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Anxiolytic Activity of Ethanolic Extract of Three Species of Indonesian Lempuyang (*Zingiber zerumbet, Zingiber aromaticum,* and *Zingiber americans*)

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Abstract

BACKGROUND: Lempuyang, or widely known as wild ginger, has been used in Indonesia as traditional medicine for a long time. However, scientific research supporting its medicinal use is still very limited.

AIM: This research was conducted to investigate the anxiolytic activity of three species of Indonesian Lempuyang (*Zingiber zerumbet, Zingiber aromaticum,* and *Zingiber americans*).

METHODS: Sixty-six male Swiss Webster mice were divided into 11 groups (negative control, positive control, and experiment groups consisting of low, moderate, and high dose of *Z. zerumbet, Z. aromaticum*, and *Z. americans* extract, respectively). Anxiolytic activity was evaluated by three methods: elevated plus maze test, open field test, and hole board test.

RESULTS: The results showed that these three species of Indonesian Lempuyang lowered the anxiety response in elevated plus maze test, open field test, and hole board test compared to negative control ($p < 0.05$). The best anxiolytic activity was achieved by moderate dose of *Z. americans* (8.5 mg/20 gbb), consistently through three methods applied.

CONCLUSION: This research supports the potential use of the ethanolic extract of Lempuyang (*Z. zerumbet, Z. aromaticum,* and *Z. americans*) as a complementary therapy for anxiety.

Introduction

Anxiety can be defined as a continuous state of worry that something bad is going to happen. The symptoms of anxiety are varied from individual to individual. Symptoms include unpleasant feeling, excessive fear, as well as physical symptoms such as sweating, headache, palpitation, abdominal pain, restlessness, and inability to stand or sit for a long time [1]. It is estimated that 4.4% of the global population suffers from depression and another 3.6% suffers from anxiety disorders. Southeast Asia has the highest prevalence of anxiety disorders, approximately 23% of the global population. In Indonesia, Ministry of Health reported that the incidence of psychiatric disorder such as depression and anxiety symptoms among individuals aged over 15 years old was estimated to be around 14 million people (approximately 6% of the population of Indonesia). In 2018, the increase in the proportion of mental disorders shows a significant amount of data compared to 2013, up from 1.7% to 7% [2].

Lempuyang, generally known as wild gingers, is a medicinal plant from Zingiberaceae family traditionally used in Indonesia to relieve symptoms of anxiety. *Zingiber zerumbet, Zingiber aromaticum,* and *Zingiber americans* are three common species of Lempuyang cultivated in Indonesia. These three species of Lempuyang can be differentiated by its rhizome organoleptics. *Z. americans* has smaller rhizome size compared to the other two rhizomes. Rhizome of the *Z. zerumbet* has a paler yellow color than the others, and *Z. aromaticum* has more fragrant than the others [3]. These three species of wild gingers are often used as an Indonesian herbal medicine and their functions can be substituted with one another [4].

Z. zerumbet, *Z. aromaticum*, and *Z. americans* contain alkaloids, flavonoids, tannins, and essential oils. The major constituent of its essential oils is sesquiterpene such as humulene, camphene, α-caryophyllene, camphene, and zerumbone [5]. *In vivo* study showed that zerumbone reduced locomotor activity, indicating its potency as an anxiolytic, relaxant, and sedative agent [6]. Anxiolytic-like effect of zerumbone was also evaluated in scopolamine-induced anxiety in rats [7].

These three species of Lempuyang were commonly used in Indonesia as traditional medicine,

however, scientific research supporting its medicinal use is still very limited. This study was aimed to evaluate the anxiolytic effect of *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* in low, moderate, and high dose using elevated plus maze, open field test, and hole board test.

Materials and Methods

Materials

Rhizomes of *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* were collected from the Manoko Experimental Plantation, Research Institute for Spices and Medicines, Indonesian Ministry of Agriculture. Fresh rhizomes were collected, washed, sliced into thin slice (approximately 2 mm), and then dried in room temperature without direct sunlight. Dried rhizomes were pulverized into powder and were kept in tight amber container. Diazepam was used as a reference drug obtained from local pharmacy (Kimia Farma, Tbk.).

Extraction and preparation of plant material

Powdered dried rhizomes of *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* were extracted using maceration method with 96% ethanol as a solvent in 1:10 proportion and stirred occasionally during the first 24 h. Extracts were remacerated with fresh solvent after 24 h and 48 h. Filtrates were evaporated in rotary evaporator (Buchi® R300) followed by water bath in controlled temperature (50°C). Dried extracts were weighed to calculate the yield of extract. Due to its lipophilic properties, extracts were prepared in o/w emulsion formulation using tween and span as an emulsifying agent.

In vivo anxiolytic test

The protocol applied in this study was approved by the Ministry of Health - Poltekkes Kemenkes Bandung Ethical Committee with approval number: 01/KEPK/EC/IX/2020 in September 2, 2020. Adult male Swiss Webster mice (*Mus musculus*) were procured from local standardized breeding house. They were habituated in institutional animal laboratory in standard environment condition for at least 7 days prior to experiment. Mice was placed in cage with normal light/dark cycle (light from 6 am to 6 pm and vice versa), therefore the experiment only conducted between 7-10 am in quiet condition.

Animals were divided into the following groups: negative control (no treatment, administered CMC-Na as vehicle), positive control (administered with diazepam 0.26 mg/kg p.o as a reference drug), three groups of ethanolic extract of *Z. americans* (EEZAm), three groups of ethanolic extract of *Z. zerumbet* (EEZZ), and another three groups of ethanolic extract of *Z. aromaticum* (EEZAr) at doses 212.5, 425, and 850 mg/kg, respectively. All treatments were administered orally 1 h prior to experiment.

Hole board test

The apparatus is composed of acrylic box (50 cm × 50 cm × 20 cm) with black opaque floor. The apparatus floor is positioned 20 cm above ground, with 16 equidistant holes with 2 cm in diameter. Mice were placed in the middle of apparatus and allowed freely to explore the apparatus during 5 min of observation. Anxiolytic effect was evaluated by counting the frequency of head dips, when both eyes crossed the floor holes.

Open field test

Open field apparatus is a simple square box apparatus (50 cm × 50 cm × 25 cm), divided into center and edge parts. Edge parts were positioned from the very edge of apparatus up to 10 cm diagonally into the central part. Mice were placed in the center area of apparatus and allowed freely to explore the apparatus during 5 min of observation. Anxiolytic effect was evaluated by counting the duration and frequency of animals in center area of apparatus.

Elevated plus maze test

Elevated plus maze apparatus is a plus (+) shaped apparatus consisting of two open arms and two closed arms, positioned 50 cm above ground. Mice were placed in the center area of apparatus and allowed to move freely and explore the apparatus during 5 min of observation. Anxiolytic effect was evaluated by counting the duration and frequency of animals in open arms of apparatus.

Statistical analysis

Data were presented as mean±standard error mean. Statistical analysis was employed using Kruskal–Wallis test followed by Mann–Whitney *post hoc* test in SPSS statistical programming. $p \leq 0.05$ was considered statistically significant.

Results

Yield extraction

Z. zerumbet, *Z. aromaticum*, and *Z. americans* were successfully extracted with maceration method

using 96% ethanol as solvent. Extraction yields *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* were 10.839%, 10.7748%, and 13.256%, respectively.

Hole board test

As shown in Figure 1, the negative control group possessed the highest average number of head dips compared to other experimental groups. Diazepam, as a reference drug, significantly decreased the average number of head dips compared to negative control (p<0.05). In the EEZAr group with various doses, it appears that increasing the dose caused a decrease in the average number head dips. In addition, EEZAr in moderate and high dose was statistically significant in decreasing the average number of head dips compared to negative control (p<0.05). In the EEZZ group, it also appeared that increasing the dose also caused a decrease in the average number of head dips. In contrast to the other two Lempuyang species groups, in the EEZAm groups, the moderate dose of EEZAm (425 mg/kg) showed the highest decrease in head dips (p<0.05), compared to the low (212.5 mg/kg) and high dose (850 mg/kg).

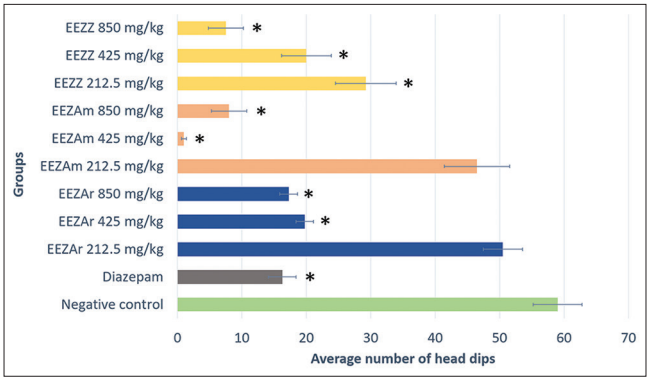

*Figure 1: Average number of head dips for each group in hole board test (*p < 0.05 against control)*

Open field test

Figure 2 shows the average entry number of experimental animals to center area of apparatus. Diazepam increased the average entry number of experimental animals to the center area. All of the Lempuyang extract groups, except for the EEZAr 425 mg/kg and 850 mg/kg groups, also showed an increasing number of entries to the center area. In this study, it appeared that there are differences in frequency patterns between experimental animals in three Lempuyang species groups. The EEZAr group showed a decreasing frequency to the center area along with dose escalation. Meanwhile, in the EEZAm group, frequency of number entries increased at moderate doses (425 mg/kg) and decreased at high doses (850 mg/kg). The EEZZ group showed an increased frequency to center area along with dose escalation.

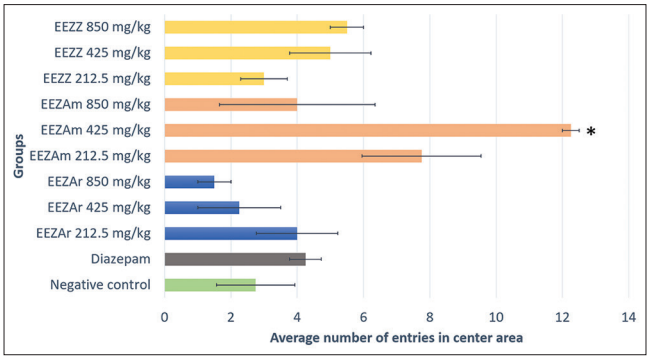

*Figure 2: Average number of entries in center area for each group in open field test (*p < 0.05 against control)*

Another parameter observed in open field test was the time spent of the animals in the center area. The average time spent of experimental animals in the center area of each group is shown in Figure 3. Figure 3 shows the anxiolytic activity of all experiment groups by increasing the time spent of experimental animals in the center area. Diazepam induced the highest increased time spent in center area compared to all the other experiment groups (p<0.05). The EEZAr and EEZZ groups showed a similar pattern of activity, causing an increased time spent in the center area along with increasing doses. In contrast to the two other Lempuyang species, in the EEZAm group, the best anxiolytic activity was shown by the moderate-dose group (425 mg/kg). At higher dose, EEZAm caused a decrease in anxiolytic activity, indicated by decrease time spent of experimental animals in the center area.

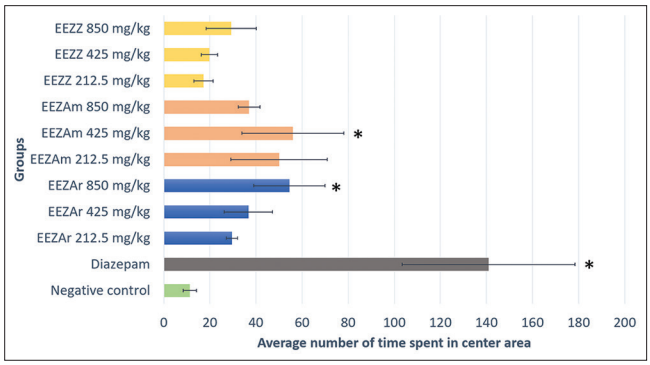

*Figure 3: Average number of time spent in center area for each group in open field test (*p < 0.05 against control)*

Elevated plus maze test

Figure 4 shows the average number of entries to open arms in the elevated plus maze. Diazepam induced the highest average number of entries compared to other groups (p<0.05). EEZAr increased an average number of entries to open arms, with the highest activity achieved by moderate dose (425 mg/kg) (p<0.05). Low dose of EEZZ and EEZAm (212.5 mg/kg) did not show an anxiolytic effect, but average number of entries appears to increase along with increasing doses.

Another parameter observed in elevated plus maze was the time spent of the animals in the open arms.

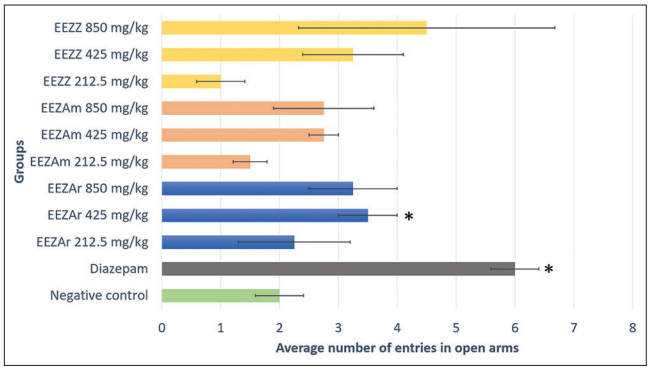

*Figure 4: Average number of entries in open arms for each group in elevated plus maze (*p < 0.05 against control)*

The average time spent of experimental animals in the open arms of each group is shown in Figure 5. Diazepam significantly increased the animal duration in open arms compared to negative control (p<0.05). At low dose (212.5 mg/kg), all three species of Lempuyang had not shown an increasing time spent of animals in open arms compared to negative control. Time spent in open arms increased in moderate (450 mg/kg) and high (850 mg/kg) dose in all experimental groups. There were different patterns of these three Lempuyang species observed in this study. The EEZAr and EEZZ groups showed increased time spent in the open arms along with increasing dose. Unlike EEZAr and EEZZ groups, moderate dose of EEZAm group (425 mg/kg) induced a longer duration in open arms compared to high dose (850 mg/kg).

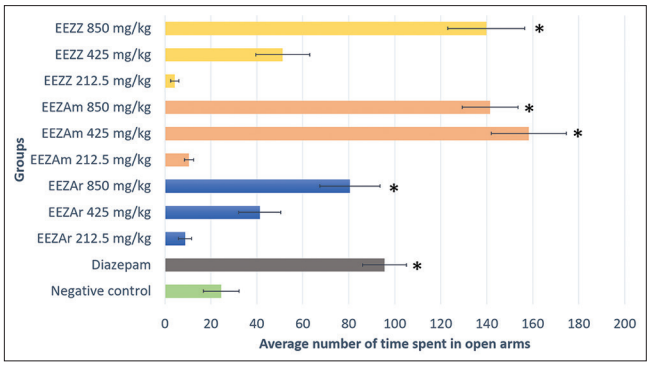

*Figure 5: Average number of time spent in open arms for each group in elevated plus maze (*p < 0.05 against control)*

Discussion

Anxiolytic effect of *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* ethanolic extract was evaluated through three methods. Methods were used in this study including hole board test, open field test, and elevated plus maze. It is necessary to evaluate the anxiolytic activity in various methods. Anxiety in rodents is a complex process, thus assessing anxiety in rodents with only single method is not preferred due to lack of validity. Multiple methods or parameters can strengthen the evaluation of the results [8].

Hole board test is often used to evaluate locomotor activity in experimental animals. Hole board test measures an animal tendency to dip its head through holes in apparatus floor [9], [10]. The hole board method is also used to measure the curiosity and exploration behavior of test animals. This method is commonly used because it offers a simple method to observe the behavioral responses of experimental animals to a new environment, and has been widely used to evaluate drug effects [11]. Drugs affecting central nervous system may affect the locomotor and activity of experimental animals on the hole board, including sedatives, hypnotics, anti-anxiety, anxiogenic, stimulants, and central nervous system depressants [10], [12], [13].

All species of Lempuyang reduced the head-dipping response in the hole board, indicating a decreasing locomotor activity of the experimental animals. In this study, decreased locomotor activity was also observed in the diazepam group. Diazepam is a group of benzodiazepine drugs with clinical indications as anti-anxiety and hypnotic sedative. At low doses, diazepam causes an anxiolytic effect, and as the dose increases, the effect increases from anxiolytic to sedative hypnotic [14]. In hole board test, anxiolytic effects are generally demonstrated by increasing head dips, meanwhile at higher dose, diazepam has sedative effect which caused a decreasing head dips. This phenomenon is commonly known as the biphasic response (increasing animal response at lower dose, and decreasing it at higher dose). It can be concluded that lempuyang possessed a similar sedative effect like diazepam, which reduced the frequency of head dips [15], [16]. Head dips are often associated with emotional status of experimental animals, and anxiolytic may decrease head dip response [13]. Experimental animals placed in hole board are known to have an elevated corticosteroid level, which may indicate anxiety state. Head dips are presumed as animal response as an attempt to find a potential escape route. It can be concluded that decrease in head dips indicates a decrease of fear or anxiety [10].

Open field test is a common method to evaluate the psychological behavior of an experimental animal. This is a simple method and provides various information regarding neurobehavioral response, such as ambulatory and emotional state in an experimental animal. This method is commonly used to evaluate the effect of sedative, stimulant, anxiolytic, drug affecting neuromuscular, and drug affecting locomotor activity [8], [17], [18]. Open field test is also often used specifically to evaluate anxiety behavior and flight response to a certain stimulus due to rodent tendency to show aversion to a new, spacious, and bright environment [19]. Rodents phylogenetically consider these stimuli as a danger situation as they might be seen by their natural predators. This test represents animal conflict between its nature of fear to open space and animal curiosity to a new environment. In situation

when animals experience fear or anxiety, animals tend to avert from center area of apparatus and prefer to be in edge area near the apparatus wall [8], [19].

Elevated plus maze is the most common behavioral test to evaluate anxiety in animals due to its simplicity and specificity and also this test does not require a specific preparation prior to treatment. This test was designed based on rodent tendency to explore new environment versus its natural fear of unprotected, bright, and elevated new environment (indicated by the open arms of apparatus). The critical parameter in this test is the time spent in open arms of apparatus. Placing rodents in open arms may induce physiological stress such as increasing defecation and elevated corticosteroid level. Administration of anxiolytic agent such as benzodiazepine can decrease animal natural fear and increase animal response to explore the apparatus and thus can be observed by prolongation time spent in open arms. In elevated plus maze, the entries number (frequency) to open arms represent locomotor activity, meanwhile duration in open arms represent the quality of anxiolytic property of a substance [16], [20], [21].

Ethanolic extract of three species of Indonesian Lempuyang (*Z. zerumbet*, *Z. aromaticum*, and *Z. americans*) provides an anxiolytic effect on experimental animals consistently through three test methods performed. In Indonesia, Lempuyang has been traditionally used as an empirical-based herbal medicine, known as Jamu [3]. Of all parts of the plant, the rhizome of Lempuyang has been extensively studied due to its plentiful pharmacological effect. Rhizome of Lempuyang contains polyphenol, alkaloid, and essential oils composed of 86% sesquiterpenes, approximately [22]. In quantitative analysis, zerumbone is a major essential oil found in rhizomes followed by humulene, caryophyllene, and zingiberene. Essential oils also contain monoterpenes borneol, α-pinene, champor linalool, γ-terpinene, and β-phellandrene [22], [23].

Zerumbone is a monocyclic sesquiterpene with three double bonds: two bonds are coupled with carbonyl group and the other is isolated. Qualitative analysis has been studied to investigate zerumbone content in various plant parts of Lempuyang, and the result showed that the rhizome contains the highest zerumbone content compared to other parts of the plant [22]. Zerumbone is a volatile compound and generally has a distinctive odor, so it is traditionally used for aromatherapy and relieving anxiety. Research shows that zerumbone can reduce the total locomotor activity (TLA) of experimental animals. A decrease in locomotor activity may indicate zerumbone activity as a sedative, anxiolytic, relaxant, or hypnotic agent [24]. However, further research is needed to evaluate the mechanism of action of zerumbone in reducing locomotor activity specifically. Anxiolytic properties of zerumbone are also demonstrated in scopolamine-induced memory impairment in rats. Zerumbone has positive effect in managing hyperactivity, anxiety, and enhancing cognitive response in rats. Zerumbone is known to mediate anxiolytic effect through its interaction with $GABA_A$ receptor in the brain, thereby increasing brain inhibitory activity [7].

In addition to zerumbone, β-caryophyllene compounds in Lempuyang also have an anxiolytic effect by acting as a selective agonist at the cannabinoid-2 receptor which plays a role in mood regulation and anxiety. Another monoterpene compound, α-pinene, is known to be widely distributed in brain tissue and has been associated with anxiolytic activity. The compounds of the terpenoid class are generally lipophilic, resulting in its high ability to pass through blood–brain barriers and can affect brain activity [25], [26].

The rhizome of Lempuyang also contains flavonoids including 3-methyl kaempferol, kaempferol-3-O-(2,4-di-O-acetyl-α-l-rhamnopyranoside), and kaempferol-3-O-(3,4-di-O-acetyl-α-l-rhamnopyranoside) [27]. Kaempferol is known to have an anxiolytic effect by reversibly inhibiting the activity of the MAO-A and MAO-B enzymes [28], [29]. MAO-A and MAO-B enzymes are two major enzymes which play an important role to degrade monoamine compounds. Inhibition of this enzyme will cause the accumulation of amine neurotransmitters in the brain related to pathophysiology of anxiety such as dopamine, serotonin, and norepinephrine [1].

Anxiolytic compounds in *Z. zerumbet*, *Z. aromaticum*, and *Z. americans*, such as β-caryophyllene, α-pinene, and kaempferol, were extracted using 96% ethanol. Ethanol is a common solvent for herbal medicine due to its safety profile compared to other organic solvents. Various studies also show that 96% ethanol can extract active components of *Z. zerumbet*, *Z. aromaticum*, and *Z. americans*. Zerumbone is a polar compound and thus can be easily extracted in 96% ethanol. A previous study concluded that 96% ethanolic extract of Lempuyang contains 28.05%–30.95% zerumbone [30]. Other terpenoids extracted by 96% ethanol solvent were α-pinene, β-caryophyllene, and α-humulene [31]. Another study suggested that flavonoid kaempferol was also extracted in this solvent [32]. Further research is needed to quantify the exact amounts of anxiolytic compounds in *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* used in this study.

The three species of Lempuyang have long been used empirically in Indonesia and have been documented in Materia Medika Indonesia, a standard reference book specifically used for herbal medicine since 1978. In this reference, the requirement for essential oil content of *Z. aromaticum* rhizome should not be lower than 0.4%, meanwhile for *Z. zerumbet* and *Z. americans*, essential oil content should not be lower than 0.5% and 0.6%, respectively. [33]. This reference states a higher requirement for essential oil content in *Z. americans* compared to the other two species, indicating higher content of essential oils. This finding is consistent with the results of the study where *Z. americans* showed the highest anxiolytic activity. But then again, further

research is needed to compare the levels of active compounds responsible for anxiolytic activity.

In *Z. americans* extract, the optimum anxiolytic activity is achieved by moderate dose (425 mg/kg). On the contrary, at higher dose (850 mg/kg), Z. americans produced less anxiolytic effect. This phenomenon tends to be similar with biphasic dose–response pattern, also known as hormetic dose–response. This phenomenon was only observed in *Z. americans* extract, but not in *Z. zerumbet* and *Z. aromaticum*. These findings suggest the following opportunities to investigate the possibility of hormetic dose–response in *Z. zerumbet* and *Z. aromaticum* in higher doses, considering that hormesis dose–response is commonly reported in the anxiolytic drugs group during preclinical test [34]. The specific mechanism of the hormetic dose–response of *Z. americans* has not been discovered yet, but hormetic mechanism of diazepam has been studied in several literatures.

Diazepam hormetic dose–response is often reported in anxiolytic test due to its sedative side effect [16]. This hormetic effect is also thought to be due to a mixed agonist–antagonist effect of diazepam interaction on two receptor sites, with high affinity for the agonist site and low affinity for the antagonist site. Diazepam is known to have an anxiolytic effect at low doses due to its ability to bind to the allosteric benzodiazepine site, type I GABA receptor subunit. The possible mechanism of hormetic effects is due to the presence of allosteric kinetic modulation at the receptor subunit, as a result of the simultaneous interaction of compounds in a receptor with unequal accelerations, causing changes in receptor affinity and density at a certain dose point. This is an adaptive response to maintain homeostasis that allows endogenous compounds to adjust in response to altered GABA regulation caused by certain drugs [34], [35].

Thus, it can be concluded that among the three Lempuyang species evaluated in this study, the best anxiolytic activity was achieved by moderate dose (425 mg/kg) of *Z. americans* ethanolic extract. The results of this study corroborate previous research conducted by Ogawa *et al*. (2014), which stated that the active compounds of Lempuyang were able to reduce locomotor activity, indicating sedative and anxiolytic activity, as well as the research of Jafarian *et al*. (2019), which stated that the active compounds of Lempuyang were able to reduce hyperactivity and anxiety and improve cognitive abilities in scopolamine-induced memory impairment in experimental animals.

The development of herbal-based drugs does not only focus on aspects of drug efficacy but also needs to consider the safety aspects of compounds. Ethanolic extract of Lempuyang with dose up to 3000 mg/kg had not shown any toxic effects in acute and chronic toxicity testing. It can be concluded that the ethanol extract of Lempuyang is practically non-toxic [27], [36]. This safety profile supports the potential use of ethanolic extract of Lempuyang as a complementary therapy for anxiety.

Conclusions

The present study concluded that 96% ethanolic extract of *Z. zerumbet*, *Z. aromaticum*, and *Z. americans* possesses anxiolytic effect in hole board test, open field test, and elevated plus maze test. Moderate dose (425 mg/kg) of *Z. americans* extract provides the highest anxiolytic effect compared to other groups. This research supports the potential use of the ethanolic extract of Lempuyang (*Z. zerumbet*, *Z. aromaticum*, and *Z. americans*) as a complementary therapy for anxiety.

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Author Contribution

All authors contributed in carrying out the experiment, discussed the results, and contributed to the final manuscript.

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