The nerve protection and *in vivo* therapeutic effect of *Acalypha indica* extract in frogs

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Abstrak

Tujuan *Membuktikan efek neuroproteksi dan/atau neuroterapi ekstrak air Acalypha indica Linn. pada katak yang dilumpuhkan dengan injeksi pankuronium bromida*

Metode *Enam puluh ekor katak (Bufo melanostictus Schneider) dibagi ke dalam dua kelompok perlakuan yaitu kelompok neuroproteksi dan neuroterapi. Setiap kelompok dibagi ke dalam 6 subkelompok yaitu kontrol negatif (diberi air); kontrol positif (pirasetam), dan 4 kelompok dosis ekstrak: 200, 300, 400, 500 mg/KgBB. Pankuronim bromida 0,2 %, pelumpuh otot, disuntikkan subkutan pada punggung katak. Untuk efek proteksi, ekstrak diberikan per oral 1 jam sebelum disuntik, sedangkan untuk efek terapi ekstrak diberikan, sepuluh menit sesudah penyuntikan pankuronium bromida. Parameter yang diukur adalah waktu (menit) timbul dan lama kelumpuhan, serta waktu untuk perbaikan.*

Hasil *penelitian menunjukkan bahwa pada dosis ekstrak 400 dan 500 mg/KgBB terjadi efek neuroproteksi yang berbeda bermakna dibanding kontrol negatif dan pirasetam ($p<0.05$). Ekstrak pada dosis 200-500 mg/KgBB menunjukkan efek neuroterapi yang berbeda bermakna dibandingkan kontrol negatif ($p=0,000$) dan tidak berbeda bermakna dibandingkan pirasetam, kecuali pada dosis ekstrak 300 mg/KgBB, menunjukkan efek lebih baik dibandingkan dengan pirasetam ($p=0,012$).*

Kesimpulan *Bahwa ekstrak air Acalypha indica Linn. memiliki efek neuroproteksi dan neuroterapi yang sama atau lebih baik dibandingkan pirasetam pada katak yang dilumpuhkan dengan pankuronium bromida. Namun, masih diperlukan uji farmakodinamik dan farmakokinetik pada dua model hewan coba yang lazim digunakan.* ***(Med J Indones 2010; 19:96-102)***

Abstract

Aim To demonstrate nerve protection and/or treatment effect of *Acalypha indica* Linn. extract on nerve paralysis induced by subcutaneus injection of pancuronium bromide on frog's back.

Methods The study was performed on sixty frogs (*Bufo melanostictus Schneider*) that divided into two groups, i.e. the neuro-protection and neuro-therapy group. Each group was divided further into 6 sub-treatment groups: negative control group treated by water and positive control group treated by piracetam, treatment groups received the extracts 200, 300, 400, 500 mg/kgBW. Pancuronium bromide 0.2% (1 : 20 dilutions) were injected subcutaneously as muscle relaxant. The protective effect was studied by giving the extract orally, 1 hour prior to injection; while the therapeutic effect of the extract was studied by 10 minute treatment after injecting pancuronium bromide solution. The parameters measured were the onset and duration of paralysis (in minutes) and the recovery time (time needed to recover into normal condition).

Results The study showed significantly different protective effect of *Acalypha indica* Linn. root water extract at 400 and 500 mg/Kg.BW compared to negative control group and positive control group (piracetam ($p<0.05$); while the therapeutic effect was obvious at the dose 200-500 mg/Kg.BW compared to negative control group ($p=0.000$). There was no significant difference compared to positive control group (piracetam), except at 300 mg/Kg.BW ($p=0,012$).

Conclusion These results have proven that the water extract of *Acalypha indica* Linn. root has comparable protective and treatment effect on nerves system, as piracetam, but further studies should be performed to provide more evidences particularly pharmacokinetic and pharmacodynamic studies on two animal models that commonly used. ***(Med J Indones 2010; 19:96-102)***

Key words: *Acalypha indica Linn, Bufo melanostictus Schneider, nerve-protection*

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In 2006, the morbidity rate of stroke incidence in patients with hypertension due to cerebral thrombosis was about 83%, while 70% of these cases were caused by cerebral hemorrhage. One third of these patients recovered successfully from remaining symptoms, but two thirds of them had still persistent neurological disorders characterized by musculoskeletal paralysis such as hemiplegia or hemiparese.[1]

To treat such persistent symptoms, various conventional chemical drugs have been used despite their high cost, uncertain or still questionable efficacy between their benefit and risk, and/or aggravating side effects such as sleeping disorder, anxiety and depression, e.g., piracetam.[2]

Hypertensive patients often turned their choice to alternative or complementary traditional herbal medicine, such as celery (*Apium graveolens* Linn.)[3], garlic (*Allium sativum* Linn.)[4], and *mahkota dewa* (*Phaleria marcocarpa* Scheff.)[5]. These herbs are consumed separately or in combination to treat hypertension but not as remedies for paralysis or hemi-/paraplegia that may have occurred as a result of the hypertension-caused stroke and its remaining post-stroke symptoms.

In some Indonesian communities, root extract with boiling water of *akar kucing (Acalypha indica* Linn.*)* has been used traditionally to reduce or even heal such remaining symptoms. However, no data has been established as a reference on the beneficial magnitude of *Acalypha indica* in the treatment of neurological disorders *in vivo*.

Previously, it was demonstrated that the aqueous root extract from *Acalypha indica* Linn. has neuro-protective and neuro-therapeutic effects on gastrocnemius muscles of frogs in *ex vivo* studies[6]. Therefore, a similiar experimental *in vivo* study in frogs was performed. The frogs were considered as an experimental animal model based on the data of these previous studies which used isolated frog nerves and muscles *ex vivo* in order to examine membrane transport and activity of neurotransmitters.[7-8]

In a preliminary study[6], pancuromium bromide was used as a model substance to induce neural paralysis and its dosage was adjusted to 2 mg/ml (0.2%) at 1:20 dilution for subcutaneous (s.c.) administration, equivalent to 0.1 mg per frog.

The dosage of root extract was determined based on the *ex vivo* studies to 6, 9, 12, 15 mg or 200, 300, 400, 500 mg/kgBW, respectively, at an average body weight per frog of 30 grams.

From the *ex vivo* studies, it was expected that the results of the present study would also demonstrate protective and/or therapeutic effects of the root extract from *Acalypha indica* L. on the frogs' nerve system *in vivo.* Therefore, this animal model was chosen as a novel access in this preliminary study.

The general objective of this study was to prove that the root extract of *Acalypha indica* Linn. (*akar kucing)* has neural protective and therapeutic effects *in vivo.*

The specific objective was to prove that the root extract of *Acalypha indica* Linn. in a dose range of 6, 9, 12 and 15 mg or 200, 300, 400, 500 mg/kgBW has protecting and therapeutic effects on the nerves innervating the frog musculoskeletal system *in vivo* paralyzed by subcutaneous injection of pancuronium bromide.

METHODS

The animal model: This *in vivo* study was performed with 60 frogs in a weight range of 30±3.0 grams at the Department of Medical Pharmacy and Department of Physiology, Faculty of Medicine, University of Indonesia, from December 2007 to April 2008. The frogs identified as *Bufo melanostictus* Schneider by Bogoriensis Laboratory of LIPI, Bogor, were provided by the Department of Physiology, Faculty of Medicine, University of Indonesia.

The extract dosage was adjusted to 200, 300, 400, 500 mg/kgBW or 6, 9, 12, 15 mg for each frog, respectively, and piracetam as the positive control at 30 mg/ frog or 1,000 mg/kgBW. Pancuronium bromide 0.1 mg per frog was administered by subcutaneous (s.c.) injection. This dosage had been optimised experimentally in a preliminary study[6].

Group I: Neuroprotective effect

This part of the study was conducted in six subgroups: negative control (water); positive control (piracetam); the aqueous root extract of *Acalypha indica* Linn. with doses of 6 mg; 9 mg; 12 mg; 15 mg per frog, were given orally an hour before s.c. administration of pancuronium bromide 0.1 mg. The onset of action and duration of paralysis (in minutes) and the time (scored) needed to recover to normal condition were the parameters measured.

Group II: Neurotherapeutic effect

This part of the study was also conducted in six subgroups: negative control (water); positive control (piracetam); the aqueous root extract of *Acalypha indica* Linn. with doses of 6 mg; 9 mg; 12 mg; 15 mg per frog, were given orally, 10 minutes after subcutaneous injection of pancuronium bromide. The duration of paralysis (in minutes) and scored recovery time to normal condition were the parameters measured.

Statistics

Each group in this study consisted of 5 frogs, thus SD was evaluated from n=5. The onset of action (only for protection), duration of paralysis and recovery time (in minutes), both for protective or therapeutic effects were analyzed statistically by using one-way Anova with limit of significance at p=0.05.[9]

RESULTS

Group I: Neuroprotective effects (Figs.1-3).

Neuroprotective effects in frogs paralyzed with 0.1mg pancuronium bromide *in vivo* showed a maximum at dose 12mg or 400mg/kgBW, but the entire dose range from 6mg to 15mg (or 200-500mg/kgBW) had equivalent or even stronger effects than the positive control group with piracetam 30mg.

Onset of paralysis

Figure 1 shows that frogs which obtained the extract one hour before injection of pancuronium bromide, experienced paralysis within 14-25 minutes, whereas in the negative control group onset of paralysis was after 9±1.4 minutes and in the positive control group of piracetam after 11±1.6 minutes. In other words, onset of paralysis was slower over the whole range dose from 6mg to 15mg of extract as compared to both, negative and positive controls. The peak of efficacy in this experiment is at 25±2.2 minutes with 12mg of extract (Fig.1). The protective effect of 12mg and 15mg (22±2.0 min.) of extract is significantly different from both negative and positive controls (p<0.05).

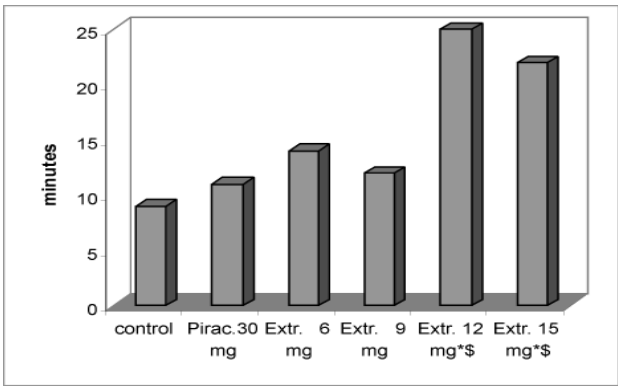

Figure 1. The onset of paralysis in the neuroprotective study (9±1.4; 11±1.6;14±1.7; 12±1.6; 25±2.2 22±2.0 min.)

* p <0.05 vs (negative) control; $ p<0.05 vs piracetam

Duration of paralysis

During an observation time of 2 hours, 12mg of extract showed a maximum protective effect on the duration of paralysis (59±2.0 min) induced by pancuronium bromide followed by 9mg with a duration time of 77±2.5 minutes. This is significantly (p<0.05) shorter than negative controls (112±5.0 min) but only 12mg of extract shortens duration of paralysis also significantly towards positive control of piracetam (81±2.8 min, p<0.05). The other treatment groups with 6mg and 15mg of the extract showed 83±3.4 and 83±3.2 min. duration time of paralysis comparable to piracetam. In other words, duration of paralysis was shorter over the whole range of dosage from 6mg to 15mg of extract as compared to negative control, with 12mg being also significantly more effective than piracetam 30mg (Fig.2).

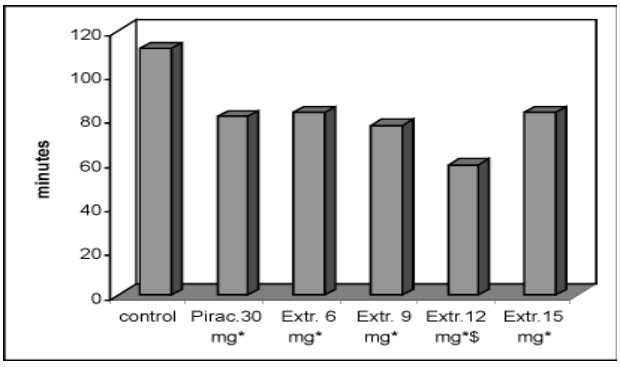

Figure 2. Duration of paralysis during 2 hours of observation (112±5.0; 81±2.8; 83±3.4; 77±2.5; 59±2.0; 83±3.2 min.)

* p <0.05 vs (negative) control; $ p<0.05 vs piracetam

Complete recovery

Complete recovery took about 2 hours in the control group which had only been given water through the nasogastric tube, i.e., these frogs did not wake up before 2 hours of observation. Based on this data, the groups were catagorised as follows: unawake after 2 hours (>120 minutes) = score 5; awake between 90-120 min = score 4; 60-90 min = score 3; 30-60 min = score 2; and less than 30 minutes = score 1.

The negative control group thus scored 4.25 and the piracetam group as positive control scored 3.8. Administration of 12 and 15mg of extract resulted in recovery scores of 2.4 and 2.5, respectively (Fig. 3), which values are significantly different from both negative control and piracetam groups ($p < 0.05$). The other two treatment groups with 6mg and 9mg of extract and a score of 3.0 were also more effective than piracetam, although the difference is not significant. However, all treatment groups differ significantly from the negative control group. Also in this experiment, the administration of 12mg of extract shows a maximum effect (Fig. 3). Statistical evaluation was performed with the original values in minutes before scoring.

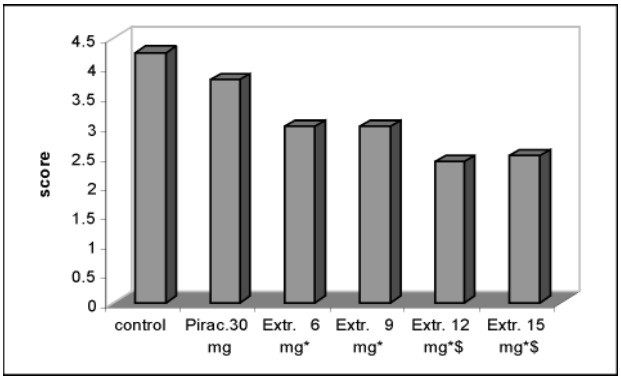

Figure 3. Recovery score within 2 hours of observation (protective effect)

* $p<0.05$ vs (negative) control; $ $p<0.05$ vs piracetam

Group II: Neurotherapeutic effect (Figs.4 and 5).

Duration of paralysis

This experiment resembles that one shown in Fig. 2, the difference is that the extract was now administered 10 min after the injection of pancuronium bromide. The results are depicted in Figure 4.

The therapeutic effect of *Acalypha indica* Linn. extract is characterized by a maximum at the group which obtained 9mg per frog. The difference in duration time of paralysis (20±1.2 min *vs* 115±5.4 min in negative controls) is highly significant (p=0.000) and also *vs* the positive control group which had received 30mg of piracetam (40±2.0 min; p=0.012).

All other values in the dose range of 6–15mg of extract per frog also demonstrated significantly shorter duration times of paralysis between 44±2.2 and 65±3.0 minutes (p=0.000-0.003) *vs* negative controls (115±5.4 min), comparable to the positive control group which had obtained piracetam (40±2.2 min; p=0.145-0.455). Hence, except for the group with 9mg of extract, no significant difference to the piracetam group was observed (Fig. 4).

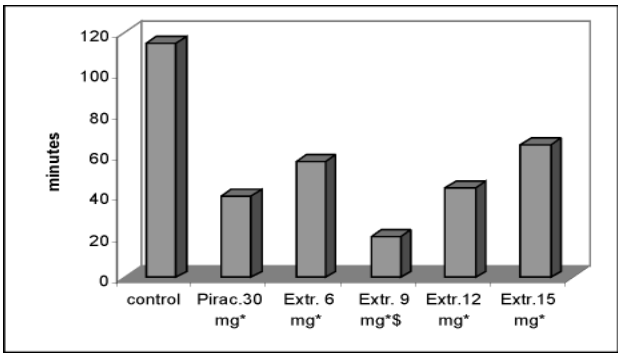

Figure 4. Therapeutic effect on duration of paralysis during 2 hours of observation. (115±5.4; 40±2.0; 57±2.6; 20±1.2; 44 ±2.2; 65±3.0 min.)

*$p<0.000$ vs (negative) control; $ $p<0.012$ vs piracetam

Complete recovery

After 2 hours of observation, all values in the dose range of 6–15mg of extract per frog demonstrated significantly shorter complete recovery times from paralysis between 27±1.4 min for the group with 12mg and 44±1.6 min for the doses of 9mg and 15mg (p=0.000-0.003) *vs* negative controls (115±5.6 min) and positive controls piracetam (58 ±2.0 min; p=0.000-0.316). These results were scored in the same way as explained in the context of Fig.3 and are depicted in Fig.5: negative control=score 4.25; piracetam=3.1; extract 6mg=2.3; extracts 9mg and 15mg=1.7; extract 12mg=1.4.

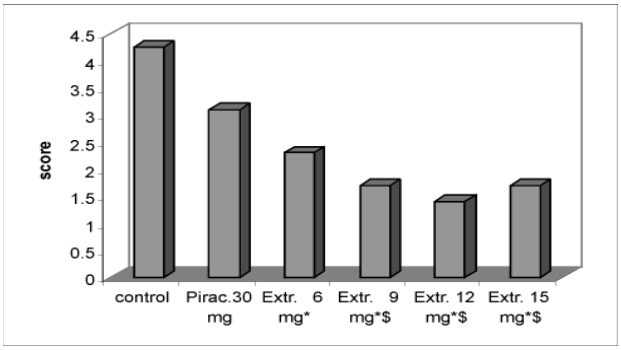

Figure 5. Recovery score within 2 hours of observation (therapeutic effect)

*p<0.000-0.004 vs (negative) control; $ p<0.000 vs piracetam

Summary

A significant neuroprotective effect of *Acalypha indica* Linn. extract on frogs paralyzed with pancuronium bromide *in vivo* has been shown at a dosage of 12mg per frog or 400 mg/kgBW, while the whole dose range of extract (6-15mg or 200-500 mg/kgBW) exerted a significant therapeutic effect compared to the negative control group with a maximum effect at 9-12mg or 300-400 mg/kgBW.

DISCUSSION

Acalypha indica Linn. (*akar kucing)*[10,11]

Nahrstedt et al. isolated acalyphin from *Acalypha indica* Linn. and published its structure in 1982 [10] and it was considered the active compound[11] although a variety of other compounds have been isolated from *Acalypha indica* [12,13]. Various extracts from this plant had been shown to have diurectic [14], antimicrobial [15], scavenging activity [16] , and antifertility [17] effects, however not all extracts were decocts from the plant's roots as proven for the antiurisemic activity in rats[18].
In our study, a standardised aqueous extract from the roots was applied.

Frogs as animal models

Frogs have been used classically for demonstration of neuro-musculoskeletal effects in the laboratories of physiology and pharmacology and numerous studies generally using this *ex vivo* neuro-musculoskeletal model support its validity [7-8]. Therefore, also our working group used this *ex vivo* model in a preliminary study.[6] The present study demonstrating protective and therapeutic effects of *Acalypha indica* Linn. (*akar kucing)* extract in living frogs provides evidence that frogs can be used as an *in vivo* animal model for innervation, both in peripheral and central nervous systems.

Piracetam

From innumerous publications on the effects of piracetam, only few which may be considered in the context of "positive control" in the present study are selected. Clinically doses of piracetam up to grams are applied in therapy and the official safety data sheet denotes $LD_{50(mouse)}$ of 2,000g/kgBW. There for, in our study the dosage of 1,000mg/kgBW was chosen, equivalent to 30mg per frog. Piracetam is thought have impact on acetylcholine via muscarinic cholinergic receptors and on NMDA glutamate receptors and may modulate ion channels and thus membrane permeability [19-22]. Furthermore, in animal models there appears to be an effect on cerebral/neural mitochondrial energy metabolism, electron transport and ATP synthesis [23-24].

Dosage finding of the extract

The effective dose in frogs *in vivo*, 20 times the dose of *ex vivo* study (200-500 mg/kg BW or 6-15 mg/frog, at an average body weight of 30 g), was lower than *ex vivo* study using neuromuscular junction's model .i.e. 10; 15; 20; 25 mg/sample, because of their body weight, both the protective and therapeutic effect [6].

Effects

In the neuroprotective set of experiments, the extract is superior over piracetam 30mg which is even statistically significant at higher extract dosages of 12-15mg. From these experiments different from the plant extract, piracetam does not exert protective efficacy.

In the therapeutic set of experiments, piracetam exerts a siginificant effect vs controls, as does the plant extract at all dosages from 6-15mg. However, at a dose of 9mg, the extract is significantly more effective than 30mg of piracetam.

In both sets of experiments, the plant extract exerts a bell-shaped pharmacodynamic action with peaks at 12mg for the protective effect and at 9mg for therapy. These dosages are also significantly superior over our reference compound piracetam at the applied dose of 30mg.

These results are interpreted that the extract acts as a competitive inhibitor to pancuronium bromide in neuromuscular junction both in neuroprotective and neurotheraptic sets of experiments, while piracetam did not show comparable neuroprotective effect. Hence, in our model, piracetam can be used as neurotherapic compound at neuromuscular junction, but its mechanism of action appears to be different from the *Acalypha indica* extract. It is not clear whether one of the "active" compounds of *Acalypha indica* like acalyphin, is responsible for the effects seen in our sets of experiments or rather their metabolites [25]. Furthermore, as often in herbal medicines, it is the specific composition of extracts that causes pharmacological effects.

The effective dose of this extract as neurotherapeutic agent was lower (300 mg/kgBW) than its neuroprotective dosage (400-500 mg/kgBW). Since the effect was better than that of piracetam the extract of *Acalypha indica* Linn. will be promising to be choosen as complementary or alternative herbal medicine to treat neurologic disorders, especially in central nervous system although the mechanism of action cannot yet fully be explained.

Although frogs can be used at least as a first or preliminary animal model as demonstrated in this study, further studies have to be conducted with another animal model using mice or rats. Furthermore, the mechanism of action of the extract as a neuroprotecting and neurotherapeutic agent has to be demonstrated yet in central and peripheral nervous systems. To this end a model not using pancuronium bromide should be used in order to exclude that the extract might just counteract this compound in terms of a competitive antagonist. The aim of future studies should also be done to improve the extract as a standardized herbal medicine for the treatment of central or peripheral neural paralysis, either in the central nervous system or in neuromuscular junction.

In conclusion, the results of this study indicate that the extract of *Acalypha indica* Linn. has neuro-protective and neurotherapeutic effects in frogs *in vivo*. From our interpretation it is suggested that the extract acts as a competitive inhibitor to pancuronium bromide in neuromuscular junction, both in neuroprotective and neurotherapeutic sets of experiments. However, further studies should be performed in other animal models to prove its mechanism of action in preclinical pharmacokinetic and pharmacodynamic studies.

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