

The Effect of Lemon Peel (*Citrus limon*) Aromatherapy Inhalation as Antidepressant on Rats using Diffuser Method

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Abstract. Background Depression is a mood disorder that affects a person's quality of life. This condition is influenced by the levels of monoamine compounds such as serotonin, norepinephrine, and dopamine in the brain. The decrease in monoamine levels leads to depressive symptoms. Lemon peel essential oil has an antidepressant potential, that contains phytochemicals, d-Limonene, which increase monoamine levels in the brain. Aromatherapy diffuser inhalation method is one kind of treatment route in depression. The mechanism of action is by breaking down the smaller essential oil particles so they penetrate quicker to the brain. Objective: To determine the effect of lemon peel (*Citrus limon*) aromatherapy inhalation diffuser method on immobility time of white rats (*Rattus norvegicus* Wistar strain) on the tail suspension test. Method: True Experimental with Post Test Only Control Group Design. Fifteen white male rats Wistar strain were divided into 5 groups. The positive control group was given stressors and saline induction intraperitoneally. In addition to stressors, the treatment group was also given lemon peel aromatherapy inhalation at a dose of 1%, 2.5%, and 5%. Measurement of immobility time was carried out by hanging the rats' tails through the tail suspension test method and the time of animals stopped moving for at least 1 second was calculated using a timer. Data were analyzed using the ANOVA test, Post Hoc test, Pearson correlation test, and Linear Regression test. Results and Discussion: ANOVA test showed a significant difference between groups ($p = 0.017$). The post Hoc test showed a significant difference ($p < 0.05$) between the positive control and the P3 group (5%). The linear regression test obtained Adjusted $R^2 = 0.598$ (inhalation of lemon peel aromatherapy gives an effect of 59.8%). The decrease of immobility time was due to the reduction of depression that was caused by the increase of monoamine level induced by d-limonene in lemon peel essential oil. Conclusion: The inhalation of lemon peel (*Citrus limon*) aromatherapy with diffuser method has the antidepressant effect that reduces the immobility time of depressed white rats (*Rattus norvegicus* Wistar strain).

Depression is a syndrome that affects a person's cognitive, behavior, and nervous system. It can cause a person's inability to carry out his daily life and work functions (Yim et al., 2009). Depression is a mood disorder that can threaten a person's life, which impacts cognitive and physical conditions so that it can reduce interest in daily life activities (Baquero&Martin, 2015).

The prevalence of depression in the world, approximately more than 300 million in 2015. Depression is also the largest contributor to a person's suicide attempt, with 800,000 cases per year. In Indonesia, the prevalence of depression was 9,162,886 cases (World Health Organization, 2017). The prevalence of depression in people aged >15 years in East Java is around 4.53%, with the lowest prevalence in

Sumenep Regency around 0.7%, while the highest prevalence is in Malang City at 10.21% (Indonesia Ministry of Health, 2018).

Depressive conditions are associated with neurotransmitter levels in the brain, such as norepinephrine, serotonin, and dopamine. Therefore, these three play an important role in the process of depression. Decreasement of the three will show symptoms of depression in a person (Stahl, 2013).

Furthermore, stressful conditions will suppress BDNF (brain-derived neurotrophic factor) function as a neuroprotector. So that when BDNF in the brain is decreased, monoamine levels will also decrease (Stahl, 2013).

D-limonene is a phytochemical substance in citrus plants that has a significant role in reducing symptoms of depression, with the mechanism as a 5-HT1A partial agonist and increasing dopamine levels in the brain (Komiya et al., 2006). Lemon, one of the plants of the citrus genus, contains high d-limonene in its peel, around 64.0 - 70.5% (Dosoky & Setzer).

Diffuser method aromatherapy is a recommended therapy in clinical matters, through the nose or by inhalation. It is considered the fastest and most effective route for therapeutic options for emotional problems, including anxiety and depression. (Price, 2011).

METHODS

This study used the True Experimental Design with Post Test Only Control Group Design Method, with simple random sampling technique, using 15 rats were divided into 5 groups, i.e., negative control, positive control, and three treatment groups with different inhalation concentrations of aromatherapy. The research took place in the Biomedical Laboratory of the Faculty of Medicine, University of Muhammadiyah Malang. The population was white male rats (*Rattus norvegicus* Wistar strain), which were taken from captivity. Samples used were white male rats (*Rattus norvegicus* Wistar strain) aged 2-3 months, weighing 150-250 grams, no anatomical defects, active movement, thick fur, and clear eyes.

Hangin Tail with Tail Suspension Test (TST)

This procedure was carried out from day

1 to day 14. All groups except negative controls would be hanged by the Tail suspension test procedure for 6 minutes every day on a 60cm hanging pole, which aims to provide stressors causing depressive symptoms (decreased movement) in experimental animals.

Making Lemon (Citrus limon) Peels Essential Oil

The first step is to cut the lemon peel with a 5mm x 5mm size, weigh 1000gr of lemon peel, and put it into the pressing machine. The pressing device is run at a pressure of 1000psia. After getting the pressing result, then put it in a beaker glass and then filter it, add anhydrous sodium sulfate to the oil yield obtained from pressing to reduce the oil's moisture content. Furthermore, Na₂SO₄ was separated from the oil phase by filtering. Then put the final oil into the container as needed. (Kurniawan et al., 2017).

Animal Grouping and Aromatherapy Exposure of Lemon Peel inhalation (Citrus limon)

The rats used were divided into 5 groups, each group consisting of 3 mice. Negative control was only given food and drink until day 15. The positive control was given a stressor and injection of normal saline (1ml / kg BW) intraperitoneally 30 minutes before assessing immobility time on day 15 (De Almeida et al., 2004). The treatment group was given stressor and inhalation of lemon peel aromatherapy (Citrus limon) with diffuser method with doses of 1%, 2.5%, and 5% concentrations respectively on the 15th day for 90 minutes (Leite et al., 2008; Komiya et al., 2006).

Calculation of Immobility Time

The immobile period (silence period) in mice is defined as mice's behavior to stop trying to escape from a threatening condition during stressor administration using the TST method for at least ≥ 1 second (Ueno et al., 2019). Measurements are made by calculating the state of rest in the mice in seconds using a timer.

Data Analysis

The data obtained in this study were

analyzed using the normality test with the Shapiro-Wilk method, then carried out the homogeneity test with the Levene test, then continued with the ANOVA test to determine the difference between treatments and observations. If the data variants were homogeneous, then continued with the Post Hoc Bonferroni test, but if the data variant is not homogeneous, continued with the Post Hoc Games-Howell test. The last step is to carry out the Linear Regression test to find out how much influence the inhalation concentration dose of lemon peel aromatherapy (*Citrus limon*) on Tail suspension test treatment (shortening immobility time).

RESULT

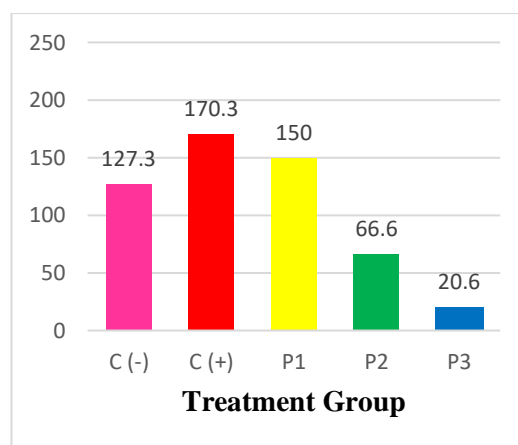
This study took 15 days. The first 7 days were the adaptation process of experimental animals, and 14 days later was the time of hanging rats' tails, which were intended as a stressor to cause depression symptoms, the last 1 day as a calculation of immobility time.

This study consisted of 3 rats per group with 5 groups of rats, namely negative control, positive control, and three treatment groups. The negative control as the normal group was used as the comparison group. The next group was a positive control, which was given a stressor in the form of a tail suspension test (TST) for 2 weeks (day 1 to day 14) and normal saline injection 30 minutes before calculating the immobility time (day 15) without giving inhalation aromatherapy lemon peel (*Citrus limon*). The three treatment groups were then given stressors through the tail suspension test for 2 weeks (day 1 to day 14). They were given inhalation of lemon peel aromatherapy diffuser method on day 15 for 90 minutes with different doses concentration; the first treatment group (P1) with a concentration of 1%, the second treatment group (P2) with a concentration of 2.5%, and the third treatment group (P3) with a concentration of 5%.

On the 15th day, observations of depression symptoms in rats were carried out by calculating immobility time using a timer in each experimental group.

Table 1 Calculation of Immobility Time on the Tail Suspension Test (TST)

Group	Immobility time (second)			Average Immobility time(second) (mean ± std.dev.)
	1	2	3	
C (-)	120	128	134	127.3 ± 7.0
C (+)	213	164	134	170.3 ± 39.8
P1	53	213	184	150 ± 85.2
P2	80	100	20	66.6 ± 41.6
P3	10	50	2	20.6 ± 25.7



Notes :

- C (-): Negative control (normal mouse)
- C (+): Stressor and injection of normal saline (1mg / kgBW)
- P1: Giving aromatherapy inhalation of lemon peel (*Citrus limon*) 1%
- P2: Giving aromatherapy inhalation of lemon peel (*Citrus limon*) 2,5%
- P3: Giving aromatherapy inhalation of lemon peel (*Citrus limon*) 5%

Graph 1 Average Immobility Time (second)

Based on the data above (table 1 and graph 1), the negative control is the normal immobility time value with an average of 127.3 seconds, while the positive control has an average of 170.3 seconds. Thus, it shows an increase in immobility time by a difference of 43 seconds compared to the negative control.

The first treatment group (P1) showed an increase in immobility time compared to the negative control (C-), but there was a shortening when compared to the positive control (C+). Group P1 had a mean immobility time of 150 seconds, which showed a shortening compared to C+, with a difference of 20.3 seconds. Group P2 shows a shortened immobility time

when compared with Group C+ with a difference of 103.7 seconds. Group P3 also showed a shortened immobility time compared to Group C+ with a difference of 149.7 seconds. In Group P2 and P3, the mean immobility time showed a shortening when compared to C-, besides that, it was found that the largest shortening of the mean immobility time occurred in Group P3 when compared to the mean immobility time of Group P1 and P2.

Table 2 Normality Test

Group	Significance
C (-)	0.736
C (+)	0.326
P1	0.463
P2	0.298
P3	0.843

Based on the data in table 2 above, it shows that Groups C-, C+, P1, P2, and P3 show p values > 0.05 . So it can be concluded that the data is normally distributed.

Table 3 Homogeneity Test

Variable	Significance
<i>Immobility time</i>	0.031

Based on the data in Table 3 shows that the homogeneity test uses the Levene test with a significance value of 0.031. Therefore, it can be concluded that the data is not homogeneous because the p -value is less than 0.05.

Table 4 Oneway Anova test

Variable	Significance
<i>Immobility time</i>	0.017

Based on the data in Table 4 above, it shows that the significance value is 0.017. Thus, it can be concluded that there is a significant difference between the immobility time of the treatment group and the control group.

Table 5 Post Hoc Games-Howell Test

Group	Treatment	Significance
C+	P1	0.985
	P2	0.348
	P3	0.046

Based on the data in Table 5 above, shows that Group P1 and P2 compared to Group C+, have a significance value of 0.985 and 0.348, respectively, which means that there is no significant difference. Still, Group P3 compared to Group C+ has a significance value of 0.046. Thus, it can be concluded that there is a significant difference only in the dose of Group P3 concentration because the significance value is p less than 0.05.

Table 6 Linear Regression Test

Variable	Coefficient(B)	Sig.
Constant	235.000	0.000
Dose	-53.233	0.002

Based on the data in Table 6 above, shows that the significance value is less than 0.05, which means that the dose of inhalation concentration of lemon peel aromatherapy (*Citrus limon*) has an effect on the tail suspension test (shortening immobility time).

DISCUSSION

This research used white rat strains of Wistar, which were given repeated stressors using a behavioral test, namely tail suspension test (TST), by hanging the mice's tails. To determine the effect of aromatherapy inhalation antidepressant lemon peel (*Citrus limon*), it is necessary to make rats used in research to be in a depressed state. In causing a symptom of depression in mice (silent movement in mice), it requires a repeated stressor treatment using the tail suspension test (TST) method by hanging the mouse tails as high as 60cm from the floor, hanging the tail using adhesive materials such as plaster which less than 1cm from the end of the mouse tail and hung for 6

minutes every day for 14 days (Ueno et al., 2019).

Repeated stressors can cause hyperactivity of the hypothalamus-pituitary-adrenal (HPA axis) and last a long time (chronic), causing the secretion of CRH (corticotropin-releasing hormone) to be higher as well, it stimulates ACTH (adrenocorticotrophic hormone) to be secreted and causes the feedback mechanism is increasingly disturbed, in the end, it will stimulate the release of cortisol by the adrenal glands and released into the plasma so that in conditions of chronic stress, cortisol levels will increase. If stress lasts long enough, it will affect the volume condition of the hippocampus and paraventricular nucleus (PVN). This feedback failure could lead to a decrease in the function of glucocorticoid receptors in the brain, so cortisol will continue to increase due to the absence of this negative feedback (Yang et al., 2015). The hippocampus is a structure in the brain that contains an amino acid that plays an important role in depression regulation, one of which is the tryptophan amino acid. When the hippocampus volume changes, it will interfere with the tryptophan levels (Steiger et al., 2010). Tryptophan is a material used to synthesize serotonin. When there is a decrease in the tryptophan, the serotonin level in the brain will also decrease because the two are closely related. Repeated stress exposure will also decrease norepinephrine (NE) through a mechanism to reduce locus coeruleus (LC) activity and reduce dopamine levels through a mechanism to reduce the tyrosine hydroxylase (TH) enzyme (Bear et al., 2016).

Reducing the amount of BDNF during depression will cause atrophy and apoptosis of neurons in the brain. In the end, it can reduce levels of serotonin, dopamine, and norepinephrine. Decreased neurotransmitter monoamine levels in the brain, especially serotonin, followed by a decrease in dopamine and norepinephrine, can trigger depressive symptoms. (Stahl, 2013).

Aromatherapy inhalation uses the diffuser method, which is the indirect type of aromatherapy, which means emitting odors in the room which will then be inhaled by someone. Inhalation toxicity will occur if you inhale

aromatherapy in a room without ventilation (Buckle, 2015). Therefore in this study using an aromatherapy box equipped with ventilation to prevent toxic effects. Generally, in 1ml, there are 20 drops of essential oil.

The use of normal saline in this study is intended as a placebo. This is in line with De Almeida et al(2004) 's research that the use of normal saline is used in the control group so that it will be useful as a placebo. According to Colloca (2018) that the use of a placebo in experimental animal studies shows effects such as providing active treatment.

Based on the results of the study, the group of mice that were only given stressor treatment without lemon peel aromatherapy inhalation (positive control) had the highest average immobility time compared to the group of mice that were given stressor treatment and lemon peel aromatherapy inhalation (treatment group), with average immobility time of 170.3 seconds.

Based on statistical results using the ANOVA test, it shows that there are significant differences between treatment groups in the experiment, with a significance value of 0.017. This shows that there is a difference in the average immobility time value in each treatment group. Because the treatment group was given lemon peel essential oil (Citrus limon) through the inhalation aromatherapy diffuser method, which has a high terpene content. One of the most important types of terpenes in lemon peels is d-limonene.

Komiya et al(2006) stated that d-limonene has an antidepressant effect by affecting the production of serotonin and dopamine in the brain of mice, where the mechanism of action is to increase serotonin levels in the synaptic gap for some time so that a lot of serotonin is in the synapse gap which then binds to 5-HT_{1A} receptors at post-synapse so that it will signal dopamine neurons to release dopamine into the synaptic cleft (Stahl, 2013).

Furthermore, to determine the differences between treatment groups, the Post Hoc Games-Howell test was carried out. The test results showed that the average immobility time in the treatment group, which was given inhalation of lemon peel aromatherapy (Citrus

limon) did not show any significant difference, either the concentration of 1% (sig. 0.993) or the concentration of 2.5% (sig. 0.146). On the other hand, the treatment group that was given aromatherapy inhalation of lemon peel (*Citrus limon*) with a concentration of 5% for 90 minutes per day significantly reduced immobility time (sig. 0.035). So it can be concluded that the largest dose, 5%, has a significant effect as an antidepressant.

Research conducted by Komiya et al. (2006) showed that the aromatherapy of 1ml of lemon peel (*Citrus limon*) inhalation or the equivalent of 20 drops of essential oil put into a vaporizer has an antidepressant effect shown by shortening the immobility time on FST (Forced Swim Test). This shows with a higher concentration of 50 drops of essential oil (5% concentration) has a better effect than the 2% concentration. Thus, the higher the dose given, the better the antidepressant effect. This is supported by Costa et al.'s (2013) findings, which state that higher doses of essential oils are needed and repeated use to produce a more effective effect on increasing the neurotransmitter monoamine in the brain.

In this study, it was influenced by factors other than the dose concentration of inhalation essential oil of lemon peel aromatherapy (*Citrus limon*), i.e., endogenous factors and exogenous factors. Endogenous factors such as different genetic susceptibility in response to a stressor treatment and the presence of hormonal factors (Crowley et al., 2006). In a state of stress, it can also trigger a compensatory response, namely, fight or flight; thus, there will be hyperactivity of the HPA axis, which will ultimately have an impact on decreasing monoamine levels in the brain (Hall et al., 2012).

The first exogenous factor is the environmental conditions of the mice before becoming experimental animals in the study. The researchers used mice from the same environment from the same rat breeders, controlled food and drink, and placed all mice in the same environmental conditions (light, temperature, humidity, etc.) to minimize the first factor, the exogenous factor. The second factor is that repeated use of essential oils can produce a more effective effect in increasing the brain's

neurotransmitter monoamine. (Costa et al., 2013).

CONCLUSIONS

Aromatherapy inhalation of lemon peel (*Citrus limon*) with the diffuser method can provide an antidepressant effect on white rats (*Rattus norvegicus* Wistar strain), which is characterized by shortened immobility time on the tail suspension test. The dose of inhalation concentration of lemon peel aromatherapy (*Citrus limon*), which has a significant effect in shortening immobility time, is a concentrated dose of 5% (P3).

SUGGESTION

It is necessary to do further research on the effect of inhalation of lemon peel aromatherapy (*Citrus limon*) with the diffuser method on the depression response of white rats (*Rattus norvegicus* Wistar strain) using other behavioral tests besides tail suspension tests, such as forced swim tests, open field tests, elevated plus maze test, and social interaction test. Furthermore, it is necessary to do further research using lemon extract (*Citrus limon*) to determine the effect of shortening immobility time in white rats (*Rattus norvegicus* Wistar strain). Further research needs to be done by providing repeated exposure to inhalation of lemon peel aromatherapy (*Citrus limon*) with the diffuser method to be more effective in increasing monoamine neurotransmitters in the brain.

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