ANTICONVULSANT EFFECT OF ETHYL ACETATE FRACTION AND UNSOLVED ETHYL ACETATE FRACTION FROM SIRSAK LEAF (*Annona muricata*, L.) ON PENTYLENTETRAZOL INDUCED IN MICE

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Background. *Ethanol extract from sirsak leaf (**annona muricata** L.) reported have anticonvulsant activities. For studying it deeply ethanol extract must do fractination by using ethyl acetate, thus resulting ethyl acetat fraction and unsolved ethyl acetat fraction.*

Objective. *This research aimed to know about anticonvulsant activities from each fraction.*

Methods. *It research conducted by using mices which divided is to 8 groups, each groups consist 7 mices which pentylentetrazol induced 90 mg/kg bw. Doses ethyl acetate fraction Groups (FE) are 100, 200, 400 mg/kg bw, and unsolved ethyl acetate fraction groups are 100, 200, 400 mg/kg bw. Control group just added Na CMC 0.5% and comparation group added by Phenobarbital dose 50 mg/Kg Bw by using peroral technique. Both ethyl acetat and unsolved ethyl acetat fractions were given quantificacy test of total flavonoid content by Chang method, calculated as quercetin. All data should be analyzed by Kruskal Wallis test which continued with Man-Whitney by using reliable level 95%.*

Outcome measured. *The parameters anticonvulsant activities are clonic and tonic onset, clonic and tonic incidence , clonic frequency, time and incidence of mortality.*

Results. *All fractions delayed the onset of tonic and reduced tonic and mortality incidence. The most decrease of mortality incidence in FE400, while longest delayed the onset of tonic in FT400. The latency of mortality possessed in FE400, FT100, FT200, FT400 with the higest at FT400. Total flavonoid of FE is 1,31% and FT is 2,6%.*

Conclusion. *In conclusion, that all fractions are appeared have anticonvulsive activities mean while not yet equal Phenobarbital in dose material treatment.*

Keywords : *Annona muricata, Ethyl acetat fraction, unsolved ethyl acetat fraction, Anticonvulsant, pentylentetrazol*

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INTRODUCTION

Epilepsy is one of the most common serious neurological disorders. Treatment with anti-epileptic drugs (AEDs) is generally chronic even life long. The drugs are synthetic molecules that exert serious adverse effects, such as drowsiness, ataxia, gastrointestinal disturbance, hepatotoxicity, and megaloblastic anemia (Namara, 2003). Plant extracts can be an important source for the development of better and safer drugs for the treatment of epilepsy. The plant material can be selected, such as sirsak (*Annona muricata*). The plant has been used medicinally in many tropical African countries for an array of human ailments, especially for parasitic infections and cancer. It has also been used in some African herbal medicine systems for its sedative, antispasmodic and convulsive seizure properties (Taylor, 2002). N'Gouemo *at al*. (1997) have reported that ethanol extract from sirsak leaf (*annona muricata* L.) has anticonvulsant activities. For studying it deeply ethanol extract must do fractination by using ethyl acetate, thus resulting ethyl acetat fraction and unsolved ethyl acetate fraction. This research aimed to know about anticonvulsant activities from each fraction.

METHODS

Material

Fresh sirsak leaves are dark green obtained from Kuningan in September 2011. Sirsak leaf were identified in the laboratory of Plant morphology, Department of Biology, Ahmad Dahlan University Yogyakarta. Comparative material, used phenobarbital tablets (KF) obtained from the Apotek Mitra Bahagia Cirebon, Na CMC obtained from the Laboratory of Pharmaceutical UAD. Pentylentetrazol (PTZ) from Sigma Co.

Animal

Healthy, male Balb C mice (*Mus domesticus*) weighing 20-30 g were used. The animals were kept and maintained under laboratory conditions of temperature, humidity and light (12-hour day/12-hour night cycle); and were allowed free access to food (standard pellet diet) and water *ad libitum*. The animals were broadly divided into 8 groups, each groups consist 7 mices which pentylentetrazol induced 90 mg/kg bw. Doses ethyl acetate fraction Groups (FE) are 100, 200, 400 mg/kg bw, and unsolved ethyl acetate fraction groups are 100, 200, 400 mg/kg bw. Control group just added Na CMC 0.5% and comparation group added by Phenobarbital dose 50 mg/Kg Bw by using peroral technique.

Phytochemical screening

The methods of Culvenor-Fitzgerald (1963) and Simes *et al*., (1995) were used to screen the Annona muricata leaf used in this study for its chemical constituents. The findings of the phytochemical screening are shown in Table 1.

Preparation of extract and fractionation

One kilogram (1 kg) of sirsak leaves was air-dried at room temperature. The plant was milled into fine powder in a blender and then macerated in ethanol 96% and extracted twice, on each occasion with 5 litre at room temperature for 3 x24 h with occasional shaking. The extract was concentrated under reduced pressure in a rotary evaporator at 50^{o}C. The resulting ethanol extract 146 g (14,6 %). The extract was separated into ethyl acetat soluble and insoluble parts. thus resulting ethyl acetat fraction and unsolved ethyl acetat fraction. Furthermore, each faction is made in 3 doses, a dose of 100, 200, and 400 mg / kg.

Thin-layer chromatography

TLC was conducted on sirsak leaf ethanol extract, ethyl acetate fraction, unsolved ethyl acetate fraction. Stationary phase such as silica gel 254 nm, mobile phase BAW upper layer (butanol, acetate, water = 4: 1: 5). Stains used are $FeCl_3$, ammonia vapor, UV light 254 by comparison quercetin.

Standardization of active substances

Standardization of the active flavonoid substances conducted by Chang method with a spectrophotometer Shimadzu UV-1800. Total flavonoid content of ethyl acetate fraction and unsolved ethyl acetate fraction of sirsak leaves is calculated as quercetin.

Evaluation of anticonvulsant property

The anticonvulsant testing method of Amabeoku *et al.*, (1998) and Visweswari *et al.*, (2010) with slight modifications. Each dose administered orally in animal experiments in accordance with the group for 7 days and 0.5% NaCMC as well as controls. Phenobarbital 50 mg / kg given orally on seventh day to 30 minutes before the administration of PTZ. PTZ 90 mg / kg dissolved in physiological saline was given ip. in all groups 30 min after administration of each fraction on the seventh day. Experimental animals were observed for 30 minutes after administration of PTZ. Parameters observed in the form of onset and incidence of clonic, onset, duration and incidence of tonic, clonic frequency, time of death and mortality.

Clonic PTZ seizures were defined as an episode of muscle spasms involving fore limbs with or without the loss of the righting reflex. Tonic PTZ seizures were characterized by an initial ventroflexion followed by full fore limb and hind limb extension (N'Gouemo, *et al.*, 1997).

Statistical analysis

Data were analyzed with the Kruskal Wallis test followed by Mann Whitney test at 95% confidence level.

RESULTS AND DISCUSSION

Results of determination to indicates that the true leaves of the sirsak (*Annona muricata*). Furthermore, phytochemical screening exhibit phenolic compounds are indicated by the formation of a greenish black color by reaction with FeCl3. Flavonoid compounds identified by reaction with Mg and concentrated HCl which gives red color. With shaking formed foam that indicate the presence of saponin compounds. Alkaloids and steroids were not identified, although some studies reported isokuinolin

Table I. hRf value and color spot from four samples

samples	hRf	Color spot without stain	Color spot with UV 254	Color spot with $FeCl_3$	Color spot With amonia
Ethanol	85	Yellow green	Slight green		Yellow brown
extract	76	Yellow green	Slight green		
	65		Slight green		
	23	Yelloe green	purple		yellow
FE	85	Yellow green	green	Black blue	Yellow brown
	76	Yellow green	Slight purple		Slight brown
	65		Slight purple		Slight brown
FT	41	yellow	Purple	Black blue	Yellow
	23	yellow	purple	Black blue	Yellow
kuersetin	76	yellow	green	Black blue	yellow

FE = ethyl acetat fraction

FT = unsolved ethyl acetat fraction

alkaloids and phytosterols (Watt and Breyer-Brandwijk, 1962, cit Adewole and Ojewole, 2009).

TLC

TLC results in the form of the value of the hRf and color spot of the four samples that ethanol extract, ethyl acetate fraction (FE), unsolved ethyl acetate fraction (FT), and quercetin can be seen in Table I.

Table I shows that the FE and FT contain polyphenolic compounds indicated by bluish black spots after being sprayed with FeCl3 (Harborne, 1987). Quercetin as a comparison also shows the same color although with different color intensity. Provision of ammonia vapor produces color yellow spots, yellow brown, and brown weak. This means there are flavonoids at all the sample. According to Harborne (1987), Flavonoids are phenolic compounds, therefore it is the color change when added alkaline or ammonia. The resulting color depends flavonoids. According Leabouf *et al*., (1982), Watt and Breyer-Brandwijk, (1962) cit Adewole and Ojewole, (2009), sirsak leaves contain flavonoid.

Quercetin as a comparison was not detected its presence in all samples, although the value of the HRF there is similarity in the three samples is 76 but of different color spots.

Results of active substances standardization

Measurement results of total flavonoid content of unsolved ethyl acetate fraction is 2.62% and ethyl acetate fraction is 1.31%. Unsolved ethyl acetate fraction is more polar than ethyl acetate fraction. Thus, flavonoids which are water-soluble compounds (Harborne, 1987) more in the unsolved ethyl acetate fraction.

Results of testing the anticonvulsant effect

The test results showed that the anticonvulsant effect of different factions and different doses up to 400 mg / kg BW can not delay the onset of clonic and do not cause a decrease in the incidence of clonic seizures that occur in mice. Based on the results of the Mann Whitney test for the onset of clonic seizures at various doses of the fraction of ethyl acetate and ethyl acetate insoluble fraction showed no significant differences due to the significant value of more than 0.05 ($p > 0.05$) compared to the control group.

Tonic incidence decreased with increasing dose, although the ethyl acetate insoluble fraction doses of 100 and 200 mg / kg have the same percentage. To the onset tonic, giving ethyl acetate fraction and insoluble fraction of ethyl acetate at different doses may prolong the onset of tonic or may delay the occurrence of tonic with a significance value of less than 0.05 ($p <0,05$). Thus, all the fractions showed anticonvulsant activity. According to Adeyemi, *et al.,* (2007) and Ojewole, (2008) the ability of plant extracts to prevent seizures or prolong the onset of tonic seizures indicate anticonvulsant activity. Observation of the duration of the tonic, ethyl acetate fraction dose of 400 mg / kg body weight can significantly shorten the duration of tonic, while the dose of 100 and 200 mg / kg have not been able to reduce the duration of tonic. For the insoluble fraction of ethyl acetate, the shortest duration of tonic occurred at a dose of 200 mg / kg, which is 20.20 seconds, but statistically the results do not differ significantly in comparison with controls. According Kasture *et al*. (2000), the anticonvulsant activity can be demonstrated by a decrease in the duration of tonic in experimental animals. Thus the only anticonvulsant activity shown by the ethyl acetate fraction dose of 400 mg / kg.

The test results showed the number of seizures that occurred an average frequency of seizures that occur higher than the control group than the treatment dose FE 100. However, this value is statistically significantly different from no more significance than 0.05 ($p> 0,05$). So, there was no effect of administration of ethyl acetate fraction and ethyl acetate insoluble fraction soursop leaves the number of seizures that occurred. Thus, the parameter number of

seizures can not provide information regarding the anticonvulsant activity of both fractions.

However, this value is not significant.

Observation of the time of death showed that the ethyl acetate fraction may extend the time of death at a dose of 400 mg / kg bw, while the insoluble fraction of ethyl acetate all treatment doses of 100, 200, and 400 mg / kg bw able to extend the time of death compared to the control. To the number of deaths, compared with the control group, all treatments can reduce the number of deaths caused by mice with PTZ-induced seizures decrease in the number of deaths was highest in ethyl acetate fraction dose of 400 mg / kg. Data of all test parameters are presented in Table II.

Of all the parameters that are used to look anticonvulsant activity, most of the parameters are the parameters of onset, incidence, and duration of tonic, time of death and the number of deaths to support the conclusion that the fraction of ethyl acetate and ethyl acetate insoluble fraction showed anticonvulsant activity.

Anticonvulsant activity of the ethyl acetate fraction and ethyl acetate insoluble fraction thought to be related to the presence of flavonoids in the both fraction. Flavonoids are a large group of plant polyphenols. Flavonoids have a variety of activities, such as antioxidant (Winarsi, 2005). The antioxidative properties due to the position of hydroxyl groups capable of scavenging free radicals. Flavonoid initially oxidized by radicals, then changed to a more stable and less reactive radicals. Thus flavonoids can stabilize reactive oxygen compounds (Korkina & Afanas'ev, 1997 cit Winarsi, 2005). Adewole and Ojewole (2009), reported a decrease in reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS) in rats administered a aqueous extract of leaves of the sirsak, it means there are antioxidant

Table II. Recapitulation of Research Data on multiple anticonvulsants Parameter Testing Activity Induced in Mice PTZ

Groups	clonic onset (sec)	Clonic Inci-dence	Tonic onset (sec)	Tonic Inci-dence	Tonic Duration (sec)	Time of death (sec)	Morta-lity	Frequensi Of seizure
control	92,43 ± 23,28	7/7 (100 %)	266,50 ± 47,91	6/7 (86%)	25,20 ± 3,11	306,17 ± 60,24	6/7 (86%)	2,43 ± 0,79
Fenobarbital	1800*	0%*	1800*	0%	0*	1800*	0%*	0
FE 100	106,14 ± 12,17	7/7 (100 %)	399,00 ± 43,71*	4/7 (57%)	24,25 ± 1,71	424,67 ± 178,00	3/7 (43%)	2,00 ± 0,89
FE 200	86,40 ± 11,46	6/6 (100 %)	564,00 ± 98,99*	3/6 (50%)	19,00 ± 4,36	561,00 ± 470,71	3/6 (50%)	3,75 ± 2,22
FE 400	131,00 ± 39,84	7/7 (100 %)	722,00 ± 83,44*	3/7 (43%)	18,67 ± 3,21*	553,00 ± 185,26*	2/7 (29%)*	3,00 ± 1,22
FE 100	72,00 ± 27,12	7/7 (100 %)	443,67 ± 28,43*	5/7 (71%)	21,00 ± 2,24	908,75 ± 201,78*	5/7 (71%)	3,43 ± 1,13
FE 200	90,57 ± 36,71	7/7 (100 %)	532,00 ± 244,99*	5/7 (71%)	20,20 ± 5,07	831,50 ± 302,97*	5/7 (71%)	4,20 ± 0,45
FE 400	98,67 ± 42,33	7/7 (100%)	976,50 ± 132,23*	3/7 (43%)	21,67 ± 2,08	1134,50 ±37,48*	3/7 (43%)	2,67 ± 1,37

*p < 0,05 to control (PTZ 90 mg/kg bw i.p + CMC 0,5%)

activity of aqueous extracts of sirsak leaves. According Winarsi, (2005) based on its mechanism of antioxidants can be divided into three, namely: primary antioxidants (endogenous antioxidants / antioxidant enzymatic); antioxidant secondary (exogenous antioxidants / antioxidant nonenzimatis) and tertiary antioxidants (antioxidants that act repair biomolecules damaged by free radicals) .

Oxidative stress is an etiological factor in the occurrence of epileptic seizures (Shin *et al.*, 2011) including PTZ-induced seizures. Several studies have demonstrate this. Bashkatova et al., (2003) reported the increase in the formation of nitric oxide (NO) fivefold, and TBARS more than twofold in the brain cortex of rats after administeration of PTZ 120 mg / kg subcutaneously. NO is a free radical with a short half-life. NO is considered a disorder of molecules that play a role in the pathophysiology of Alzheimer's, Parkinson's, stroke, trauma, and seizures. Other studies have shown that administration of PTZ resulted in an increase in malondialdehyde (MDA), and decreased glutathione (GSH) (Mehla, *et al.*, 2010; Ilhan, *et al.,* 2005; Gupta, YK *et al.*, 2003) and increased catalase (Sharma *et al*., 2010). PTZ also known as a selective blocker of the chloride ion complex for GABA-A receptor and cause a decrease in function GABAergik (Ilhan, *et al.*, 2005).

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) such as O_2-*,*OH, *NO cellular level with a higher cellular antioxidant defenses. ROS generation ubiquitous nature as a result of aerobic metabolism such as oxidation in mitochondria. In order to scavenge ROS, different defense systems in the brain in the form of enzymatic, nonenzymatic, and antioxidants in the form of food. If ROS are not effectively eliminated, it can cause oxidative injury is peroxidation of cell membrane phospholipids, proteins (receptors,enzymes) and DNA. Brain tissue is very vulnerable to ROS because, (1) The brain produces very high ROS as a result of aerobic metabolism is high, whereas relatively few enzymatic antioxidant defenses, (2) with a lipid-rich brain is vulnerable to oxidative damage, and (3) DNA nerve damaged in adults ineffective repaired because there is no DNA replication in the brain.

In this study the ethyl acetate fraction showed a stronger anticonvulsant activity mainly on the parameter number of animal mortality. Ethyl acetate fraction dose of 400 mg / kg body weight can reduce the number of mortality dropped 86% in controls to 29%. On the other hand, levels of flavonoids from the ethyl acetate fraction lower than the insoluble fraction of ethyl acetate. Therefore, the estimated potential of antioxidant fraction of ethyl acetate and ethyl acetate insoluble fraction is not the only cause of the anticonvulsant effects of the two fractions.

CONCLUSION

All fractions are appeared have anticonvulsive activities mean while not yet equal phenobarbital in dose material treatment.

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