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# Inhibitory effect of asiatic acid on excitatory synaptic transmission in the rat hippocampus

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## Abstract

**An *in vitro* excitatory post-synaptic potential stimulation technique was used to examine the effects of asiatic acid (a. acid), an isolated compound of *Centella asiatica* on excitatory post-synaptic potential (EPSP) in the rat hippocampal slices. Hippocampal slices 350 µrn thick were perfused with oxygenated artificial cerebrospinal fluid, and electrodes were placed in the *cornu ammonis* (CA1) region to record EPSP responses to stimulation of Schaffer collateral/commissural fibers. Gamma-aminobutyric acid (GABA) receptors properties were measured with or without a. acid and all exposure to known GABA A or GABA B channel blockers; bicuculine or phaclofen. The major effect of a. acid was a dose and time dependent increase in the intensity and duration of GABA A blocker mediated inhibition compared with GABA B blocker, which was no response. Furthermore, the inhibitory concentration ($IC_{50}$) value for a.acid was measured 14 µM. This depressant effect was not reversible after a 30-min washout of the a. acid and these experiments confirmed that a. acid having a selective GABA B receptor not for GABA A receptor.**

**Key words**: *Centella asiatica*, Gamma-aminobutyric acid, Asiatic acid

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## Introduction

*Centella asiatica*, is a slender and creeping perennial herbal plants with weak aroma used by diverse ancient people from different cultures in Malaysia and other Asian countries. In the last decades, *C. asiatica* was identified as to have the properties of cholinergic activity, anti oxidant activity or anti inflammatory activity. A few studies have shown that *C. asiatica* has cholinomimetic, anti inflammatory and antioxidant properties. The water extract of the herb reveales significant antinociceptive activity which is statistically similar to aspirin. The extract also reveales significant anti-inflammatory activity which is statistically similar to the non-steroidal anti-inflammatory drug, mefenamic acid**.** The *C. asiatica* extract has been shown to have potentially cholinomimetic activities *in vivo*. Another significant effect of *C. asiatica* is that it acts as an anti oxidant. *C. asiatica* also accelerates nerve regeneration upon oral administration and contains multiple active fractions increasing neurite elongation *in vitro* [1].

Analytical studies have shown that *C. asiatica* contains triterpenoids, essential oils, amino acids and other compounds, such as vellarin. The terpenoids include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, centellose, brahmic, centellic, madecasic acids and a.acid [2]. A. acid is a pentacyclic triterpene compound found in *C. asiatica* which has been traditionally used for skin diseases. A. acid has been shown to promote fibroblast proliferation and collagen synthesis and to stimulate extracellular matrix accumulation in a rat wound model [3]. In addition, a.acid like other triterpenes has been reported to possess other biological effects including hepatoprotection and protective effects against β-amyloid-induced and glutamate-induced neurotoxicity [3].

On the other hand, γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. There is evidence that there are two different GABA receptors in the brain: GABA A and GABA B receptor [4] [5]. GABA A receptors is coupled with benzodiazepine re-

ceptors and $C1^-$ channels. On the other hand, GABA B receptors are coupled with G protein. The activation of GABA B receptors decreases the amplitude of $Ca^{2+}$currents and increases the $K^+$ conductance. GABA A and GABA B receptors have somewhat different physiological actions [6]. GABA A receptors is mainly involved in anxiety and convulsion [4]. In contrast, GABA B receptors are mainly related to depression and analgesia [7]. Regarding the abundance of the receptor subtypes, GABA B receptors represent 30 % of the total GABA receptors [8]. However, GABA B receptors represent a capable target for potential drugs that might enhance cognitive functions [9] since antagonists of GABA A receptors often induce epileptic paroxysms and convulsions.

GABA A as well as GABA B receptors play an important role in learning and memory [4]. Activation of GABA receptor is known to affect memory and learning through agonist or antagonist effects. In a study done by [6], shown that baclofen which was a selective agonist for GABA B induced the deficit learning and the effect was dose dependent. Baclofen is a stereospecific agonist of GABA B receptors, that is an important drug used for pharmacotherapy of spasticity in humans. In the previous study, baclofen was demonstrated that in certain experimental configurations baclofen interfered with learning in rodents in memory paradigms (including spatial congnition) when administered systemically, intracerebroventricularly or intrahippocampally [10,11]. Furthermore, previous studies reported that pre-training injections of baclofen (GABA B agonist) as well as muscimol (GABA A agonist) had detrimental effects on passive avoidance learning in rats when the test session was conducted 24 h later [6]. The study also found that, muscimol-reduced latency disappeared when muscimol was re-injected before the test session: muscimol induced state-dependent learning. Alternatively, the rats injected with baclofen before the training and test sessions showed reduced latency: baclofen failed to induce state-dependent learning.

To date, there is no other report that shows the effects of a. acid on the excitatory postsynaptic potential (EPSP) to detect the neuronal activity on exposure of a. acid and this may provide direct evidence that GABA A and GABA B receptors may influence learning and memory in a different manner. To obtain the result, an experiment was designed to examine the effect of a. acid, a compound isolated from *C. asiatica* involvement of Gabaergic systems through view of EPSP and correlated with their actual properties to well-know GABA blocker.

## Materials and Methods

### *Sample preparation*

*C. asiatica* plants were purchased from a local producer in Kuala Terengganu, Terengganu Darul Iman. The samples were then authenticated by botanical herbal expertise from Universiti Malaysia Terengganu (UMT). The collected samples were calculated the weighted. Thereafter, the whole plants were ground with an electric grinder to obtain powder form before undergoing extraction phase.

### *Extraction and solvent partitioning*

The ground samples were extracted with methanol (MeOH). Thereafter, the extractions were repeated for 3 times to ensure the entire bioactive compound from the samples [12].

The filtered extracts were transferred into a round-bottle flask (A-Tech Global Science Limited, 100 ml). The solvents from the extracts were evaporated under a reduce pressure using a rotary evaporator (RE 3000A). The weights of the crude methanol extract were measured and recorded.

The extracts then underwent solvent partitioning technique. Hexane partitioning technique was chosen since the a. acid was detected in hexane partitioning. Hexane extract and reference compound were diluted in 10mg/ml and 1mg/ml in methanol prior to thin layer chromatography (TLC) analysis. Hexane extract was then undergone to column chromatography (CC) to isolate a. acid.

### *Column Chromatography (CC)*

The preparation of column chromatography was performed as previously described by Dhoukeng *et al.,* 2005. Briefly, the column (35.0 x 6.5 cm) was filled with silica gel (Merck 9385). Silica gel was mixed with solvent to obtain a slurry mixture. The mixture was stirred slowly to eliminate air bubbles and filled into the column. The column was tapped constantly while packing with the slurry mixture of silica gel. The column was stabilized with the lowest polar of the desired solvent system. The samples were impregnated to silica gel (1:1) and were introduced to the top of the silica gel bed.

The elution process was preceded in increasing polarity (step gradient). The fractions were collected in 100 ml each (first CC process) and 5 ml each (for repeat CC process). The interest fraction was monitored by TLC. Fractions with the same TLC pattern/profile were combined. The fraction contained the same chemical compound was purified by repeating CC (70.0 x 1.0 cm). The compound was confirmed with various chromatography techniques (Infrared spectrophotometer (IR) and Nuclear Magnetic Resonance (NMR). As for IR spectrometer, the model Perkin Elmer Spectrum 100 Fourier Transform Infra-red (FT-IR) Spectrometer using KBr discs which were used with the absorption bands were measured in $cm^{-1}$. For NMR, 1H-NMR and 13-NMR spectra were recorded on Varian Unity Inova 500 Spectrometer.

***Electrophysiology (Excitatory-Postsynaptic Potential)***
The preparation of hippocampal slices were performed as previously described [13]. Briefly, hippocampal slices were prepared from young adult (4-6 weeks) male Spraque-Dawley rat from Laboratory Animal Research Unit, Universiti Sains Malaysia, (LARUSM). All electrophysiological experiments were performed in accordance with the guidelines of the Ethical Committee on the Use and Care of Animals (Animal Ethics Committee, Universiti Sains Malaysia). Animals were anesthetized with isoflurane and decapitated.

The brains were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 125, KCl 2.5, $NaHCO_3$ 25, $CaCl_2$ 2, $MgCl_2$ 1, *D*-glucose 25, $NaH_2PO_4$ 1.25 (pH 7.4), and bubbled with a 95% $O_2$/5% $CO_2$ mixture. Transversal slices of the hippocampus (350 μm thick) were prepared using a microtome, (Microm HM650V, Germany). After incubation in a holding chamber with aCSF (22 - 25 °C) for at least 60 min, the slices were placed in the recording chamber and superfused with aCSF at a flow rate of 1.5 ml/min and were incubated for 30 minutes to decrease the stress of the brain.

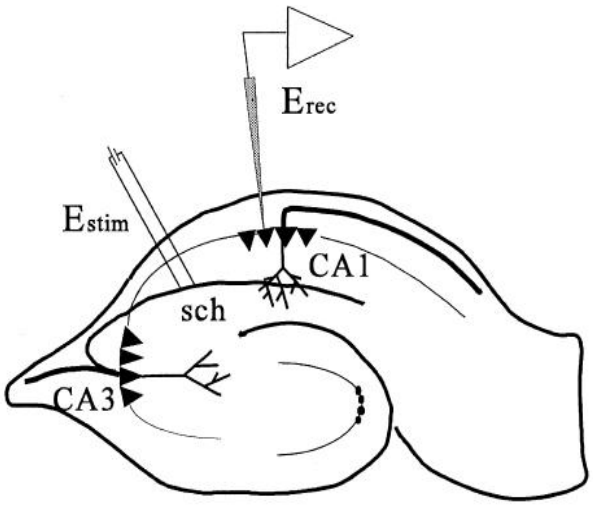



***Figure 1****. fEPSPs recording of activity in the hippocampal slice. fEPSPs were elicited by Schaffer collateral (sch) stimulation through a polar stimulation electrode and recorded at CA1 region as extracellular field potentials with ACSF-filled glass recording electrodes (0.5–1.5MΩ) placed in the stratum radiatum of the CA1 region.*

fEPSPs were elicited by Schaffer collateral stimulation through a polar stimulation electrode and recorded as extracellular field potentials with ACSF-filled glass recording electrodes (0.5–1.5MΩ) placed in the stratum radiatum of the CA1 region (Figure 1) using SEC-10LX (NPI, Germany). The synaptic response to a standard test stimulus (0.033 Hz) was monitored until a stable recording was obtained, and the input–output relationship was then determined. The stimulus strength (0.2–2.5 mA) producing a response of approximately 50% of the maximal response amplitude was determined and used for all subsequent experiments. For purpose of viability, only synaptic potentials with more than 0.2 mV and without superimposed population spikes were used for the experiments by stimulating the hippocampus CA1 region. After stable baseline recorded of the responses of the brain slices for 20 min, GABA blocker (bicuculine for GABA A receptor blocker or phaclofen for GABA B receptor blocker) bath-applied was follow by a.acid to the hippocampal slices for 60 min and then was washed out for another 30 min. The evoked synaptic responses were recorded every 15 s during bath application of asiatic acid and for another 30 min after washout.

The evoked synaptic responses were recorded and analyzed with a personal computer using custom-developed software (Cellwork version 5.0 and Igor Pro, version 2.30D, Germany). The fEPSPs were quantified by measurements of the amplitude of the synaptic responses. Each of the amplitudes of the fEPSPs obtained during a.acid application was normalized to the average amplitude of the 10 min baseline recordings of the fEPSPs acquired before a. acid application. The significance of the differences between the means was calculated for different points in time (t=20, 40, 60, 80, 100, 120 and 140 min), using a t-test or Mann–Whitney rank sum test subsequently, 20 min after stable baseline of EPSP and a. acid applied. Values were considered significantly different if $p \leq 0.05$. In the text, values are shown as mean ± S.E.M.

***Inhibition Concentration ($IC_{50}$) of A.acid***

Using the Prism program (GraphPad Software, Inc. CA, USA) on a Compaq computer, concentration response curves were fitted to the equation: $y = a\,x/(x + b)$, where y is the drug effect, a is maximum effect, x is concentration of drug and b is the $IC_{50}$ value (concentration of drug producing 50% of maximum inhibition). An $IC_{50}$ value for a a.acid was calculated for each brain slices with GABA A blocker since the GABA B had no significant effect on fEPSP amplitudes, there was no $IC_{50}$ for a.acid with GABA B blocker. The mean $IC_{50}$ (and S.E.M.) was obtained by averaging results from several concentration of the a. acid in each brain slices

## Results

***Isolation of a. acid***
The dried and finely ground leaves of *C. asiatica* (10 kg) were extracted at room temperature with methanol (MeOH) for three days and concentrated under vacuum

to afford 445 g of crude extract. The *C. asiatica* extract (250 g) was dissolved in water and successively extracted with hexane, chloroform, ethyl acetate (EtOAc) and *n*-butanol to yield, respectively, 95, 75, 45 and 18 g in solvent partitioning.

Hexane was subjected to a column chromatography (75×5.2) filled with silica gel (63–200, 60 A°) and eluted with a gradient of *n*-hexane in dichloromethane, dichloromethane in chloroform, and chloroform in methanol. Ninety-eight fractions of 100 ml were collected and regrouped on the basic of analytical TLC in fifty fractions.

Column chromatography 12 g was subjected to repeated column chromatography (60 cm×3 cm) filled with silica gel (32–63, 60 A° ) eluted with a gradient of methanol in chloroform to yield 2 compounds. Compound 1 was

amorphous with a white powder and finally identified as a. acid (30 mg) according to various chromatography techniques with previous publication of Infrared spectroscopy (IR) and Nuclear Magnetic Resonance (NMR).

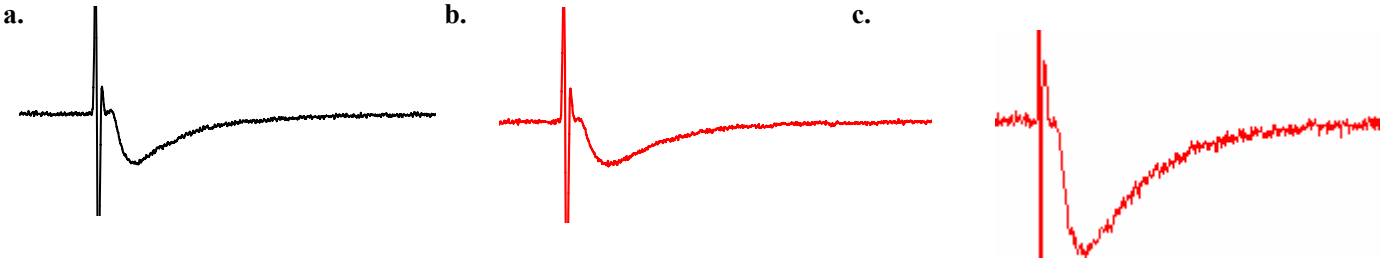
**Effects of a. acid on excitatory synaptic transmission in hippocampal slices**

***Effects of a. acid with GABA B blocker on fEPSP.***
In the CA1 region of hippocampus, extracellular potentials were elicited by stimulating the Schaffer collateral-commisurral fibers and recorded in the stratum radiatum. Original recordings with and without application of 100 µM are shown in Fig. 2d. Perfusion of a. acid had no significant effect on fEPSP amplitudes, either in slices with concentration a. acid 30 µM (Fig. 2a, n=6) or from 100 µM (Fig 2b, n=7). The fEPSP amplitudes are listed in Table 1. The significance of the differences between the means was calculated for different points (t=20, 40, 60, 80, 100, 120, 140 min), using the paired Student's t-test. Values were considered significantly different if $p< 0.05$.

***Table 1***. *Mean fEPSP amplitudes ± SEM compared to the baseline reference. Bold numbers are significant compared to the control. Perfusion of a. acid had no significant effect on fEPSP amplitudes with phaclofen, either in slices with concentration a. acid 30 µM or from 100 µM. Bath application of a. acid affected the amplitudes of the fEPSPs with bicuculine evoked in a concentration-dependent manner in slices from 1, 3, 10, 30 and 100 µM a. acid. The onset of the blocking effect varied with the concentration of a. acid.*

**fEPSP**

| Point in time (minutes) | t=20 | t=40 | t=60 | t=80 | t=100 | t=t120 | t=140 |
|---|---|---|---|---|---|---|---|
| Control (without a.acid) | 100±3% | 101±3% | 102±2% | 102±2% | 102±2% | 103±2% | 103±2% |
| 30 µM a.acid/phaclofen | 100±3% | 99±3% | 100±2% | 99±3% | 101±3% | 102±1% | 103±3% |
| 100 µM a.acid/phaclofen | 100±3% | 100±2% | 101±3% | 100±3% | 101±4% | 102±1% | 102±3% |
| 1 µM a.acid/bicuculine | 99±3% | 99±4% | 97±3% | 98±3% | 98±2% | 98±2% | 97±2% |
| 3 µM a.acid/bicuculine | 100±3% | 100±4% | 96±3% | 89±3% | 89±4% | 88±1% | 88±2% |
| 10 µM a.acid/bicuculine | 99±2% | 96±3% | 89±3% | 87±3% | 86±3% | 82±3% | 75±3% |
| 30 µM a.acid/bicuculine | 99±2% | 94±2% | 88±3% | 81±4% | 74±3% | 60±3% | 51±4% |
| 100 µM a.acid/bicuculine | 100±1% | 83±3% | 71±2% | 61±4% | 46±3% | 29±2% | 10±2% |




with 30 µM a. acid +phaclofe

with 100 µM a. acid +phaclofen

control (without a.acid)

**d.**

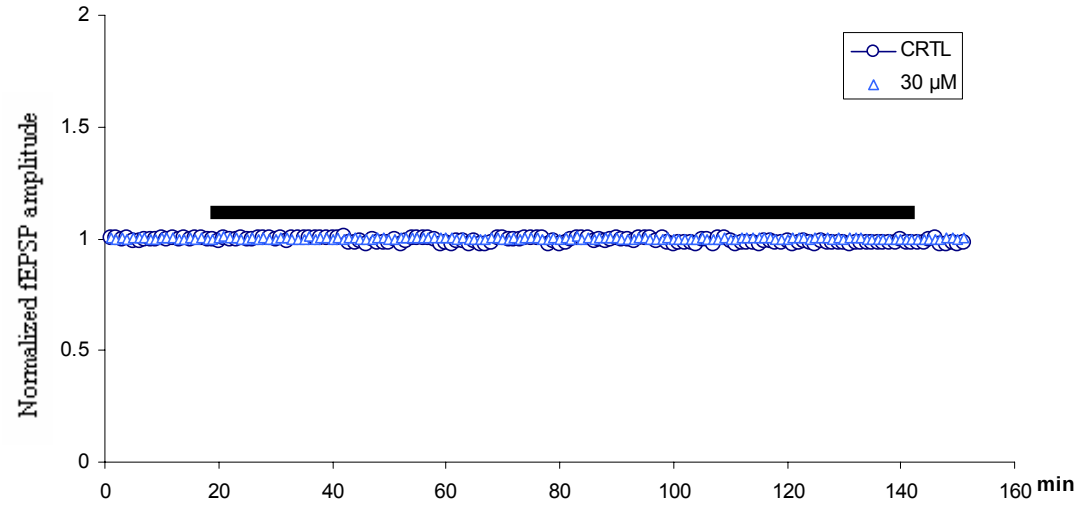
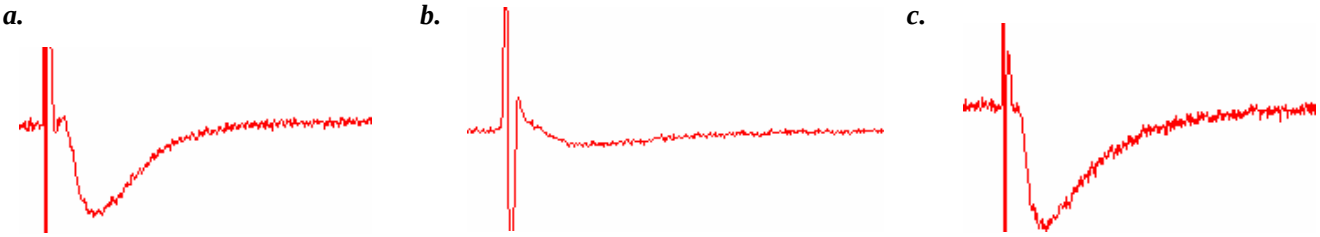



***Figure 2**. Effect of a.acid on fEPSPs. Typical traces of fEPSPs in the CA1 dendritic layer after stimulation of Sch without and with administration of 30 μM (a), 100 μM (b) and control (c). Diagrams of mean values±S.E.M of the fEPSP amplitudes (normalized to the average of 10-min baseline response) under control conditions (without a.acid, blue triangles) and after administration of 30 μM (black dots) (d). Black bar demotes the perfusion with a. acid*

***a.*** ***b.*** ***c.***



with 30 μM a.acid + bicuculine | with 100 μM a. acid + bicuculine | control (without a.acid)

***d.***

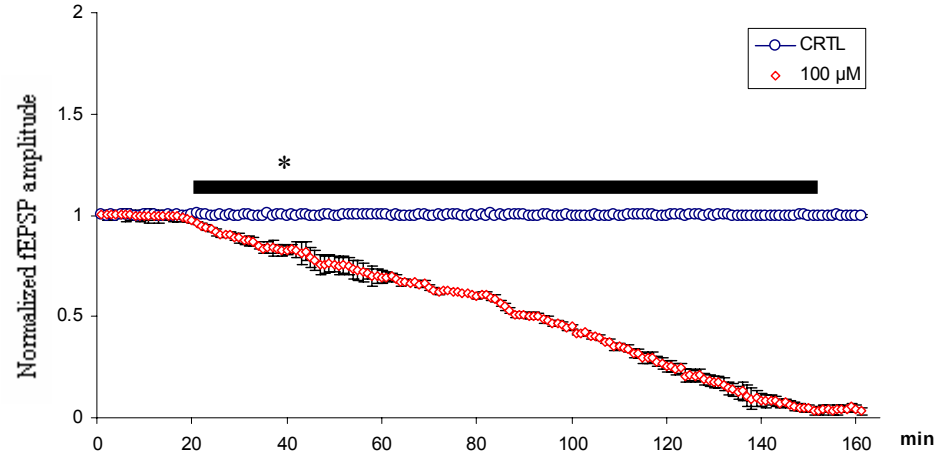



***Figure 3**. Effect of a. acid on evoked fEPSPs. Typical traces of fEPSPs in the CA1 dendritic layer after stimulation of Sch with administrations of 30 μM (a), 100 μM (b) and control (c). Diagrams of mean values ± S.E.M of the fEPSP amplitudes (normalized to the average of 10-min baseline response) under control conditions (without a. acid, blue dots) and after administration of 100 μM a. acid (red triangles) (d). Black bar demotes the perfusion with a. acid. Asterisks indicate a significant difference between the values obtained and the control values.*

From that result of the inhibition effects of a.acid in dose-dependent manner, the concentration response curwere fitted and further examined the inhibitory effect of a. acid on the fEPSPs evoked in the CA1 sub region. A. acid decreased fEPSPs in a dose-dependent manner with an $IC_{50}$ of approximately 14 μM as depicted in Fig. 4.

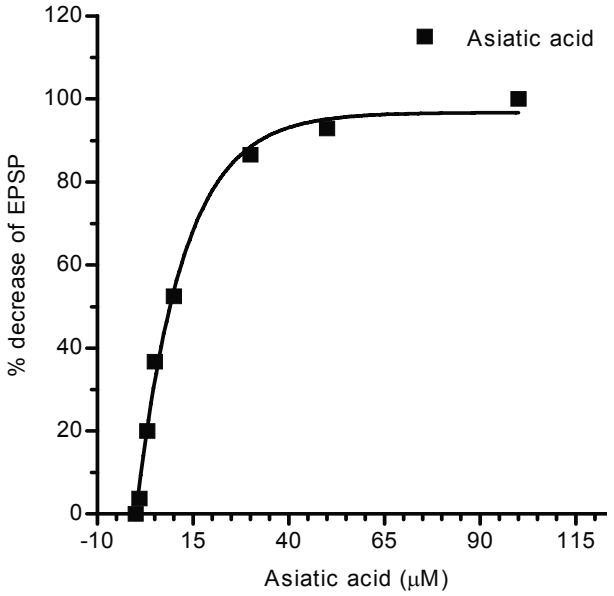



***Figure 4**. Inhibition of a. acid on fEPSPs evoked potential in the CA1 region of hippocampal of rats. Result is from one experiment run in triplicate.*

***Effects of A. acid with GABA A blocker on fEPSP.***
Bath application of a. acid affected the amplitudes of the fEPSPs evoked in a concentration-dependent manner in slices from 1, 3, 10, 30 and 100 μM a. acid. The onset of the blocking effect varied with the concentration of a. acid. The fEPSP amplitudes are listed in Table 1. The significance of the differences between the means was calculated for different points (t=20, 40, 60, 80, 100, 120, 140 min), using the paired student's t-test. Values were considered significantly different if $p< 0.05$.

Original recordings with and without application of 100 μM are shown in Fig. 2d. Application of 1 μM produced a slight decrease in the fEPSP amplitudes to about 97% of the control amplitudes, which had no significant 140 min after application. Application of 3 μM led to a sensible reduction of the fEPSP amplitudes to about 88% of the control values, which was significant 80 min after application. Thus, application 10 μM led to a moderate reduction of the fEPSP amplitudes to about 75% of the control values, which was significant 60 min after application. Application of 30 μM (Fig. 2a, n=7) led to greater reduction of the fEPSP amplitudes to about 51% of the control values, which was significant 60 min after application. Lastly, application of 100 μM (Fig. 2b, n=7) led to a greater reduction of the fEPSP amplitudes to about 10% of the control values, which was significant 40 min after application. The depressant effect was not reversible after a 30-min washout of the a. acid.

## Discussions

The aim of this study is to investigate the effects of a. acid, a compound isolated from *C. asiatica* extract with blockers of GABA A and GABA B, on Gabaergic transmission in the hippocampus slices. From this experiment it was found that the a. acid blocks excitatory transmission at the hippocampal CA1 synapse in a concentration-dependent manner for GABA A. The blocking effects were considerably greater in slices with concentration of 100 μM with bicuculine. In contrast, a. acid, even at the high concentration of 100 μM, exerted no effects with GABA B blocker, phaclofen.

The impairment of excitatory synaptic transmission at the Schaffer collateral-CA1 synapse by a. acid indicated that this effect was caused by the direct or indirect action of this substance on Gabaergic receptors. A. acid interacts with specific GABA B receptors and lead to the opening of GABA B receptor that affected the potassium ions of the neuronal cell to outflow from the cell. This led to hyperpolarization of the neuronal cell resulting decrease of EPSP within time. The depressant effects were irreversible after a 30-min washout of the a. acid.

The viability question may rise from this study whether the irreversible effect came from a. acid properties or by its viability of slices itself. The viability of the hippocampal slices was confirmed when only the synaptic potentials with more than 0.2 mV and without superimposed population spikes were used for the experiments by stimulating the hippocampus CA1 region before wash in the a. acid. Thus, the control slices with the recording slices for the same length of time under the same conditions, with bicuculine or phaclofen and without a. acid also were performed.

The results of this study demonstrate a dose-dependent of EPSPs after wash in the a. acid in the recording chamber. The inhibitory effect of a. acid on the fEPSPs was examined. A. acid at doses 1, 3, 5, 10, 30 and 100 uM decreased fEPSPs in a dose-dependent manner with an $IC_{50}$ value of approximately 14 uM. Doses 1 and 3 uM were found slightly decrease from the EPSPs which was not significance compared with baseline. Doses 100 uM greater reduction of the EPSPs about 100 % of the amplitudes and this shown that a. acid 100% activated the GABA B channel in 100 uM.

It was found that a. acid acted as an agonist to GABA B since it led to a sensible reduction of the fEPSP ampli-

tudes. It may induce deficit of place learning with same effect to baclofen (GABA B agonist) as reported [14]. The GABA B receptor agonist baclofen presumably increased presynaptic inhibition in the hippocampus [15], possibly via an increased $K^+$ conductance and/or a decrease in voltage-dependent $Ca^{2+}$conductance as shown in hippocampal neurons [16] [17]. Since a. acid was also proven to be able to increase similar potassium conductances similar to baclofen effect, it could act via a similar process to decrease facilitation of perforant path synaptic transmission.

*C. asiatica* has been described as possessing central nervous system activity, such as improving intelligence. Previous studies indicated that whole plant of *C. asiatica* was beneficial in enhancing memory [18] and extracts of *C. asiatica* was reported to improve general mental ability of mentally retarded children [19] [20]. Furthermore, fresh leaf juice of *C. asiatica* can improved passive avoidance task in rats [21]. These result support that *C. asiatica* can enhance memory and learning in human and animals.

GABA B receptors may also play a neuroprotective role through modulation of cholinergic activity [22]. Indeed, activation of GABA B receptors that inhibited mAChR-induced synchronous glutamatergic activity in both as the rat piriform cortex [23] and hippocampus [24]. Local perfusion with the GABA A agonist muscimol dramatically reduced striatal ACh release, while the GABA B agonist baclofen caused only minor decreases in ACh release. This suggests that GABA tonically regulates striatal ACh release primarily through stimulation of inhibitory GABA A receptors [25]. It seems here that, GABA B could be the main target in memory and learning research in future.

For the known GABA B agonist, baclofen, the $IC_{50}$ estimated at 7 μM [26]. As compared to a. acid, it's less potent for GABA B receptor. A profound inhibition of GABA B neurotransmission for a. acid was observed in a dose-dependent manner. In some slices an almost complete block was found with concentration 30 μM of a. acid. For a. acid, the $IC_{50}$ estimated 14 μM. From that, the inhibiton of EPSP with less than 14 μM were minimal but still can mediate depression of synaptic transmission and contribute to the inhibition controlling neuronal excitability.

Based on the result, a. acid is different from *C. asiatica* extract. A. acid seemed to give inhibitory effect to learning and memory according to the EPSPs result in this study. A. acid may synergistically react with other compounds to potentate a positive response to the memory and learning rather than a. acid alone to give a negative response to the EPSPs. Meanwhile, it was also suggested that the glutamate receptors activity may not be enough to counter-react with activation of GABA B receptors. Although this finding showed that a. acid selectively agonist to GABA B, there is a need to confirm with single cell recording for future study. These findings may support that a. acid gives an effect on GABA B receptor systems that are related to learning and memory similar to baclofen effect when examine in future for the effect of GABA B on animal behavioral study.

**Conclusion**

The present study demonstrates that the a. acid extracted from *C. asiatica* has profounf effects to GABA B receptors agonist with the $IC_{50}$ for a. acid values for EPSPs was 14 μM. A. acid seems to have good potential for drug discover as especially for GABA B agonist group. Further experiments are needed to postulate the real effect of a. acid to the neuronal activity in future.

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