Anxiolytic effect of aqueous extract of *moringa oleifera* leaves in balb/c mice

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ABSTRACT

Background: *Anxiety control is required to achieve successful dental treatment especially in children. Dental anxiety is exaggerated fear or worry associated with dental treatment and it is followed with a sense of losing control. Moringa oleifera has been claimed as a potential plant to treat anxiety. A large amount of flavonoid in Moringa oleifera leaves is known to have anxiolytic effect due to their interaction with gamma-aminobutyricacid (GABA) receptors like benzodiazepine molecule. The aim of this study was to determine the anxiolytic effect of aqueous extract of Moringa oleifera (AEMO) leaves in balb/c mice.*
Method: *Subject of this study was 25 mice divided into 5 groups. Diazepam as a positive control (K+), CMC-Na 0.5% as a negative control (K-), aqueous extract of M. oleifera leaves 375 mg/kgBW (P1), 500 mg/kgBW (P2), and 625 mg/kgBW (P3). Tests were conducted using Elevated Plus Maze. Before and after data were analyzed with paired T-test, differences of anxiety score between groups was analyzed with One-way ANOVA.*
Result: *Group P2 and P3 significantly reduce anxiety in mice ($p < 0.05$). The anxiolytic effect showed by group P2, P3 and K(+) were significantly different ($p < 0.05$) compared to group K(-).*
Conclusion: *Aqueous extract of Moringa oleifera leaves produced its anxiolytic effect at 500mg/kgBW and 625mg/kgBW peroral.*

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INTRODUCTION

Dental practice is inseparable from the anxiety experienced by patients. Dental anxiety is fear or worry about something terrible related to dental treatment, which is usually followed by losing control.[1] Research by Imamullah (2022) showed that 75% of 6-8 years old children experienced dental anxiety measured by Dental Anxiety Scale.[2] Anxiety control is required to achieve successful dental treatment. Distraction method such as instrumental music lowered psychological anxiety before dental treatment.[3] However, patients with extreme anxiety and uncooperative need pharmacological approach such as N_2O sedation or oral anxiolysis with benzodiazepines.[4] Diazepam is one of benzodiazepine family that often used as an oral sedation drug and gold standard to treat anxiety.[5] Benzodiazepines are anxiolytic (anxiety-reducing) drugs with a high level of effectiveness, but have side effects such as insomnia, muscle relaxation, coordination problems, and headaches.[6]

Herbal plants, known as phytomedicine, are widely applied as an alternative in the field of medicine.[7] One of the plants that is considered to have the potential to treat anxiety is *Moringa oleifera (M. oleifera)* .[8] Large groups of flavonoids such as kaempferol, quercetin, routine, chrysin and apigenin contained in *M. oleifera* leaves have anxiolytic effects.[9] Those compounds act as a central nervous system depressor through its interaction with the gamma-aminobutyricacid receptor (GABA) as in the diazepam molecules.[10]

A series of animal trials are needed on this alternative agent for pharmacological evaluation and initial screening of dose determination before testing in humans. Mice have organ components that can represent other mammals, especially humans.[11] Elevated plus maze (EPM) are the most common tools and have been validated to analyze anxiety based on the innate nature of mice that fear of high open areas.[12] The longer the mice were in the open arm and the more often the mice entered the open arm showed the anxiety reduction of the mice.[13] Previous research by Ingale and Gandhi (2016) using EPM showed that the intraperitoneal administration of aqueous extract of *M. oleifera* leaves at 375mg/kgBW and 500mg/kgBW effectively reduced anxiety of mice.[14] Intraperitoneal administration has high risk of injection errors that can cause organ damage to death. Oral administration is considered safer, but has lower bioavailability, because the drug will pass through the digestive tract before being forwarded to the systemic blood circulation.[15] Acute toxicity study conducted to determine median lethal dose (LD50) of the aqueous extract of *M. oleifera* leaves showed no mortality (non-lethal) at a maximum dose of 6400 mg/kgBW (p.o) in mice.[16] This study will examine the effect of aqueous extract of *M. oleifera* leaves at doses of 375mg/kgBW, 500mg/kgBW, and 625mg/kgBW peroral on the anxiety of mice measured with EPM.

RESEARCH METHOD

This study has obtained ethical clearance from the Ethics Commission at Faculty of Dentistry Gadjah Mada University, No.0055/KKEP/FKG-UGM/EC/2019. The study was conducted in March-April 2019 at the Physiology Laboratory, Faculty of Medicine Gadjah Mada University. Research materials used including *Moringa oleifera* leaves, Na-CMC, diazepam tablets, and aquabides. The tools used are gastric probe, EPM and a recording device connected to a computer. The research subjects consisted of 25 balb/c strain mice (*mus musculus)* which were divided into 5 treatment groups; negative control (K-) with 0.5% Na-CMC, aqueous extract of *Moringa oleifera* (AEMO) leaves 375 mg/kgBW, 500mg/kgBW, 625mg/kgBW, and

diazepam 1mg/kgBW as a positive control (K+). All treatment were carried out orally on mice.

Aqueous extract of *M. oleifera* is processed by maceration method. Extract was administered peroral using a gastric probe. Anxiety test on mice was measured using an EPM[14,17], performed before and 60 minutes after oral administration. Mice were placed in the middle of the maze facing one of the open arms and allowed to explore for 5 minutes. Duration in the open arms and the total entry into open arm are used as anxiety parameter of the mice. Before and after data were analyzed with paired T-test, differences of anxiety score between the groups was analyzed with One-way Anova followed by LSD Test at 95% confidence level using SPSS 0.17.

RESULTS

This study aims to evaluate the effect of aqueous extract of *M. oleifera* leaves peroral on anxiety in mice measured with EPM. The EPM is used to measure the anxiety of mice based on the duration of mice in the open arms and the frequency of entries into the open arms. An increase number in this parameter indicates anxiolytic effect.

The results of the study on 25 mice showed that positive control group, AEMO 500mg/kgBW, and AEMO 625mg/kgBW showed significant increase in average duration in the open arms of EPM ($p<0.05$), as listed in Table 1. Average frequency of entry into the open arms also had significant increase in positive control group, AEMO 500mg/kgBW, and AEMO 625mg/kgBW ($p<0,05$). The highest increase was seen in positive control group (Table 1). This showed that aqueous extract of *M. oleifera* 500 mg/kgBW and 625 mg/kgBW produced anxiolytic effects or decreased anxiety in mice. Group of AEMO 375mg/kgBW and placebo did not show a significant difference ($p >0.05$) before and after treatment.

Table 1. Average and Standard Deviation of Duration in Open Arms (seconds) and Frequency of Entry into Open Arms of EPM Tool Before and After Treatment and paired T-test

Parameter	Treatment Group	n	Mean			t	*p*
			Before	After	Difference		
Duration in Open Arms of EPM (seconds)	Placebo (K-)	5	78.6±61,3	71.2±63,8	-7.4±25,8	-0.641	0.557
	Diazepam (K+)	5	87.4±26,9	180.6±17,5	93.2±12,9	16.141	0.000
	AEMO 375 mg/KgBW	5	92.6±57,7	79.6±39,3	-13.0±19,9	-1.456	0.219
	AEMO 500 mg/KgBW	5	61.8±41,5	115.4±31,7	53.6±11,4	10.44	0.000
	AEMO 625 mg/KgBW	5	58.0±54,6	143.4±75,2	85.4±22,5	8.483	0.001
Frequency of Entry into Open Arms of EPM	Placebo (K-)	5	9.2±6,7	7.2±6,1	-2.0±4,0	-1.118	0,326
	Diazepam (K+)	5	6.0±3,7	15.0±2,9	9.0±2,4	8.216	0,001
	AEMO 375 mg/KgBW	5	9.4±6,4	9.4±4,4	0.0±3,5	0.000	1,000
	AEMO 500 mg/KgBW	5	7.6±4,7	11.2±3,6	3.6±2,4	3.343	0,029
	AEMO 625 mg/KgBW	5	5.8±3,2	12.2±4,9	6.4±3,0	4.693	0,009

To compare changes in anxiety between the groups, a one-way Anova test was performed with the results listed in Table 2. In both parameters, the highest difference score or the highest decrease in anxiety occurred in the diazepam group (K+), followed by the AEMO group at 625mg/kgBW and 500mg/kgBW. The Anova test on both parameters showed $p<0.05$, meaning that there were significant

differences in anxiety changes between the five treatment groups. LSD test was continued to compare differences between groups (Table 3).

Table 2. One-Way Anova Test of Average Differences of Duration in Open Arms and Frequency of Entry to Open Arms of EPM

Parameter	Treatment Group	Mean	F	*p*
Duration in Open Arms (seconds)	Placebo (K-)	-7.4±25,8	33.751	0,0001
	Diazepam (K+)	93.2±12,9		
	AEMO 375 mg/KgBW	-13.0±19,9		
	AEMO 500 mg/KgBW	53.6±11,4		
	AEMO 625 mg/KgBW	85.4±22,5		
Frequency of Entry to Open Arms	Placebo (K-)	-2.0±4,0	10.222	0,0001
	Diazepam (K+)	9.0±2,4		
	AEMO 375 mg/KgBW	0.0±3,5		
	AEMO 500 mg/KgBW	3.6±2,4		
	AEMO 625 mg/KgBW	6.4±3,0		

Table 3. LSD Test Between Treatment Groups at Duration in Open Arms and Frequency of Entry into Open Arms of EPM

Treatment Group	Duration in Open Arms	Frequency of Entry into Open Arms
	p	*p*
Placebo - Diazepam	0,000	0,000
Placebo - AEMO 375 mg/KgBW	0,652	0,327
Placebo - AEMO 500 mg/KgBW	0,000	0,011
Placebo - AEMO 500 mg/KgBW	0,000	0,000
Diazepam - AEMO 375 mg/KgBW	0,000	0,000
Diazepam - AEMO 500 mg/KgBW	0,004	0,013
Diazepam - AEMO 625 mg/KgBW	0,531	0,207
AEMO 375 mg/KgBW - AEMO 500 mg/KgBW	0,000	0,086
AEMO 375 mg/KgBW - AEMO 625 mg/KgBW	0,000	0,004
AEMO 500 mg/KgBW - AEMO 625 mg/KgBW	0,017	0,175

The results of LSD test showed significant difference in the parameter of duration in open arms and frequency of entry into open arms between the placebo group and other groups ($p<0.05$), except AEMO 375mg/kgBW ($p>0.05$). Compared to diazepam group, AEMO 625mg/kgBW did not show significant difference ($p>0.05$), but significant with other groups ($p<0.05$) in both parameters. This shows that aqueous extract of *M. oleifera* leaves at a dose of 625mg/kgBW statistically has an equivalent effect to Diazepam (1mg/kgBW) in reducing anxiety of mice.

DISCUSSION

M. oleifera leaves are one of the medicinal plants that are considered beneficial in treating anxiety. This study was designed to examine the effects of aqueous extract of *M. oleifera* leaves on

anxiety of mice using an animal behavior pharmacological test. The anxiolytic effect of aqueous extract of *M. oleifera* leaves was evaluated using the EPM.

Anxiolytic effect occurred in the group of mice given Diazepam 1 mg/kgBW, aqueous extract of *M. oleifera* leaves 500mg/kgBW and 625mg/kgBW. The highest anxiety reduction was found in diazepam group, followed by aqueous extract of *M. oleifera* leaves 625 and 500 mg/kgBW in both parameters. Aqueous extract of *M. oleifera* leaves at a dose of 625mg/kgBW produced an equivalent effect to diazepam in reducing anxiety of mice. Aqueous extract of *M. oleifera* leaves 375 mg/kgBW orally did not produce anxiolytic effect on mice.

Previous studies by Ingale and Gandhi (2016) using aqueous extract of *M. oleifera* leaves 375 and 500 mg/kgBW intraperitoneal showed a significant increase in the duration and total entry into open arms of EPM compared to placebo.[14] The results of this study corroborate these findings, which aqueous extract of *M. oleifera* leaves 500 and 625mg/kgBW peroral significantly increase the number of both parameters compared to placebo, while doses of 375mg/kgBW did not show significant difference with placebo. This can be due to the oral administration of the extract that will pass through the digestive tracts before being passed on to the systemic blood circulation so its bioavailability is lower.[15]

Ingale and Gandhi (2016) in their study confirmed the involvement of GABA in anxiolytic activity of AEMO.[14] The role of the GABA inhibitory neurotransmitter is known as a center for anxiety regulation, and this neurotransmitter system is a target of benzodiazepines and other related drugs used to treat anxiety disorders. Benzodiazepines such as diazepam bind and activate the $GABA_A$ receptor, causing an increase in the frequency of the opening of the Cl-channel, resulting in hyperpolarization of the membrane, which in turn leads to decreased activity or inhibition of neurons in the brain.[19] However, further research is needed to better understand the role of GABA in AEMO.

Phytochemical test revealed the presence of large amounts of flavonoids contained in aqueous extract of *M. oleifera* leaves.[14] Furthermore, it is known that most of the flavonoid groups such as kaempferol, quercetin, routine, chrysin and apigenin contained in *M. oleifera* leaves bind to $GABA_A$ receptors so that they can reduce anxiety as in the benzodiazepine molecules.[9] Thus, the results of this study indicate that aqueous extract of *M. oleifera* leaves has an effect on reducing anxiety at doses of 500 and 625 mg/kgBW in mice, with the dose of 625mg/kgBW resulting the highest anxiety reduction.[20]

CONCLUSION

It was concluded that aqueous extract of *M. oleifera* leaves at 500mg/kgBW and 625mg/kgBW peroral effectively reduce anxiety of mice. Aqueous extract of *M. oleifera* leaves at the dose of 625 mg/kgBW has an equivalent effect to Diazepam (1mg/kgBW) in reducing anxiety of mice.

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