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Preliminary Phytochemical Screening, Acute Oral Toxicity and Anticonvulsant Activity of the Rhizomes of Acorus gramineus Soland.

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Abstract : The present of study is carried out to investigate the preliminary phytochemical properties, acute oral toxicity and anticonvulsant activity of the rhizomes of *Acorus gramineus* Soland. (*A. gramineus*). Standard methods were applied to screen phytochemicals from the methanol rhizomes extracts. The performance of acute oral toxicity study following Organization for Economic Cooperation and Development (OECD) 425 guidelines had been run before the anticonvulsant activity evaluation of resisting pentylenetetrazole (PTZ) induced seizure in mice was figured out. In the dose at levels of 100, 200, 300 and 400 mg/kg body weight, the treatment's impact was estimated through an experimental mice model and compared with phenobarbital (100 mg/kg p.o.) as positive control. Histopathological analysis is also applied for verifying the direct effects of *A. gramineus* extract on mice's brain. Carbohydrates, flavonoids, alkaloids, tannins, phenolic compounds, anthraquinones, steroids and terpenoids are qualified in phytochemical screening. The oral median lethal dose of the rhizome extracts was estimated as upper 5000 mg/kg body weight. In PTZ-induced seizures, the extracts significantly retarded the latency of convulsant ($p < 0.05$) in at dose of 300 and 400 mg/kg p.o., decreased the frequency of convulsant and increased up to 100% protection resistant to death. The histopathological analysis revealed the impacts of extract on brain tissue in the dose of 300 and 400 mg/kg p.o. The findings proved in this study suggest that the methanol extracts of *A. gramineus* rhizomes is dependable and high potential in anticonvulsant activity in PTZ-induced seizure in mice.

Keywords: *Acorus gramineus*, Anticonvulsant, Phytochemical, Acute Oral Toxicity, Lethal Dose (LD50), Pentylenetetrazole, Histopathological.

I. INTRODUCTION

Medicinal plants have been widely used for thousands years ago as the most effective therapy for many diseases. In oriental countries, until now, medicinal plants still use in parallel with western medicine in the treatment of disease. The evidence shows that more than 80% of the population in developed countries is still using the traditional medicine found in the locality and the surrounding area [1]. *Acorus* is a genus of monocot flowering plants, which is the sole genus of the ancient surviving line of monocots, among them, the common species are *A. gramineus* [2]. In medical books of ancient China recorded, *A. gramineus* used to therapy of diarrhoea, gastralgia, cough, bronchial asthma, neurasthenia, fever, convulsions, rheumatism, osteodynia, arrhythmia [3] and particularly epilepsy in combination. Moreover, the extract was used in the traditional Chinese prescription and its beneficial effects on memory disorders, on learning performance and anti-aging effect in senescence have been reported [4]. That is the reason why it has been widely used to this day as a special kind of panacea for neurological diseases [5].

The brain is made up of millions of nerve cells called neurons. They generate electrical impulses and messages to control the human thoughts, emotions and movement. Epilepsy is known as the brain disorder when sudden bursts of electrical activity in the brain disrupt normal pattern of these impulses.

Symptoms of epilepsy are disturbing of normal neural pattern activity, causing strange sensations in emotion and behavior and can lead to the critical cases of seizures, muscle spasms and loss of consciousness [6]. The normal electrochemical activity of the brain that results in seizures is terribly disrupted when patients contracted epilepsy. Everyone has a risk of suffering seizure under the certain circumstances. It is only when there is a tendency to have more than one recurrent seizure that epilepsy is diagnosed [7]. More and more people are with epilepsy, according to Government. Epilepsy (Seizures Disorders) (2013) statistics, the ratio is 0.05% (about 50 per 100,000) of the population in the developing countries with epilepsy while that number in the developing countries is 0.1% (about 100 per 100,000) of the population [8]. In Vietnam, people with epilepsy accounted 0.44 – 0.55% of the population, however, only about 29/189 (0.15%) patients were treated with antiepileptic drugs (AEDs) in accordance periodically (as reported by WHO) [9]. Moreover, epilepsy doesn't spare the developed countries such as Ireland in which one in 115 people is with epilepsy (over 0.008% population). Almost epilepsy patients have no family member with epilepsy [10]. Antiepileptic drugs (AEDs) are widely used in the treatment for epilepsy that sometimes cannot control seizures in some patients. In addition, the medication also causes hypersensitivity reactions, weight loss, and drug interactions which is the reason of causing toxicity central nervous system [11]. Over the course of strict control, AEDs in commercializing have been discovered that it does not really control seizures effectively on more than 25% of patients [12]. According to the studies, the commonly used anti-epileptic medications such as phenytoin, carbamazepine and sodium valproate come with a list of serious side effects, particularly neurological toxicity [13]; it is not to mention the risk of high latent drug interactions in combination with the use of other drugs like predisposes [14, 15, 16]. Therefore, conducting research and testing of new agents potentially for effective treatments as well as real safety toxicity in anti-epilepsy is absolutely necessary. That is why this study was undertaken to find a non-toxic agent capable of treating epilepsy.

II. MATERIALS AND METHODS

Plant materials

The rhizomes of *A. gramineus* were collected in a mountain of Ban Don, Buon Don district, Buon Me Thuot city of Vietnam in April, 2014. This medicinal plant was identified and certified by Dr. Hoang Le Son, Head of Applied Biochemistry Department - International University (IU) - Viet Nam National University-Ho Chi Minh City, Viet Nam. The specimen was deposited and in the herbarium of Applied Biochemistry Laboratory, Department of Applied Chemistry, School of Biotechnology, International University, Viet Nam National University-Ho Chi Minh City, Viet Nam with voucher No. HB-BIO-14-04-28.

Chemicals

Pentylenetetrazole (PTZ), code P6500-25g was purchased from Sigma-Aldrich (St. Spruce, Saint Louis, MO63103, USA), absolute methanol was procured from Merck Millipore Vietnam Co., Ltd. Phenobarbital (100 mg, Sanofi-Aventis industries, USA). All drugs and chemicals were stored in accordance with the most stringent regulations and fresh prepared with distilled water to the desired concentration.

Experimental animals

The Albino Swiss mice *Mus musculus var*., weighing 20-30 g, were purchased from Pasteur Institute of Ho Chi Minh City in good health and mood. They were fed in individual clean cages which let them free access to standard diet and water ad libitum. During the study, mice were kept in the best condition under a temperature controlling system and environmental condition with 12 h of light and dark cycle. All the animals were fed on laboratory conditions to acclimatize for a week prior to the beginning of

experiments. Moreover, all experimental animals were treated kindly and gently, without any abuse and violence following the animal use ethics as international acceptance. All authors hereby declare that the experiment was strictly complied with the regulations of "Principles of laboratory animal care" (NIH publication No.85-23, revised 1985), as well as specific rules and laws of nation applied [17].

Extraction

Fresh rhizomes of *A. gramineus* were dried and ground into a fine powder. The powdered rhizomes (120g) were extracted with 480 mL of 80% methanol (50-80%) in Soxhlet apparatus. The process was carried out for 72 hours continuously [18]. Then the crude extract was filtered and evaporated in vacuum by rotary evaporator until forming a brownish residue. The residue, also known as total extracts, was cold stored in the refrigerator for the best condition until the next use. The efficiency of the extraction was 28.55%.

Phytochemical screening

Phytochemical analysis of the extract was conducted for the detection of active pharmaceutical constituent [19].

Oral acute toxicity test

In vivo, acute toxicity of the plant extract was performed in healthy female albino mice labelled individually with the weight among 20 - 30 g. Before taking the dose, the mice had to be fasted overnight and each mouse was taken its specific dose depended on their current weight. Dried extracts were fed in the aqueous form and be prepared to the desired concentration with distilled water. Following the Organization of Economic Cooperation and Development (OECD/OCDE) Test Guidelines on Acute Oral Toxicity supported by the computer-guided Statistical Programme-AOT425statPgm, version 1.0, the procedure was effectuated. The Up and Down Lethal Dose 50 (LD50) protocol and classifying category of toxicity based on the provisions of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) as adopted by the United Nations Economic and Social Council in July 2003 were conducted.

Limit test

At the beginning, each mouse was absorbed a single dose of 2000 mg/kg through oral administration by feeding tube, which the gavage is dedicated to feeding mice. Then, food was retained during 3 - 4 hours. As the first observed phase, animals were under the observation continuously for 30 minutes, and during the next 24 hours periodically as the second phase. In case of surviving animals, four additional mice were administrated and observed the same dose and the same method to complete the protocol. All the alive animals were remained for further observation in the next 14 days.

Then, the extracts were given to every single mouse in a single dose up to 5,000 mg/kg p.o. The same procedure was carried out as the previous one for this step. As the first observed phase, animals were under the observation continuously for 30 minutes, and during the next 24 hours periodically as the second phase. In case of surviving animals, four additional mice were administrated and observed the same dose and the same method to complete the protocol. If all of mice were still alive, it is able to conclude “non-toxicity property” without performing the main test. If not, when all the animals died, the main test was figured out.

Anticonvulsant activity methodology

Mice were randomly arranged into 6 groups with 5 animals per group (n = 5). Group I were fed equal amounts of distilled water and was regarded as negative control. Group II, III, IV and V were

respectively received an amount of extract at doses of 100, 200, 300 and 400 mg / kg, p.o.. Meanwhile, group 6 represented as possitive control, received phenobarbital at dose of 100 mg/kg, p.o.. All doses of extract and AEDs were given to mice 60 minutes before the injection of PTZ (85mg / kg, i.p.) at lethal dose and then the convulsant phenomenon was observed strictly and continuously. The mice were observed and recorded the latency and frequency of convulsant for every single mouse which placed in individual cage during 1 hour. The values were performed through the formula Mean ± Standard Error of the Mean (SEM). Manifestations of seizures were evaluated based on the scale of 3 levels with 6 different expressive points according to Racine's scale, the scale widely used in epilepsy research dedicated to animal models [20, 21].

Table 1: 6-points scale for anticonvulsant activity.

Light seizures	Intermediate seizures	Heavy Seizures
0.5: Immobility, piloerection, salivation, narrowing of eyes, face and vibrissae twitching, ear rubbing with forepaws	**1.5**: Clonic movements of forelimbs and mild whole body convulsions, exophthalmia, aggressive behavior	**2.5**: Rearing and falling, eye congestion
1.0: Head nodding and chewing movements	**2.0**: Rearing and running with stronger tonic-clonic motions including hind limbs, tail hypertension, lock jaw	**3.0**: Loss of postural tone with general body rigidity

Statistical analysis

Statistical data is applied by SPSS software, version 16.0, following the term Mean ± S.E.M. One-way ANOVA and Paired samples T-test was used as tools for data analysis. For all the tests a 'p' value of 0.05 or less was considered as statistical significance.

Histopathological studying

The brain morphology is not a key part of this study; its task is just able to show the impact of *A. gramineus*'s extracts on brain directly [22]. Moreover, it also makes a comparison not only within doses but also between extracts and commercial drug under the expert's assistant of M.D, Hoang Van Thinh, Head of Pathology Department of CR hospital, Hochiminh city. All the images of brain anatomy were taken under the zoom length 40x and 200x to ensure the consistency of this study.

III. RESULTS

Phytochemical screening

Phytochemical screening of the methanolic rhizomes extract of *A. gramineus* revealed the presence of carbohydrates, flavonoids, tannins, alkaloids, phenols, quinones, cellulose and terpenoids.

Table 2: Phytochemical screening of the methanolic rhizomes extract of A. gramineus

No.	Phytochemical tests		Extracts			
	Phytochemical constituents	Phytochemical test	Methanol	Ethanol	Aqueous	Ethyl acetate
1	Carbohydrates	Fehling's test	+	+	+	+
		Barfoed's test	+	+	+	+
2	Flavonoids	Ammonium test	+	+	+	-

		Aluminum Chloride test	+	+	+	-
		Lead Acetate test	+	+	+	-
		Ferric Chloride test	+	+	+	-
3	Alkaloids	Wagner's test	+	+	-	+
		Dragendroff's test	+	+	-	+
		Hager's test	+	+	-	+
4	Saponins	Frothing test	-	-	-	-
5	Tannins	Ferric Chloride test	+	+	-	-
		Lead Acetate test	+	+	-	-
6	Phenolic compounds	Ferric Chloride test	+	+	+	+
7	Anthraquinones	Borntrager's test	+	+	+	+
8	Terpenoids	Salkowski test	+	+	+	+
9	Steroids	Lieberman-Burchard's test	-	-	-	-
10	Oils and Fats	Sudan test	-	-	-	-
		Saponification test	-	-	-	-

"+": positive test result, "-": negative test result.

Acute oral toxicity test

The LD50 value of the methanolic rhizomes extract of *A. gramineus* was exposed to be higher than 5000 mg/kg of body weight. This study expresses that the rhizomes extract of *A. gramineus* is safe or non-toxic even in greater acute doses.

Table 3. Dose progression and results for Limit test (X = Died, O = Survived).

Test seq.	Animal ID	Dose (mg/kg)	Short-term	Long-term
1	1	2000	O	O
2	2	5000	O	O
3	3	2000	O	O
4	4	2000	O	O
5	5	2000	O	O
6	6	2000	O	O
7	7	5000	O	O
8	8	5000	O	O
9	9	5000	O	O
10	10	5000	O	O

Table 4. Acute toxicity hazard categories and (approximate) LD50 values defining the respective categories

Acute oral toxicity	Category				
	1	2	3	4	5
LD50 value	LD50 ≤ 5 mg/kg	5 mg/kg < LD50 ≤ 50 mg/kg	50 mg/kg < LD50 ≤ 300 mg/kg	300 mg/kg < LD50 ≤ 2000 mg/kg	2000 mg/kg < LD50 ≤ 5000 mg/kg

Anticonvulsant activity of *A. gramineus*

The impact of methanolic rhizomes extract of *A. gramineus* on PTZ-induced seizures in female mice is shown in table 5.

Table 5. Effect of methanol extracts of A. gramineus rhizomes on PTZ-induced seizure mice

Group (n = 5)	Latency of Convulsant (second)	Recurrent of Convulsant (time)	Number of Convulsant	Number of Death	Protection
I	45.106 ± 3.268	4.20 ± 0.374	5	5	0%
II	83.842 ± 10.871	3.80 ± 0.490	5	2	60%
III	94.758 ± 21.372	2.40 ± 0.400	5	2	60%
IV	181.413* ± 27.877	1.20* ± 0.374	4	0	100%
V	655.067* ± 24.710	0.60* ± 0.245	3	0	100%
VI	202.982* ± 30.358	1.00* ± 0.316	4	0	100%

*Each value reprresents mean ± S.EM; *p < 0.05 compared with the control

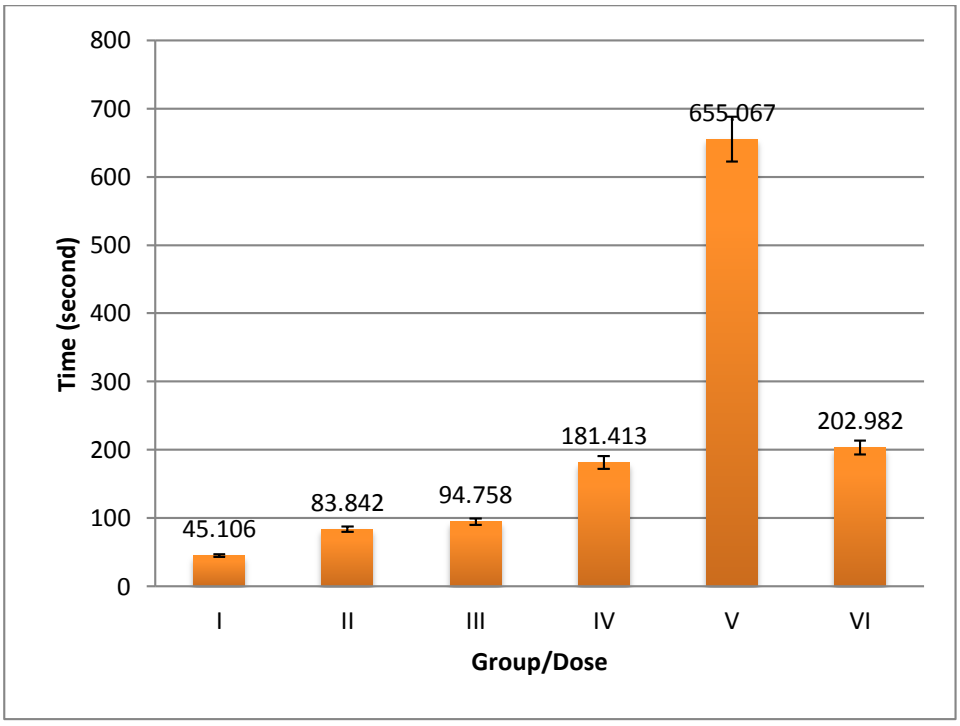

Figure 1: Showing the anticonvulsant activity of A. gramineus in the doses of 100, 200, 300, 400 mg/kg on the latency of convulsions in mice.

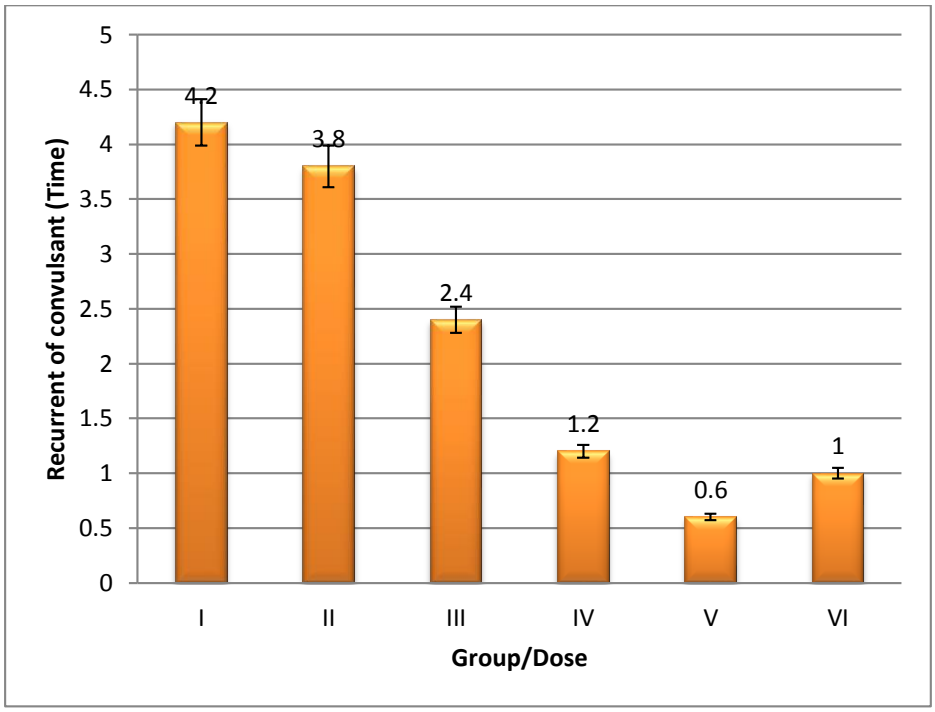

Figure 2: Showing the anticonvulsant activity of A. gramineus in the doses of 100, 200, 300, 400 mg/kg on the recurrent of convulsions in mice.

Based on the data presented above, we can absolutely confirm that *A. gramineus* is capable of very high resistance to seizures, an important symptom of epilepsy. For the delay of convulsant, shown in Figure 1, methanolic extracts of *A. gramineus* rhizomes can extend stability of mice from 45.106 ± 3.268 in Group I by 4 times (181.413 ± 27.877) in group IV, and even 14 times (655.067 ± 24.710) in group V.

As expected, similar to the results of the anti-seizure frequency also yielded positive results. As what has been presented in Figure 2, Total seizures have really plummeted from 4.20 ± 0.374 in Group I to 1.20 ± 0.374 in Group IV and dramatically decreasing into 0.60 ± 0.245 in group V. But not be able to completely block the shock attack, but the results are really interesting when compared to the commercial drug being used to treat epilepsy (Phenobarbital).

In summary, at doses of 300 and 400 mg/kg of body weight, survival rate was 100% homologous compared with drugs on the market. Thus, extracts of *A. gramineus* rhizomes have very high potential in preventing seizures and can be applied in the treatment of disease. However, these predictions should be tested to ensure reliability.

Histopathological Studying

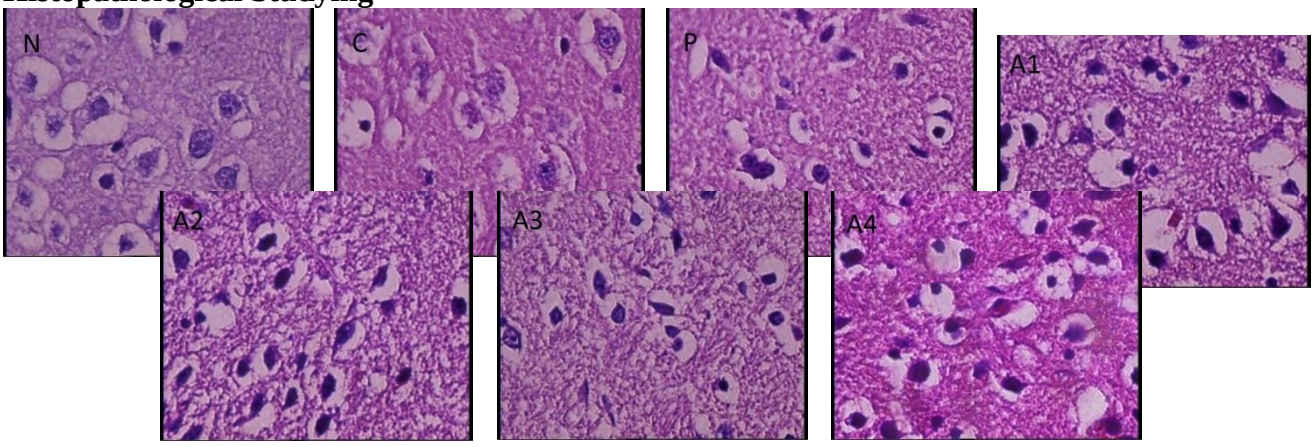

Figure 3. Showing histopathological changes in seizure of mice's brain in dose of normal (N); negative control (C); positive control (P1); 100mg/kg (A1); 200mg/kg (A2); 300mg/kg (A3) and 400mg/kg (A4) respectively.

Because of being an acute test, the damage of PTZ was just in the cortex. Generally, it is very easy to observe that the distribution of neurons and glial cells were in the cortex uniformly in N. Compared to C, A1, A2; these specimens were influenced heavily by PTZ which neurons concentrated in regions with decreasing order from C to A2. Moreover, density of glial cells was lower and only about 30%* compared to the specimen N. In contrast, similar to the distribution of neurons in the cortex of the N, the specimens A3, A4 and P distributed neurons and glial cells evenly, not too dispersed or focused on a specific area of cortex, particularly A4 and P.

In detail, N with all manifestations of a normal nerve tissue is as the neurons arranged orderly around the cortex tissue without any destroyed nuclei; the glial cells are covered with the ideal distance between them. It is an excellent model to compare the differences with the specimens which were carried out for this research following perennial clinical experience of Dr. Thinh. The specimen C was devastated, particularly swollen neurons; the nuclei of nerve cells was pyknosis leading to distort the cell's structure and nuclei were skewed to one side of the cell additionally. Besides, a number of neurons' nuclei had been completely karyorrhexis and karyolysis; 70%* of the cells were traumatized including three phenomena with increasing levels like pyknosis, karyorrhexis and karyolysis. Simultaneously, the glial cells also appeared sparse compared to specimen N. A1 and A2 are the specimens having significantly changes, number of injured cells are slightly decrease, no longer the phenomenon of nuclei karyolysis. Minority of nuclei, these are karyorrhexis, are replaced by nuclei pyknosis and normal nuclei in nerve cells. Although the nuclei are still deformed like the racket, rhombus, triangle, cells damage were reduced to approximately 50%* in specimen A1 and 35%* in specimen A2. The above phenomena have really plummeted in the specimens A3, A4 and P; nuclei have not been distended. There are no karyorrhexis or karyolysis and just a small number of pyknosis. The nuclei are in the center of nerve cells, in spite of remaining some nuclei skewed to one side. It is very easy to recognize the similarities between samples A3 and P when they have the same damaged cells accounted for 15%*. Beyond expectations, A4 forms a distinct difference when only around 5%* of damaged cells. The histopathological analysis really points out the high efficiency of the *A. gramineus's* rizhomes extracts in reducing seizures not only phenomena of convulsant but also the impact on nerve cells.
* All the numbers in the histopathological analysis were under the expert computing by Dr. Hoang Van Thinh following his professional formula.

IV. DISCUSSION

The presence of carbohydrates, flavonoids, alkaloids, tannins, phenolic compounds, anthraquinones, steroids and terpenoids, which organic compounds are often used widely in the production of pharmaceuticals and cosmetics, were revealed during the test. Alkaloids is a popular metabolite on the most plants for pharmaceutical applications such as antiarrhythmic effects (quinidine, spareien), antihypertensive effects (indole alkaloids many), anticancer activities from (demericindoles, vincristine and vinblastine) andantimalarial actions (quinine) [23, 24]. The natural therapy is one of the features of tannins, particularly in Japan and China; astrigents, against diarrhea, diuretics, against stomach and duodenal tumors are the hallmarks of tannins used in Asian medical [25, 26]. It will be a major shortcoming without mentioning the role of flavonoids in medical industry; anti-inflammatory activity, antimicrobialactivity, anti-allergicactivity, antioxidant activity, ostrogenic activity, enzyme inhibition, cytotoxic antitumor activity and vascular activity are contained [27]. These are typical examples to clearly describe practical value of *A. gramineus* for medicine and economics.

The oral median lethal dose of the extracts was estimated as over 5000mg/kg body weight leading to wide range of applications for cosmetic, pharmaceutics, therapeutic by using rhizomes extracts of *A. gramineus.*

The data from this study illustrate that *A. gramineus* absolutely can reduce the frequency and increase latency of convulsion under the effect of PTZ-induced seizures. Inhibiting the activity of gamma amino butyric acid (GABA) at GABA-A receptors is the core mechanism of PTZ in exerting convulsion [28]. The enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsion, respectively [29]. Phenobarbital is a well-known conventional antiepileptic drug that is able to generally inhibit sodium currents and enhance GABA-ergic inhibition. Because the extracts decreased reoccurrence and delayed latency of PTZ seizures, it is probable that it may be interfering with GABA-ergic mechanisms to exert its anticonvulsant impact.

Many organic compounds have been confirmed their presences in *A. gramineus* extracts through phytochemical tests. It is totally believed that flavonoids, available in *A. gramineus*, play a vital role in anticonvulsant activity based on previous studies carried out in other species such as *Solanum nigrum* [30, 31]. However, further research is clearly necessary to clarify the specific compounds responsible for the anticonvulsant activity.

The results are not only similarity in the statistical analysis but also identical in histopathological analysis, in P2 and A3; that is the first step for further research on the effects of *A. gramineus* extracts of anticonvulsant activity, especially in field of histopathological analysis. However, it is necessary to carry out further studies on the effects of the extracts to other parts of the brain, not only the cortex. Consequently, *A. gramineus* is a potential plant to be invested and developed into a commercial product of high value in pharmaceutical market.

V. CONCLUSION

The 80% methanolic extracts of *A. gramineus* rhizome at the dose of 300 and 400mg/kg p.o. possesses the anticonvulsant activity against the effects of PTZ-induced seizure in mice; it is really safe for oral administration further. Findings were figured out in this study can be a foundation for further research or investigations for developing of antiepileptic medicines in the future.

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