**Acalypha indica** root extract increases post-hypoxic rat hippocampal tissue culture cell viability via phospholipase A\(_2\) inhibition

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**Abstract**

**Background:** Phospholipase A\(_2\) (PLA\(_2\)) is involved in inflammation and cell death following stroke, and inhibition of its activity may promote neuroregeneration. This study aimed to observe the influence of *Acalypha indica* Linn root extract towards relative cell viability and PLA\(_2\) enzyme level in post-hypoxic hippocampal tissue culture.

**Methods:** Experimental *in vitro* study using 24 primary neuronal cell cultures obtained from Sprague Dawley rats exposed to hypoxia with 5% O\(_2\)/5% CO\(_2\)/N\(_2\) balanced gas for 24 hours. Post-hypoxia, *Acalypha indica* Linn root extract was added at doses of 10, 15, and 20 mg/mL to three treatment groups. No treatment was given to the control group. Each group consists of six samples. After 72 hours of incubation, relative cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) examination, and phospholipase A\(_2\) enzyme level was determined using ELISA.

**Results:** PLA\(_2\) enzyme level of rat hippocampal tissue culture treated with *Acalypha indica* Linn root extract at 10, 15, and 20 mg/mL were significantly lower than that of control (5.55 ng/mL, 6.85 ng/mL, 7.42 ng/mL vs. 7.96 ng/mL, p < 0.05).

**Conclusion:** *Acalypha indica* Linn root extract increases the relative cell viability and decreases the PLA\(_2\) enzyme level of post-hypoxic mouse hippocampal tissue with the optimal dose of the extract at 10 mg/mL. (Med J Indones. 2013;22:136-40. doi:10.13181/mji.v22i3.581)

**Keywords:** *Acalypha indica* Linn, cell viability, hypoxia, neurogenesis, phospholipase A\(_2\)

Stroke is the leading cause of morbidity and a common cause of mortality worldwide.\(^1\) Thrombolytic therapy is currently the primary therapy for ischemic stroke\(^2\) due to its effect in improving neurological function; the only proven therapeutic approach that has this effect.\(^3\) Unfortunately, this therapy has a 3-hour therapeutic window, which is very brief,\(^2\) and increases the risk of hemorrhagic transformation.\(^4\) Thus, neurorestorative therapy with longer therapeutic window that can improve neurological function to restore and optimize the brain function is necessary.\(^3,4\) Neurorestorative therapy enhances the development of new nerve cells (neurogenesis) in brain tissue following ischemic stroke.\(^4\)

One mechanism of stroke is the activation of phospholipase A\(_2\) (PLA\(_2\)) enzyme, which will produce arachidonic acid (AA), leading to inflammation and eventually cell death.\(^5\) There are at least 22 different PLA\(_2\) enzymes that have been identified in various mammalian tissues, broadly classified into three families: calcium-dependent cytosolic (cPLA\(_2\)), secretory (sPLA\(_2\)), and calcium independent (iPLA\(_2\)).\(^5\) Cytosolic PLA\(_2\) (cPLA\(_2\)) and secretory PLA\(_2\) (sPLA\(_2\)) are involved in neuroinflammation and neurodegeneration.\(^5,8\) Inflammation has been proven to inhibit basal and post-hypoxic neurogenesis.\(^10\) In cerebral hypoxia, there is an increase in cytosolic PLA\(_2\) (cPLA\(_2\)) in astrocytes and microglia.\(^8,11\) hippocampus,
Acalypha indica increases post-hypoxic hippocampal viability

one of the site containing neural stem cells needed for neurogenesis, is also very susceptible to hypoxia and a prominent site of PLA2 expression. Nowadays, all PLA2 inhibitors that have been studied are non-specific so that the synthesis of a specific inhibitor for cPLA2 is highly required. Studies have shown that "knock-out" (cPLA2/-) mice had smaller infarcts and fewer neurological deficits compared to wild type following cerebral ischemia. Drugs that target PLA2 enzyme have also shown neuroprotective effects.

Acalypha indica Linn (akar kucing) is an indigenous Indonesian plant that can easily be found in all regions of Indonesia. People have been empirically using the roots of the plant to cure nerve paralysis caused by stroke. The plants have many active chemical compounds; some that have been identified are kaempferol (flavonoids), beta-sitosterol, HCN, gamma-sitosterol, and acalyphin. Yolanda, et al have studied Acalypha indica Linn’s neurotherapeutic effect on post-hypoxic nerve cells. Nirmal, et al have proven that acalyphin and stigmasterol exert their anti-inflammatory effects by forming PLA2 inhibitor complexes. Decreasing the inflammatory response can increase the mobilization of endogenous adult stem cells population in the central nervous system, thereby increasing neurogenesis and neurological function. This study aims to observe the effect of Acalypha indica Linn root extract on PLA2 enzyme in post-hypoxic hippocampal tissue in vitro, and its subsequent impact on cell proliferation.

METHODS

Study design and sample

An in vitro experimental study was conducted on primary neuronal cell cultures, which were taken from the hippocampus of adult Sprague Dawley rats aged 9-10 weeks weighing 150-200 grams. The experiment was conducted at the Oral Biology Laboratory, Faculty of Dentistry Universitas Indonesia from September to October 2011. The animals were sacrificed by cervical dislocation. The total number of samples was 24, divided equally into 4 groups (1 control group and 3 treatment groups) based on Federer formula. Ethical clearance was obtained from the Committee of the Medical Research Ethics of the Faculty of Medicine, Universitas Indonesia prior to the study.

Culture of nerve cells

Nerve cells were cultured in 10-cm petri dishes that have been coated with poly-L-lysine (Nacalai Tesque, Jakarta, Indonesia) for 1 hour. The nerve cells were then seeded into 1x10^6 cells/dish. Cultures were incubated with 5% CO2 at 37°C. Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO, Invitrogen) was used for culture medium and was replaced every 72 hours during the incubation period.

Cell harvest and plating

Nerve cells cultures were harvested on day 10 and identified morphologically. The number of cells was counted using trypan blue and a hemocytometer. The nerve cells were then seeded into 24 well-plates at 5 x 10^5 cells/well. The multiwell culture plates had previously been coated with poly-L-lysine for 1 hour.

Exposure to hypoxia

Directly after plating, nerve cell were then exposed to hypoxia by administering 5% O2 / 5% CO2 / N2 balanced gas. The gas were administered for 5 minutes in a vacuum container, then the vacuum container with the hypoxic cells was incubated with 5% CO2 at 37°C for 24 hours.

Treatment with Acalypha indica Linn extract

Each treatment groups of nerve cells cultures were given Acalypha indica Linn root extract at 10, 15, and 20 mg/mL. The control group was given nothing. Acalypha indica Linn root extract was obtained from the Department of Pharmacy, Faculty of Medicine Universitas Indonesia. The plates were re-incubated with 5% CO2 at 37°C for 90 hours.

Measurement of relative cell viability and proliferation

Relative cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). MTT assay was performed in duplicate. PLA2 enzyme level was measured using Mouse Phospholipase A2, PLA2 ELISA Kit from EIAab (PT. Indogen Intertama, Jakarta, Indonesia). PLA2 level examination was performed in duplicate. The MTT examination was performed on cells, while the PLA2 enzyme level examination was done on the supernatant. MTT examination was performed to confirm the findings of the our previous study.

Data analysis

Normality of data was tested using Shapiro-Wilk test. Due to abnormal distribution of data, comparison of data means was analyzed using non parametric test (Kruskal-Wallis) test and continued with Mann-
Whitney test. Data processing was performed using Statistical Package for the Social Sciences (SPSS) version 11.5 software.

RESULTS

Results of relative cell viability were in accordance with our previous study. Relative mean PLAs enzyme level in the control group was 7.96 ± 0.060 ng/mL (n = 6). In groups treated with Acalypha indica Linn extract at doses of 10, 15, and 20 mg/mL, mean PLA2 enzyme level was 5.55 ± 0.970 ng/mL (n=6), 6.85 ± 0.590  ng/mL (n = 6), 7.42 ± 0.090 ng/mL (n = 6), respectively (p = 0.04 in all treatment groups vs control; Figure 1).

The lowest level of PLA2 enzyme was seen in group treated with extract at dose of 10 mg/mL, and increased significantly in groups treated with extract at doses of 15 and 20 mg/mL (p = 0.016 and 0.04, respectively). Significant difference was also found in the PLA2 enzyme level between groups treated with extract at doses of 15 and 20 mg/mL (p = 0.04; Figure 1).

DISCUSSION

MTT test results were in accordance with the author’s previous study, indicating that Acalypha indica Linn root extract has an effect on increasing the number of viable post-hypoxic nerve cells with an optimum dose at 10 mg/mL.

The mechanism of the increased cell proliferation is thought to be due to the decrease in PLAs enzyme activity by acalyphin which will in turn decrease the inflammatory response and mobilize the endogenous stem cells to proliferate. This mechanism is investigated in this research reflected by the measurement of PLAs enzyme level. Our results indicated that the PLAs enzyme level in all treatment groups were significantly lower than control, with the lowest PLAs enzyme level found at the dose of 10 mg/mL. This result showed that Acalypha indica Linn root extract decreased the PLAs enzyme level in post-hypoxic nerve cell culture with the optimum dose at 10 mg/mL. This result also complies with the MTT assay result.

In this study, PLAs data were obtained from the supernatant of the nerve cells culture, therefore the detected PLA2 enzymes were non-specific and most likely be the PLAs enzyme secreted by cells (sPLA2). Other than sPLA2, cPLA2 is also regarded as the inflammatory precursor in cerebral hypoxia. Further research based on this finding is needed to examine the level of cPLA2 enzyme in post-hypoxic hippocampal tissue using a more specific marker.

Figure 1. Comparison of PLA2 enzyme level among control and treatment groups given Acalypha indica Linn root extract with consecutive doses of 10, 15, and 20 mg/mL. Data was given in means ± SD from 6 different samples. * showed significant difference compared to control group.
Structurally, Nirmal, et al. have shown that acalyphin exerts its anti-inflammatory effects by forming an inhibitory complex in Asp-49, Lys-69, and Gly 30 from the active site of sPLA$_2$, Russell viper, and with Ca$^{2+}$ ion from the active site of sPLA$_2$, bovine pancreas. The mechanism of this inhibitory complex formation has not been investigated in the central nervous system, thus further research is needed to assess whether or not in the central nervous system Acalypha indica Linn root extract would generate a similar sPLA$_2$ inhibitory complex mechanism similar to that in Russell’s viper and bovine pancreas.

In this study, the doses used were 10, 15, and 20 mg/mL based on experiences from previous studies. It was discovered that the optimum dose of Acalypha indica Linn root extract to increase relative cell viability and decrease PLA$_2$ enzyme level was 10 mg/mL. This result suggests that a lower dose than 10 mg/mL might give the same or better result, hence further study to determine the lowest optimum dose of Acalypha indica Linn root extract for neurorestorative therapy post-hypoxia is needed.

In addition to reducing the inflammatory response, Acalypha indica Linn’s root extracts may also increase cell viability by increasing neurotrophic factors such as BDNF, VEGF, and bFGF; which are required for the survival of neuroblast. Increased neurotrophic factors as a mechanism of increased relative cell viability caused by Acalypha indica Linn root extract has not been studied, hence further research is needed to assess whether Acalypha indica Linn root extract can enhance post-hypoxic cell viability through enhancement of neurotrophic factors by examining the levels of growth factors (e.g. BDNF).

The limitation of this research lies in the morphologic identification of the neural cells. This method cannot fully guarantee 100% identification of neural cells with certainty because other cells also such as glial cells and fibroblasts also reside in the hippocampus, which may be identified through this method. Thus, further research is needed with more precise identification of the neural cells using specific markers or antibodies such as neuron-specific enolase type 2.

From this research we can conclude that Acalypha indica Linn root extract at doses of 10, 15, and 20 mg/mL with the optimum dose of 10 mg/mL can increase relative cell viability of rat hippocampal cells post-hypoxia, in vitro.

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