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The effect of ethanolic extract and juice of malacca (*Phyllanthus emblica* L.) fruit in rat model stroke

Anastasia Setyopuspito Pramitaningastuti¹ and Maria Immaculata Iwo²

¹Sekolah Tinggi Ilmu Farmasi "Yayasan Pharmasi" Semarang, Indonesia

²Department of Pharmacology and Pharmacy Clinic, Institute Technology of Bandung, Indonesia

ABSTRACT

Stroke is a leading cause of death in Indonesia. Among Indonesia's natural resources, Malacca fruit (*Phyllanthus emblica* L.) is suspected to have potential as an alternative medicine for stroke. The objective of this study was to observe the recovery effect of ethanolic extract and juice of Malacca fruit toward rat stroke model. Plant extract was characterized and determined its chemical compound by phytochemical screening method. The dose of ethanolic extract and juice of Malacca fruit was 50 and 100 mg/kgBW. Induction of rat model of ischemic stroke was made by binding to the common carotid artery to the left. Test parameters include tests of motor function, inhibition of platelet aggregation, and extensive measurements of brain injury in rats. Motor function test was observed after post-operative on 1st, 3rd, 7th, 10th, 14th, and 21st day i.e. Forelimb Placing Test (FPT), Forelimb Use Asymmetry Test (FUAT), Corner Turn Test (CTT), and rotarod test to determine antistroke effect of both extracts. Platelet aggregation inhibition test performed on day 14 and 21 by adding Adenosine diphosphate (ADP) as an inducer of platelet aggregation. After 21 days post-operative, the lesion area in the brain was determined. Data obtained was analyzed statistically. Research data of ethanol extract and fruit juice Malacca showed that fruit juice dose of 100 mg/kgBW could improve motoric function rat model of stroke which significantly marked by FPT improvement and rotarod test on day 3 until the 21st. Data inhibition of platelet aggregation results showed that the absence of significant inhibition. Area injuries were only seen in the positive control group. An average brain injury of positive control was 7.59 ± 1.7 . Fruit juice Malacca dose of 100 mg/kgBW had more potential as alternative medicine therapy of stroke than the ethanol extract of the fruit Malacca.

Keywords: *Phyllanthus emblica* L. fruit, Forelimb Placing Test, Forelimb Use Asymmetry Test, Corner Turn Test, stroke

INTRODUCTION

Stroke is a disruption in the supply of blood vessels of the brain [17]. Stroke can be divided into ischemic stroke and hemorrhagic stroke. Ischemic stroke is a stroke caused by blockage of an artery to the brain resulting in a lowering of blood supply to the brain, whereas hemorrhagic stroke is a stroke that occurs due to rupture of blood vessels in the brain.

According to data from the American Heart Association, stroke is the cause of one in 20 deaths in the United States in 2011. On average, every 40 seconds, someone in the United States has a stroke, and someone dies of one approximately every 4 minutes [15]. According to Riset Kesehatan Dasar (Riskesdas) in 2013, the prevalence of stroke in Indonesia based on the diagnosis of health personnel has increased from 8.3 per 1000 in 2007 to 12.1 per 1000 in 2013 [1]. Based on the prevalence data, it is necessary to prevent and treat stroke for reducing mortality caused by stroke.

Stroke therapy can be done properly if the type of stroke that occurs already known. Drugs used for the treatment of ischemic stroke among other antithrombotic, anticoagulant, and anti-cholesterol. The main drugs used in hemorrhagic stroke is a drug to reduce symptoms or risk factors such as hypertension. In addition to these drugs, the neurological decline can be treated with therapy motor functions of the body. The use of synthetic drugs still provides considerable side effects and development of drugs to treat stroke primarily to improve the body's motor functions are still a little. Therefore, prevention and treatment of stroke are directed to the use of herbal medicine that is expected to have relatively small side effects.

Indonesia is known as a country rich in natural resources, therefore, the study of herbal medicine developed quickly. Malacca (*Phyllanthus Emblica* L.) is one of the types of plants that grow in Indonesia. This plant has long been known empirically by the people of Indonesia and even other countries such as India will useful as a memory enhancer, neuroprotective, antimicrobial, antidiabetic, and antiinflammatory [4]. Based on the empirical properties of these plants on the performance of the brain, this study was conducted to determine the effect of ethanolic extract and fruit juice Malacca against a rat model of stroke.

MATERIALS AND METHODS

Animals

Wistar male rats (180-250 g, 2-3 months old) obtained from the Animal Laboratory of the School of Pharmacy at Institute of Technology Bandung (ITB). Animals were fed with standard animal food pellet and water ad libitum.

Extraction

Malacca fruit obtained from the Pandaan, East Java. The plant was identified in Herbarium Bandungense, School of Life Sciences and Technology ITB. This study using the ethanol extract and fruit juice Malacca. Ethanolic extract prepared by extracting the dried fruit powder Malacca using 95% ethanol with reflux method. The extraction was filtered and the filtrate was concentrated using a rotary evaporator to obtain a concentrated extract. Malacca fruit juice is made by extracting fresh fruit pulp using a juicer, then filtered and dried by freeze-drying process.

Experimental Group

There were seven groups in study: normal group, CCAO (given Na CMC 0,5%, the vehicle), CCAO + aspirin 30 mg/kgbb, CCAO + ethanolic extract Malacca fruit 50 mg/kgbb (CCAO +EEMF 50), CCAO + ethanolic extract Malacca fruit 50 mg/kgbb (CCAO + EEMF 100), CCAO + juice Malacca fruit 50 mg/kgbb (CCAO + JMF 50), and CCAO + juice Malacca fruit 50 mg/kgbb (CCAO + JMF 100).

Cerebral ischemia induction

The method used was common carotid artery occlusion (CCAO) to induce an ischemic stroke, as performed in a previous study [3]. Rats were anesthetized with ketamine dose of 100 mg/kg intraperitoneally. Induction model of stroke performed by dissecting the ventral neck along the 1 cm to isolate the left common carotid artery using the surgical instrument. The common carotid artery that has been isolated tied at 2 different places using the USP 4-0 silk thread, then neck skin sutured with thread chromic USP 3-0 and using a sewing needle size 16. Induced pain in animal models of stroke can be reduced with the use of analgesics (Meloxicam) dose of 1 mg/kgBW are given orally 1 hour prior to induction (pre-surgery) and 24 h after induction (post-surgery), while the antibiotic powder (nebacetin) given at the skin surface to minimize infection surgical wounds. Operations carried out in sterile laboratory animals.

Behavioral Test

Behavioral Test includes forelimb placing test (FPT), forelimb use asymmetry test (FUAT), corner turn test (CTT), and rotarod test. Behavioral test performed on days 1, 3, 7, 14, 10, 14, and 24.

Forelimb Placing Test (FPT)

FPT is done by recording the placement of the limbs of a rat on the table. The stimulus was given to the nose hairs right or left and calculated response to the right or left upper limb of the stimulus by putting the rat limb movement on a surface. FPT determination performed 10 times on each leg and the calculated percentage [7].

Forelimb Use Asymmetry Test (FUAT)

Forelimb use during exploratory activity was analyzed by videotaping rats in a transparent cylinder (20 in cm diameter and 30 cm in height) for 5 minutes depending on the degree of activity during the trial. Scoring was done by an experimenter blinded to the condition of the animal using a video cassette recorder with slow-motion and clear stop frame capabilities. Behavior was quantified by determining the occasions when the unimpaired (ipsilateral) forelimb was used as a percentage of total number of limb use observations on the wall (I); the occasions when the

impaired forelimb (contralateral to the blood injection site) was used as a percentage of total number of limb use observations on the wall (C); and the occasions when both forelimbs were used simultaneously (or nearly simultaneously during lateral side-stepping movements) as a percentage of total number of limb use observations on the wall (B). A single overall limb use asymmetry score was calculated as follows: $Limb\ Use\ Asymmetry\ Score = [I/(I+C+B)] - [C/(I+C+B)]$ [7].

Corner Turn Test (FPT)

The rat were left to explore the room angle of 30°. To get out of the corner, the rat can be turned left or right. This test is performed 10 times with each of the test range is 30 seconds. Calculation of the percentage of veering to the right. Behavior that is included in the calculation is the behavior of rats turned with full rearing [7].

Rotarod test

Before the surgery, rats were trained to walk on the cylinder running on rotarod apparatus (Ugo Basile, Italy, rod diameter 6 cm). Rotarod apparatus remains activated at a speed of 8 rpm and rats were trained to be able to run at that speed for 1 minute. Then the rats were trained to walk on the acceleration of 4-40 rpm [19]. Time and rpm (rotation per minute) when the rats fell from the rod was recorded and used to determine the final score mileage rats (meters).

Inhibition of Platelet Aggregation

The rat blood is drawn as many as 900 mL and collected into tubes Eppendorf which already contains 100 mL of sodium citrate 3.18% w/v, then the blood plasma is separated with the aid of a centrifuge at a speed of 1600 rpm. A total of 250 mL of plasma was transferred into a cuvette and added a physiological sodium chloride. The plasma mixture absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 600 nm, as the initial uptake. Inducer of platelet aggregation that is Adenosine 5'-diphosphate sodium salt (ADP) 5µM of 30 mL inserted into the plasma mixture, then incubated for 20 minutes at a temperature of 37° C and measured absorbance at the same wavelength and recorded as the final uptake.

Size measurement Brain Injury in Rat

Measurement extensive injuries to the brain is the end point of the study parameters. rats brains were taken and cut with an appropriate thickness is 2 mm. Brain slices were stored at -20° C for 20 minutes then colored using a solution of triphenyl tetrazolium chloride 0.1% in the Petri dish and allowed to stand for 30 minutes at 37° C. Pieces of the brain were transferred into 10% buffered formalin. After 1-3 days of incubation in formalin [9,11]. Wide injuries of the brain were observed using the Image-J software.

Statistical analysis

Statistical analysis was performed by using Statistical Package for Social Science (SPSS) ver.20, one-way ANOVA was followed by LSD for post hoc analysis. Differences were considered significant at $p < 0.05$.

RESULTS

Behavioral test

Forlimbs Placing Test (FPT)

Figure 1 shows that all test groups were induced CCAO on the first day had a stroke due to a decrease in right limb motor function in the rat, but only the CCAO+aspirin and CCAO+JMF50 were not significantly different with CCAO group. While CCAO+JMF100, CCAO+EEMF50, and CCAO+EEMF100 did not differ significantly decreased to the normal group. This shows that on the first day of CCAO+JMF100, CCAO+EEMF50, and CCAO+EEMF100 already provides an improvement in motor function rats. But after day 1 that on day 3 and so began to look for the right limb motor function recovery.

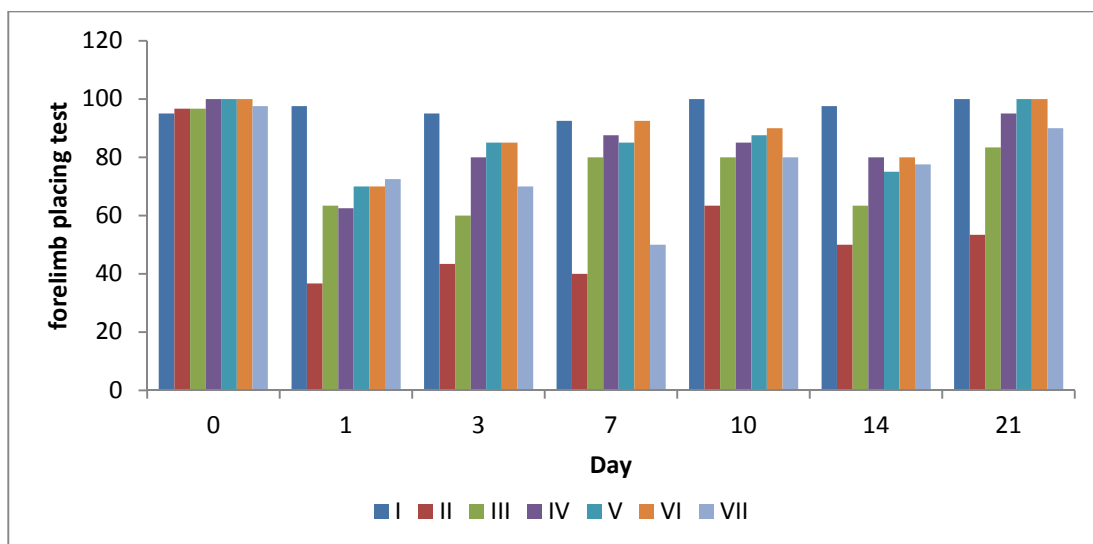


Figure 1 forelimb placing test (FPT) on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica* L.) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII)

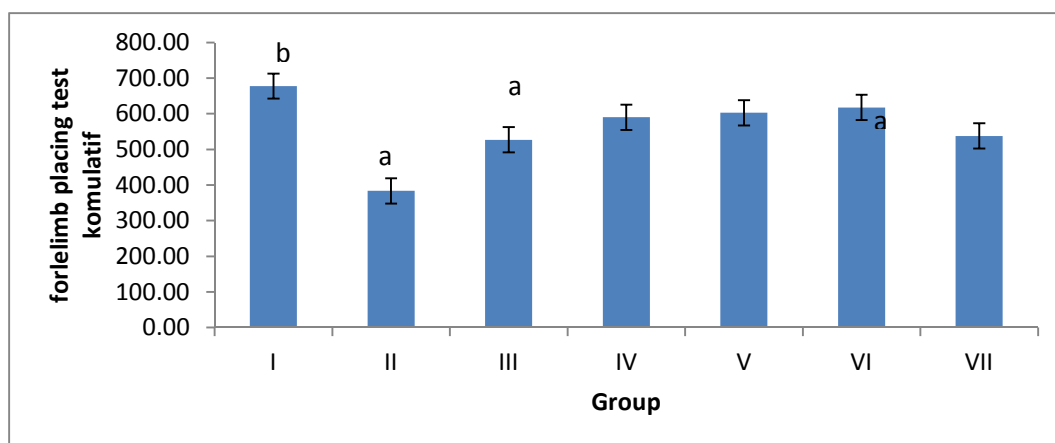


Figure 2 forelimb placing test (FPT) cumulative on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica* L.) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII). a = $p < 0,05$ compare to normal group and b = $p < 0,05$ compare to CCAO group

Figure 2 shows that the CCAO+JMF50, CCAO+JMF100, and CCAO+EEMF50 no significant difference when compared to the normal group. This indicates that the group is experiencing right limb motor function recovery in CCAO+aspirin group and all the test group.

Corner Turn Test (CTT)

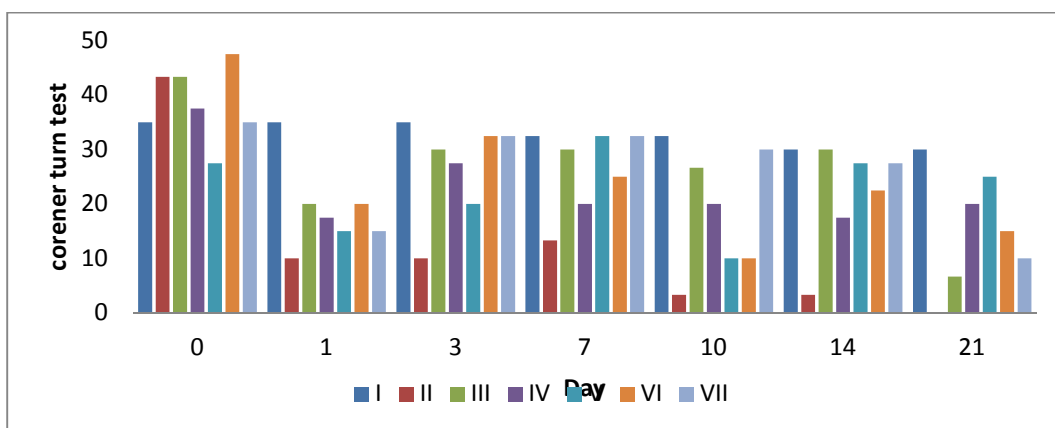


Figure 3 corner turn test (CTT) on the effect of ethanol extract and juice of Malacca (*Phyllanthus emblica L.*) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII)

Figure 3 shows that all test groups were induced CCAO on the first day had a stroke due to indicate a decline right limb motor function in the rat. After the first day on day 3 to day 14 begins to look for the right limb motor function recovery in CCAO+aspirin and all the test group.

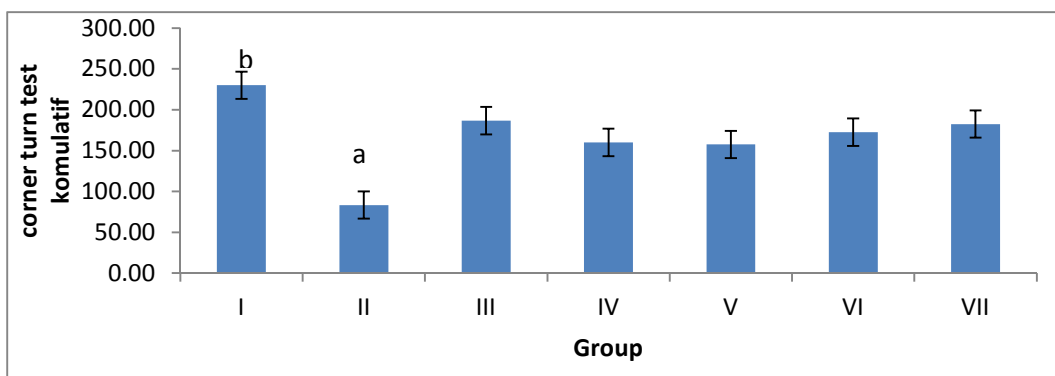


Figure 4 corner turn test (CTT) cumulative on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica L.*) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII). a = p<0,05 compare to normal group and b = p<0,05 compare to CCAO group

Figure 4 shows that aspirin group and all the test group increased motor function right leg.

Forelimb use asymmetry test

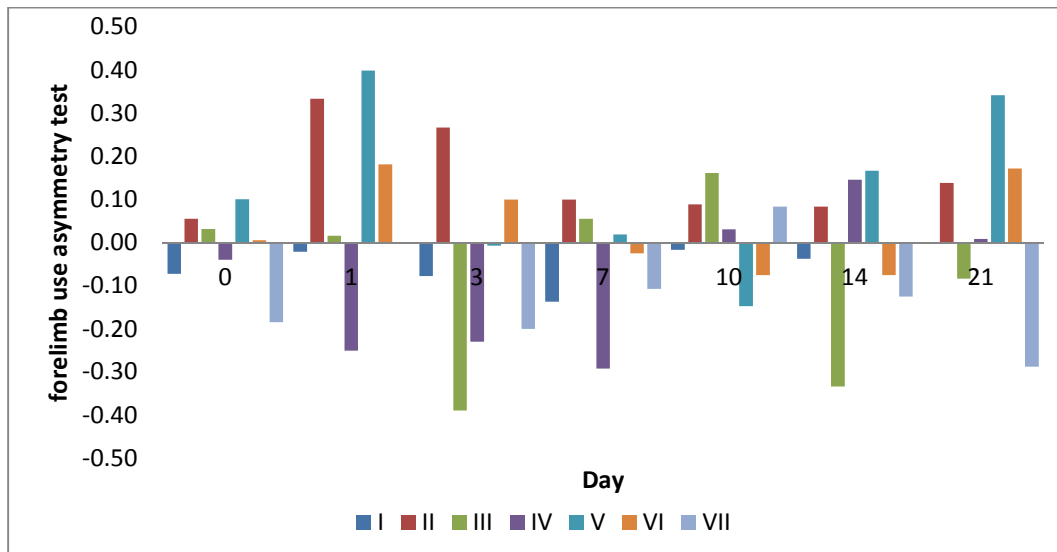


Figure 5 forelimb use asymmetry test (FUAT) on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica L.*) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII)

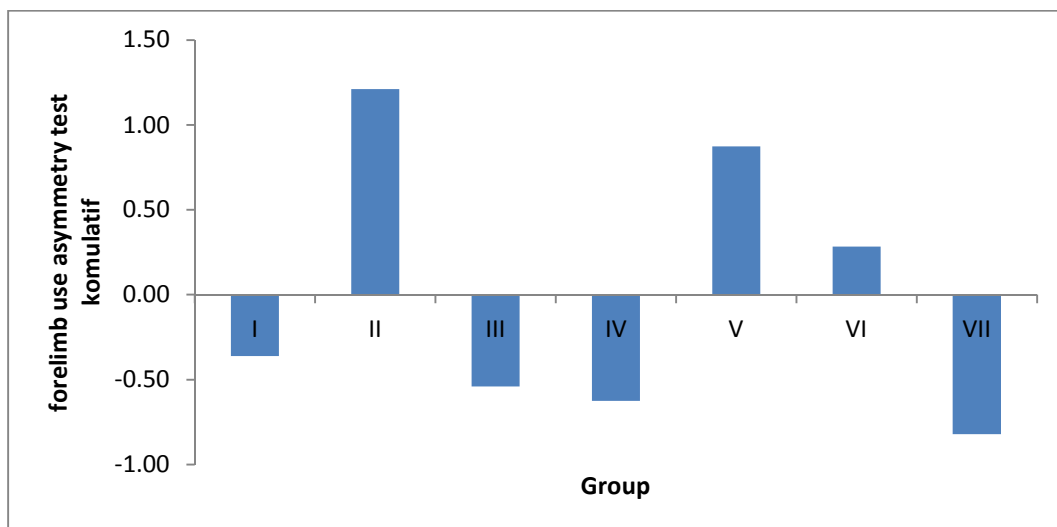


Figure 6 forelimb use asymmetry test (FUAT) cumulative on the effect of ethanol extract and juice of Malacca (*Phyllanthus emblica L.*) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII). a = $p < 0,05$ compare to normal group and b = $p < 0,05$ compare to CCAO group

FUAT data indicate the absence of significant improvement right leg function rats in the CCAO+aspirin group and all the test group.

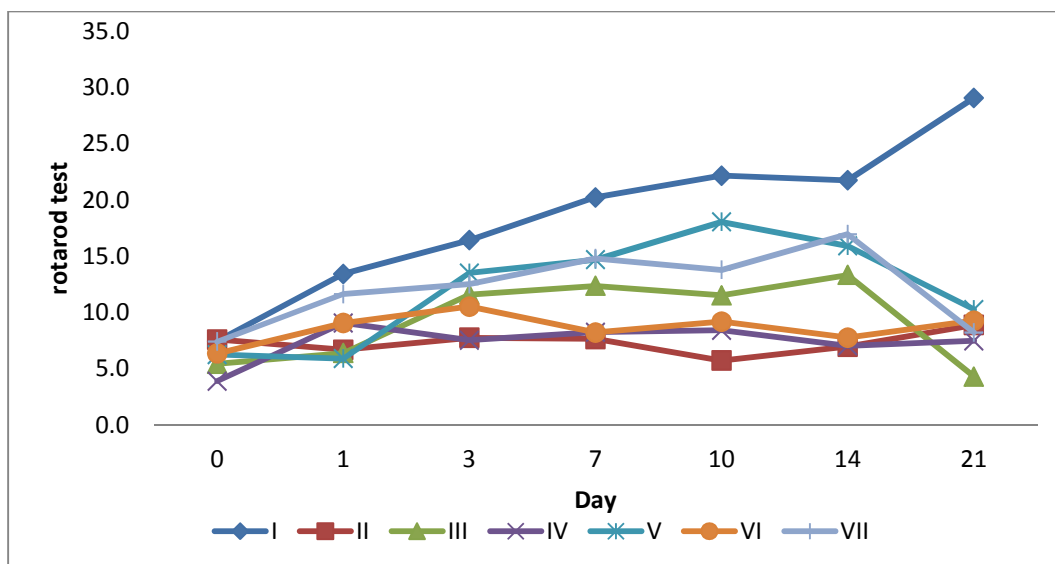


Figure 7 rotarod test on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica* L.) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII)

Figure 7 shows that all test groups on day 1 to day 7 have yet to show a recovery in motor function rats. Recovery of motoric functions seen at day 10 and day 14. CCAO+EEMF100, CCAO+JMF100, and CCAO+aspirin group showed recovery of motor function, characterized by the absence of a significant difference with the normal group statistically.

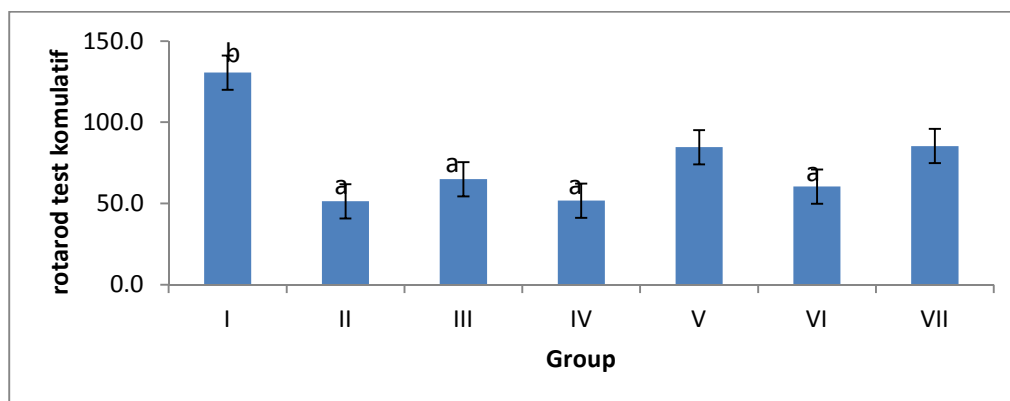


Figure 8 rotarod test cumulatif on the effect of ethanol extract and juice of Malacca (*Phyllanthus emblica* L.) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII). a = p<0,05 compare to normal group and b = p<0,05 compare to CCAO group

Figure 8 shows that CCAO+EEMF100 and CCAO+JMF100 group had no significant difference when compared with normal group, this means that the group has experienced an increase in motor function.

Inhibition of platelet aggregation

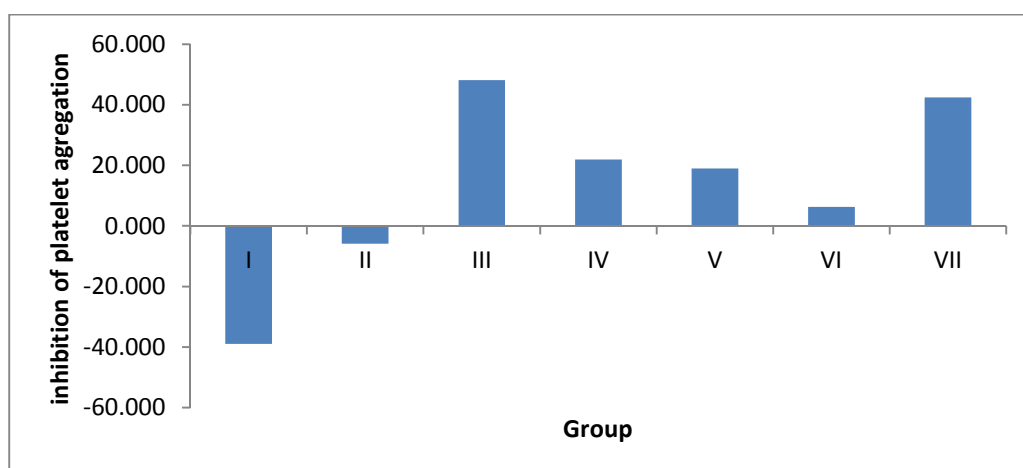


Figure 9 inhibition of platelet aggregation on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica* L.) fruit in rat model stroke. Normal group (I), CCAO group (II), CCAO+aspirin (III), CCAO+EEMF50 (IV), CCAO+EEMF100 (V), CCAO+JMF50 (VI), CCAO+JMF100 (VII). a = $p < 0,05$ compare to normal group and b = $p < 0,05$ compare to CCAO group

Data from the inhibition of platelet aggregation at day 14 showed no significant inhibition of CCAO+aspirin and all the test group compared with the normal group.

Size measurement Brain Injury in Rat

In figure 10 shows that only the CCAO group are white areas indicate brain injury. It shows that the results of behavioral tests on day 21 is quite consistent with the results of the staining of the brain. After analysis using Image-J software, obtained an average yield wide brain injury on CCAO group was 7.59 ± 1.7 .

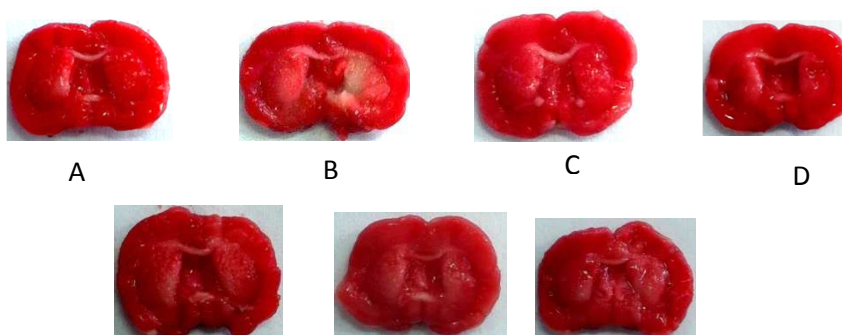


Figure 10 brain slices on the effect of ethanolic extract and juice of Malacca (*Phyllanthus emblica* L.) fruit in rat model stroke. Normal group (A), CCAO group (B), CCAO+aspirin (C), CCAO+EEMF50 (D), CCAO+EEMF100 (E), CCAO+JMF50 (F), CCAO+JMF100 (G).

DISCUSSION

Phyllanthus emblica L. is included in the division Magnoliophyta, class Magnoliopsida, and tribe Euphorbiaceae. Synonyms of *Phyllanthus emblica* L. is *Emblica grandis* Gaertner, *Emblica officinalis* Gaertner, and *Emblica arborea* Raf. Malacca fruit contains vitamin C, niacin, nicotinic acid, iron, calcium, carbohydrates, minerals, protein, fat, and fiber.

The human brain is divided into two hemispheres, the left hemisphere, and the right hemisphere. Each hemisphere receives the sensory message and gives orders to the motor in the opposite part of the body. Left hemisphere affects the body's motor functions right and the right hemisphere affects body's motor function left [17].

In this study used a mouse model of ischemia by binding the left common carotid artery. Binding of the left common carotid artery is expected to inhibit the flow of blood toward the left brain hemispheres so that the right part of the body's motor functions disturbed, which is characterized by a decrease in limb function in test behavior.

Moreover, inhibition of the left common carotid artery causes ptosis in the left eye. Ptosis is the medical term for a decrease upper eyelid when the eyes open. Ptosis in the left eye suspected to arise because of the disruption in the

oculomotor nerve fibers, which is one of the cranial nerve fibers that are at the center of the brain [17]. All rats were induced CCAO in this study experienced ptosis in the left eye.

From the four parameters (FPT, CTT, Fuat, and rotarod test) showed that both doses of CCAO+EEMF and CCAO+JMF may improve motor function of rat stroke because it gives meaningful results at FPT, CTT, and the rotarod test. CCAO+JMF100 showed a fairly constant recovery in each parameter.

Plasma absorbance measurement aims to observe the platelet activity before and after the administration of a solution of adenosine 5'-diphosphate sodium salt (ADP). ADP is a major inducer of aggregation, transformation and platelet secretion. The platelet activity can be seen from the changes in plasma absorbance measured by spectrophotometry at a wavelength of 600 nm. Platelet aggregation inhibition test did not show any significant inhibition of platelet aggregation.

CONCLUSION

CCAO+JMF100 group could potentially improve motor function in terms of a rats model of stroke motor the test such as forelimb placing test (FPT), corner turn test (CTT) and rotarod test. Platelet aggregation inhibition test did not show any significant inhibition of platelet aggregation. Brain slices of CCAO group are white areas indicate brain injury was 7.59 ± 1.7 .

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