *Areca catechu* nut in reducing stress in animals test (Andriyani and Novianty, 2023; Bhat et al., 2016; Dar et al., 1997; Neldi and Novianty, 2023; Novianty et al., 2024; Sumarni and Novianty, 2023; Ya'la and Novianty, 2023). The rodents are commonly used due to their genetic similarity to humans (Fianti, 2017) . Previous studies have followed the protocols of the forced swim test without modification, where the animals are forced to swim after acute antidepressant medication (Porsolt et al., 1978). Clinically, the therapeutic effect needs several weeks (Cryan et al., 2005). The forced swim test revealed the effectivity of antidepressant acute medication and commonly sub-chronic medication could increase the antidepressant potential of the tested compounds (Petit-Demouliere et al., 2005). Based on Abbas et al., (2012) ethanolic extract from areca nut gave the maximum action after sub-chronic medication in Sprague Dawley rats.

Recently, the antidepressant properties of areca nut extract in nonpolar solvents like hexane have not been investigated. Thus, the current study explored the antidepressant activity of n-hexane extract from old areca nut using acute and sub-chronic FST, with fluoxetine as the positive control. The toxicity effect that occurred after long-term treatment was also learned.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

n-Hexane was purchased from Merck (Indonesia). Kalxetin® containing Fluoxetine hydrochloride 20 mg/capsule was generously provided by Kalbe Farma Indonesia. As a vehicle for medication delivery, the drug was dissolved in saline 0.9%; the extract was dissolved in saline with 10% Tween-80.

2.1.2. Animals

The mice employed in this investigation were male Swiss albino strain mice, eight to ten weeks old, weighing 20 to 25 g. Commercial food and water were unlimited access in a plastic box with five to six mice per cage. For the seven days leading up to the experiment (acclimatization period), the mice were given standard care with a 12-hour cycle of light and darkness at 25±2 °C. The experiment was conducted a day following the acclimation phase from 08:00 am to 03:00 pm. The techniques were approved by the committee for animal experiments at Riau University's Medical School (approval no: B/004/UN19.5.1.1.8/UEPKK/2023).

2.2. Methods

2.2.1. Preparation of n-hexane extract

Areca nuts weighing 500 g were provided by a local farmer (Pekanbaru, Riau Province, Indonesia). The nuts were ground into a powder, macerated in 2.5 L of n-hexane for 24 hours, and then filtered with paper filters to obtain the macerate. The residue was applied to the subsequent maceration. Up to four iterations of these stages were done. The combined macerate was thickened by using a vacuum rotary evaporator.

Metabolites were observed to be present. The existence of alkaloids was determined using Dragendorff's and Mayer's reagents, ferric chloride to determine phenolic, alkaline test to identify flavonoid, and the Liebermann-Burchard to steroid/terpenoid test. To determine the presence of saponin, the solution was agitated until froth formed.

2.2.2. Forced swim test

The forced swim test (FST) was conducted after being adjusted in Porsolt's method. The mice were subjected to acute and sub-chronic FST based on the length of drug therapy (Abbas et al., 2012). The animals were compelled to swim for 15 min during the pre-test session. They were put inside the FST tank, a glass cylinder (11 cm x 20 cm) containing 10 cm of 25°C water. For the next approach, the injured animals during the pre-test were eliminated.

The following day, a test session occurred for 10 min. The duration of immobility as a parameter was observed during the last 8 min (Ueno et al., 2022). The mouse was thought to have been struggling and moving sporadically to maintain its head above the water.

We calculated the percentage of reduction immobility time to determine the dose with the maximum action as an antidepressant using formula:

$$Reduction\ immobility\ time\ (\%) = \frac{A-B}{A}\ x\ 100\% \qquad (1)$$

A is the immobility time in the control group, while B is the immobility time in mice injected with antidepressants.

2.2.2.1. Acute FST

The observation was carried out on the day after the pre-test session. The animals were medicated with 0.9% saline (vehicle control, 0.1 mL/20 g), fluoxetine (positive control 20 mg/kg), and hexane extract from old areca nut with various doses 50, and 100 mg/kg. The drugs were delivered intraperitoneally one hour prior to the test. The dose and administration route were chosen according to previous reports (Abbasi-maleki et al., 2020; Khulbe et al., 2013; Shahamat et al., 2016).

2.2.2.2. Sub-chronic FST

The extract at 50 mg/kg, fluoxetine 20 mg/kg, and saline 0.1 mL/20 g was administered to mice for 7 consecutive days, every day 24 h after the first administration. Re-test was conducted on the 7th day after acute FST.

2.2.3. Sub-chronic toxicity test

The long-term treatment was the subject of a toxicity test to determine any toxic effect that may induced from drug samples. Thus, the following day after sub-chronic FST, the mice were euthanized. The cervical dislocation was used to carry it out at the expense of the test animals, and a bulge in the peritoneal area (the place where the medicine was administered) was explored to indicate the growth of a tumor-like. Mice were skinned in the peritoneal area to observe inflamed intestines and liver tissue. This test was performed as described by Abbas et al., (2012).

2.2.4. Statistical analysis

The data was expressed using mean ± standard error of the mean (SEM) (n = 3 per dose). In order to determine statistically significant differences between the experimental groups, one-way ANOVA followed by Tukey's test was used. A statistical difference existed between each group if $p<0.05$.

3. Results and discussion

3.1. Results

3.1.1. Phytochemical analysis

The crude extract had a thick yellow tint after multiple extraction stages. The extract weight was around 84 g. The yield of the extract approximate 16.8%. According to the phytochemical test as shown in Table 1, the old areca nut powder showed positive results for steroids, phenolics, and saponins. In comparison, only saponins and steroids were present in the hexane extract.

Table 1. The result of phytochemical investigation

Metabolites	Reagent	Results	
		Old areca nut powder	n-Hexane extract
Alkaloid	Mayer and Dragendorff	-	-
Phenolic	$FeCl_3$	+	-
Terpenoid/ Steroid	Liebermann-Burchard	-/+	-/+
Flavonoid	NaOH 10% + HCl	-	-
Saponin	H_2O	+	+

Note: (+) = Secondary metabolites present; (-) = No secondary metabolites

3.1.2. Effect of n-hexane extract on immobility time in the acute FST

As can be seen in Table 2. n-Hexane extract from old areca nut declined the duration of immobility during FST at the doses of 50 mg/kg for a single dose following intraperitoneal administration. Based on ANOVA followed by Tukey's test, the extract had a similar action in declining immobility time to the commercial drug fluoxetine (20 mg/kg) at 50 mg/kg, the immobility time between those groups was not significantly differed. The dose 50 mg/kg caused significant reduction of 21.79% in the immobility time, which also induced the highest decline in immobility time.

Table 2. Effect of n-hexane extract from old areca nut in acute forced swim test

Medication	Immobility time (Sec)	Reduction immobility time (%)
Control (Saline 0,1 mL/20 g)	408.33 ± 9.82[b]	-
Fluoxetine (20 mg/kg)	324 ± 6.35[a]	20.65
n-Hexane extract (50 mg/kg)	319.33 ± 13.59[a]	21.79
n-Hexane extract (100 mg/kg)	390.66± 10.17[b]	4.32

Note: Based on Tukey's test, the different notation (a, b, and c) in the same column tells the duration of immobility significantly differ with $p<0.05$

Effects of n-hexane extract and the positive control in decreasing the immobility period during FST compared with vehicle control are presented in Fig. 1. The extract at 50 mg/kg dose induced the maximum action compared with the control ($p<0.001$-highly significant). Fluoxetine as positive control presented a similar result. In contrast with the extract at 100 mg/kg dose, the duration of immobility was declined, but the differences with vehicle group were not significant ($p>0.05$). As a result, the later medication, sub-chronic FST, was made using n-hexane extract from old areca nut, a dosage of 50 mg/kg.

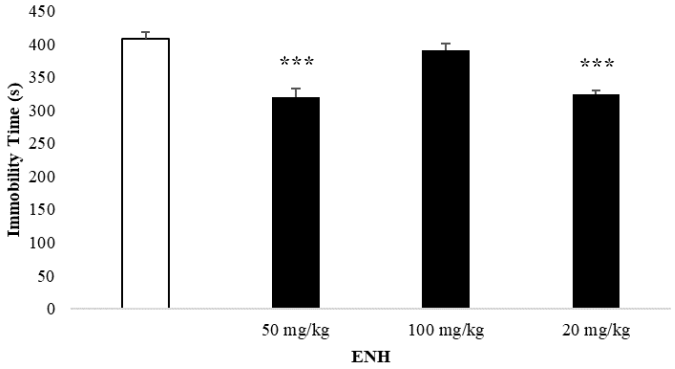

Fig. 1. The effects of n-hexane extract (ENH) from old areca nut and fluoxetine (Flx) on immobility period in mice during the acute FST. ***$p<0.001$-highly significant compared with control (C) group (n =3 per dose)

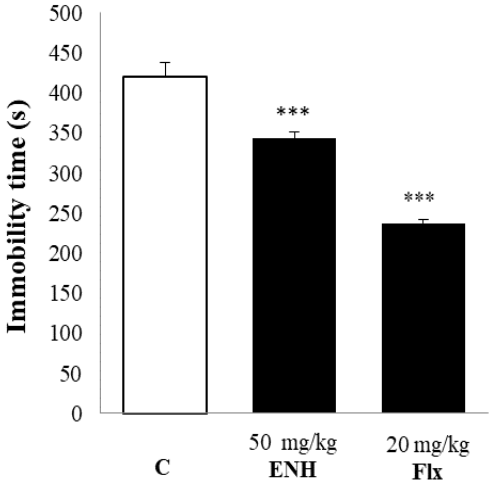

Fig. 2. The effects of n-hexane extract (ENH) from old areca nut and fluoxetine (Flx) on immobility period in mice during the sub-chronic FST. *$p<0.05$-significant; ***$p<0.001$-highly significant compared with control (C) group (n = 3 per dose)

3.1.3. Effect of n-hexane extract on immobility time in the sub-chronic FST

The effects of treating n-hexane extract (50 mg/kg) from old areca nut after 7 consecutive days significantly differed with fluoxetine (20 mg/kg), where the fluoxetine gave the better action, as seen in Table 3. Compared to acute FST result, the reduction in immobility time was raised to 43.53% in the fluoxetine group. While, the duration of immobility in the extract group after sub-chronic therapy was higher than in acute treatment. However, the extract still abled declining immobility time.

Table 3. Effect of n-hexane extract from old areca nut in sub-chronic forced swim test

Medication	Immobility time (Sec)	Reduction immobility time (%)
Control (Saline 0,1 mL/20 g)	420.33 ± 17.14[c]	-
Fluoxetine (20 mg/kg)	237.33 ± 4.63[a]	43.53
n-Hexane extract (50 mg/kg)	343 ± 8[b]	18.39

Note: Based on Tukey's test, the different notation (a, b, and c) in the same column tells the duration of immobility significantly differ with $p<0.05$

The immobility period decreased significantly ($p<0.05$) in the extract group compared with the control group (Fig. 2), as described above. While the fluoxetine reduced the immobility time highly significant ($p<0.001$).

3.1.4. Effect of n-hexane extract from old areca nut on toxicity in mice

The effect of n-hexane extract and fluoxetine given sub-chronically did not seem harmful. There was no tumor-like growth in the peritoneum (where the drug was delivered). In animal studies, the extract did not cause the accumulation of yellow liquid in the peritoneum, intestine inflammation, or liver scarring, as can be seen in Fig. 3.

3.2. Discussion

The Forced Swim Test (FST) is one of the most common tests to assess depressive behavior using test animals. During forced swimming, the mice tried to swim and climb. When mice stop moving and show a passive movement, this behavior is called failure to survive or stress (Slattery and Cryan, 2012). The time mice exhibited passive or inertial behavior was called immobility time and was recorded during the test. Antidepressant compounds can reduce immobility time on FST, where mice move actively. It is due to the role of antidepressants which can increase serotonin, norepinephrine, and dopamine levels in the brain (Armario, 2021).

The physical environment (such as noise and light), biological characteristics (such as sex, age, and strain), as well as handling are several variables that may have an impact on animals' behavior during FST (Bogdanova et al., 2013). The test animals used in this study were mice (*Mus musculus*). They were used because they have a relatively short life cycle, are easy to handle, are anatomical and physiological, and have genetic structures similar to humans (Fianti, 2017; Hermann et al., 2019). Male mice were used in this investigation because they were not affected by hormones or pregnancy like female mice, which made the sample consistently manageable, and the results were greater in precision (Nadi et al., 2021). Mice were acclimatized for 7 days before being tested to adapt to the test environment (Cannizzaro et al., 2002).

In this investigation, the medication was diluted using saline as a solvent to facilitate easier absorption. Consider that saline 0.9% is commonly used in research as a drug delivery medium and vehicle control (Chien et al., 2012). Fluoxetine, as a positive control, is an SSRI (Selective Serotonin Reuptake Inhibitor), which is a class of drugs that inhibits the action of SERT (Serotonin transporter). SERT is a protein found in membranes that transport serotonin. It pumps serotonergic activity from the synapse back into the presynaptic nerve terminal. Thus, depressed people have lower serotonin concentrations in the presynaptic and synaptic. By inhibiting serotonin reuptake through the allosteric site, the inhibitor decreases the affinity of serotonin to bind to the substrate site in SERT. Then, the level of serotonin increased (Stahl, 2013). Therefore, by utilizing fluoxetine, it can be seen the comparison of the activity from the hexane extract of old areca nut with standardized antidepressant drugs.

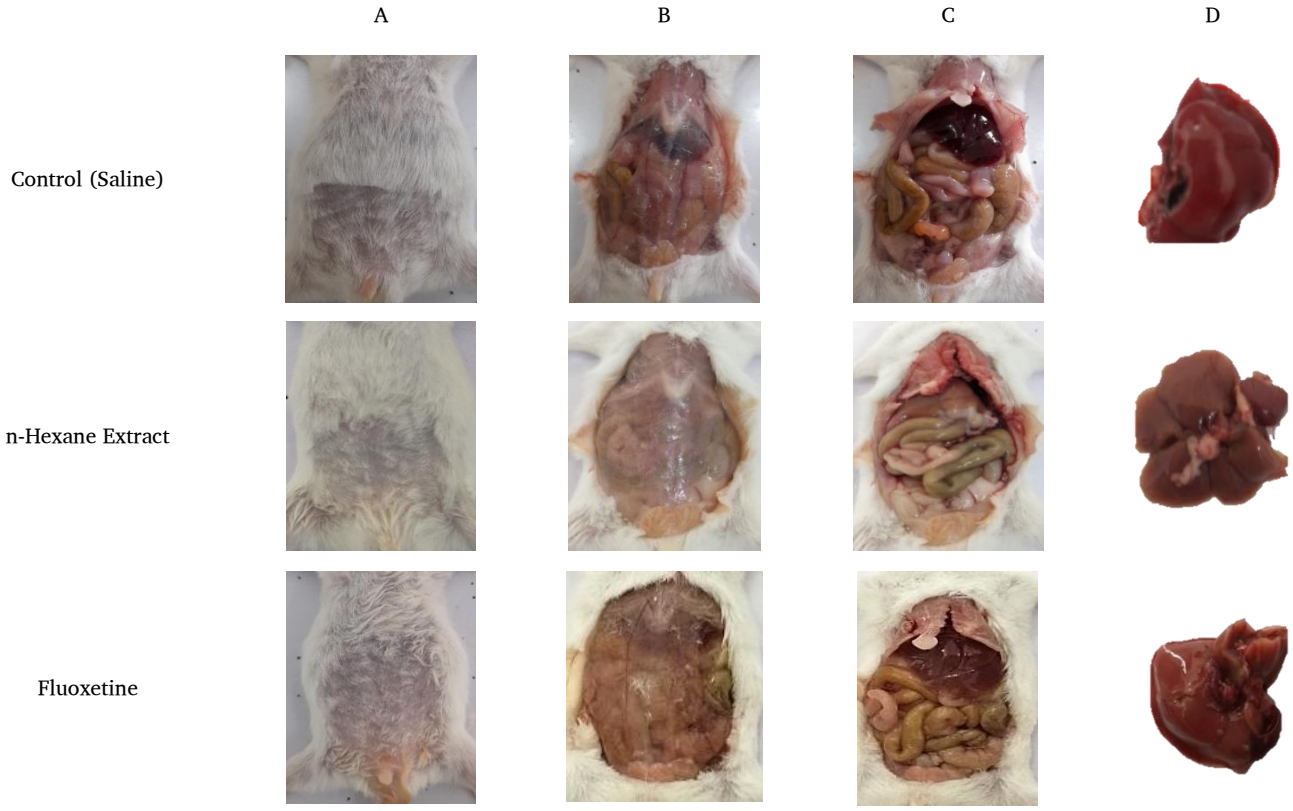

Fig. 3. Effect of saline, n-hexane extract, and fluoxetine on toxicity in mice. (A) Peritoneum cross section (B) Peritoneum after skin removal (C) Intestine section (D) Liver

Various administrations of antidepressants in depressed patients only showed significant symptom reduction through chronic treatment. However, the behavioral model of mice has good predictive validity for antidepressants and sensitivity to acute administration (Bhat et al., 2016). According to this study, n-hexane extract from old areca nut exhibited antidepressant properties after single-dose treatment (acute FST). As shown in Fig. 1, an inverted U-shaped dose response appeared. This pattern had been seen in earlier reports (Abbas et al., 2012; Xia et al., 2007). Additionally, mice treated with the ethanolic extract from areca nut acutely at

doses of 40 to 160 mg/kg produced antidepressant-like action during FST (Bhat et al., 2016). Administered methanolic extract at doses of 100 to 400 mg/kg has decreased the immobility period significantly using the mice model (Mansour et al., 2021). Furthermore, with doses of 13 and 20 mg/kg, the ethanolic areca nut fractions in hexane and aqueous presented a decreasing immobility period in male Wistar rats during FST (Dar and Khatoon, 1997). In the current report, the best response in reducing immobility time was exhibited by extract at 50 mg/kg, which behaved similarly to fluoxetine, as presented in Table 1. Abbasi-maleki and Mousavi (2017) reported similar results from further studies. The duration of immobility of the fluoxetine group and *Carthamus tinctorius* did not differ significantly.

Based on this study, the extract at the dose of 50 mg/kg had the greatest response in reducing immobility time, whereas the 100 mg/kg dose had less impact on treating depression in test animals. We suspect that our findings may be explained by the hypothesis of how substrate concentration affects enzyme activity. The optimum dosage for lowering the test animals' level of depression thought to be 50 mg/kg. At this dosage, the protein's allosteric sites will all bind to metabolites contained in the drug solution, decreasing the protein ability bind its substrate (neurotransmitter). As a result, depression will be treated and neurotransmitter levels will rise. When the dose is increased to 100 mg/kg, it is suspected that the metabolites can no longer interact with allosteric sites because they are already in a saturated condition. The substrate will then bind back to the protein as a result, lowering the levels of neurotransmitters in brain, precisely at the presynaptic and synaptic. Consequently, the group of test animals treated with the dose 100 mg/kg of n-hexane extract experienced an increase in immobility time.

Clinically, the effect of therapeutic antidepressants takes several weeks (Cryan et al., 2005). Sub-chronic FST provides a better system for antidepressant screening where animals receive treatment for several days (Berton and Nestler, 2006). In sub-chronic studies, all trials were performed 24 hours after the last injection to avoid acute effects of the drug on animal behavior (Socała et al., 2016). As antidepressant plant extracts are given to animals for seven days in a row, the immobility time during sub-chronic FST is lower compared to the acute FST result (Morais et al., 2018; Xia et al., 2007). Abbas et al., (2012) discovered that the number of neurotransmitters in the rodents' hippocampi increased after administering ethanolic extract of areca nut during sub-chronic medication. In the current study, the mice that received the optimal dose (50 mg/kg) sub-chronically showed lower immobility time and significantly differed from the control group. While the hypothesis that sub-chronic treatment had superior efficacy than acute can not be confirmed, proving by the test subject that were medicated sub-chronically demonstrated more immobile than it was treated acutely. Throughout the observation, we guessed the environment where the mice were placed affected the stress level. Mice experiencing sub-chronic treatments will need to be confined for extended periods, while the animal house has quite in bad conditions, such as stinky. Based on Ueno et al., (2018) visual and olfactory cues are used by mice to lead their behavior during FST. Sorge et al., (2014) confirmed the mice exposed to isolated odors became more stressed. As a result of the animal home situation, increasing the immobility period indicated the depressive sign. In addition, the mice's depression was exacerbated by the sounds of buildings near the animal house.

When taken for a long time, certain drugs cause serious negative effects. The ethanolic extract of areca nut, which contained tannin and saponin components, has been proven to cause tumors in Sprague Dawley Rats, scarred liver, and inflamed intestines (Abbas et al., 2012). Some metabolites in nonpolar extract, steroid and saponin in n-hexane extract did not have toxic effects as polar extract did. The latest study found that organoleptic observation of the liver and intestines after 7 days of medication showed that the saponin and steroid in the hexane extract did not cause any harmful effects in mice. The drug toxicity test conducted on mice is a model to forecast how the effects may manifest in people (Gad, 2014).

Several people with depressive symptoms have monoamines (serotonin, dopamine, and noradrenaline) lower than normal people (Stahl, 2013), and the antidepressant able to balance it (Ellhwuegi, 2004). The secondary metabolites of saponins and steroids, which interact, were assumed to be the source of the antidepressant activity in the areca nut n-hexane extract. It had been confirmed the antidepressant property of saponins in ethanolic extract of areca nut induced raising the concentration of biogenic amine such as norepinephrine and serotonin (Abbas et al., 2012) and able to stimulate the serotonergic system (Li et al., 2012). Besides that, the steroids, such as β-sitosterol isolated from areca nut, could increase serotonin levels similar to fluoxetine, an SSRI drug (Peng, Liu, Wu, et al., 2015; Zhao et al., 2016). *In vitro* experiment confirms that steroids can interact with Serotonin transporter (SERT) in allosteric regions. It was causing the SERT's activity to be inhibited (Chang and Chang, 1999). Saponin and steroids were responsible for the antidepressant effects of n-hexane extract from old areca nut via inhibiting serotonin reuptake. Further experiments are needed to confirm the antidepressant mechanism of metabolites from areca nut hexane extract.

4. Conclusion

The old areca nut n-hexane extract revealed antidepressant effects in acute and sub-chronic FST. The extract's primary constituents, saponins and steroids, interact synergistically to decline the duration of immobility. When administered acutely, the extract exhibited a similar activity to fluoxetine. However, the extract group had a lower percentage of reduction immobility time than fluoxetine when delivered sub-chronically. Furthermore, the metabolites from the extract did not induce some toxic effects after sub-chronic treatment. The result of this study suggests that traditional medicine, such as n-hexane extract from old areca nut, can treat depression.

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Conflict of interest

The authors declare there is no conflict of interest in this research.

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