Isobolographically Additive Anticonvulsant Activity between *Centella asiatica's* Ethyl Acetate Fraction and some Antiepileptic Drugs

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Objective: *To investigate interaction between orally given Centella asiatica's ethyl acetate fraction (EACA) and intraperitoneally administered antiepileptic drugs (AEDs), namely, phenytoin, valproate and gabapentin.*
Material and Method: *Isobolographic analysis was used to evaluate the interaction between EACA and AEDs in terms of protection of mice in the pentylenetetrazole test. Rotarod test was used to evaluate neurotoxicity.*
Results: *When given alone, the median effective dose of phenytoin, valproate and gabapentin were found to be 13, 104, and 310 mg/kg BW, respectively, whereas the corresponding values in the presence of EACA were 5, 29 and 79 mg/kg BW. Together with isobolographic analysis, the results obtained indicated an additive effect among all combinations tested. In relation to neurotoxicity, combination of gabapentin and EACA demonstrated a broader margin between the effective dose and the neurotoxic dose while the other two combinations did not.*
Conclusion: *The present finding suggested a potential of Centella asiatica to be developed as an adjunctive medication for epileptic patients.*

Keywords: *Centella asiatica, Anticonvulsant activity, Isobologram*

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Worldwide, approximately 1-3% of the population suffers from epilepsy. For most of the patient population, monotherapy with conventional antiepileptic drugs (AEDs) represents the mainstay of treatment[1]. In spite of optimal choice and application of currently available AEDs, about 30% of patients are resistant to the standard medication. In such cases, the addition of a second drug to the established monotherapy seems to be the most adequate treatment regimen. The adequate combination of two antiepileptics might be

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advantageous if it fully controls the seizures and simultaneously, if there are no or inconspicuous adverse effects related with antiepileptics in polytherapy[2].

Centella asiatica (CA) is a plant in Family Umbelliferae, Subfamily Hydrocolyte. Its synonyms are Indian Pennywort (English), Hydrocolyte asiatique (French), Tsubo-kusa (Japanese), Luei Gong Gen (Chinese) etc.[3]. This plant is known in Thai as Bua Bok (บัวบก).

A recent study reported that the aqueous extract of CA decreased the pentylenetetrazole (PTZ)-kindled seizures as evident by decreased seizure score[4]. In addition, it was shown that the alcoholic extract of CA increased the level of GABA, which is a key function of antiepileptic agent, in the central nervous system (CNS) in rats in a dose-dependent manner[3]. From these results, it is likely that CA may be beneficial as adjuvant to AED. Therefore, the present study aimed to investigate an interaction between extract of CA, and some currently available antiepileptic drugs in animal models.

Material and Method

Plant material and preparation of the extracts

CA was purchased from Nakornpathom Province which provides pesticide-free CA, Thailand. The aerial parts were washed with running tap water, dried and coarsely ground. The coarse powder of the plant was macerated with hexane for 7-10 days and filtered. The marc was then remacerated with another portion of hexane until the filtrate was nearly clear. The combined filtrate was concentrated under reduced pressure by rotary evaporator to syrupy mass and then evaporated with water bath until no traces of hexane were left to yield a syrupy crude of hexane fraction. After hexane extraction, the marc was remacerated again and again (totally 3 times) with ethyl acetate, methanol and boiling with water to yield ethyl acetate, methanol and aqueous fraction, respectively, by the same procedure.

Animals

Experiments were performed on male ICR mice weighing 18-25 g. They were obtained from the National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. They are acclimatized in the ventilated room of the laboratory at the ambient temperature of 25°C on a natural light/dark cycle for at least one week prior to the experiments. Standard food (C.P. mice food) and tap water are provided *ad libitum*.

All animal care and handling were conducted with the approval of the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Administration of the tested substance

Each fraction of CA extract was dissolved in a vehicle (Tween 20:water; 2:5) and given to the animals one hour prior to the injection of PTZ. A gavage tube was used to deliver the substance by the oral route which is the clinically expected route of administration of CA. The volume of administration was kept at 0.2-0.3 ml / 25 g BW of the animal.

Route of administration and pretreated time of AEDs were selected according to their respective time to peak effect previously reported. Phenytoin[5], valproate[6], and gabapentin[7] were given intraperitoneally at 90, 60 and 120 min, respectively, prior to the injection of PTZ.

Determination of the median effective dose (ED_{50}) of *Centella asiatica's* ethyl acetate fraction (EACA) and AEDs against PTZ - induced seizure test (PTZ test)

Seizures were induced by a subcutaneous (sc) injection of PTZ (70 mg/kg BW) in 0.9% sodium chloride. The volumes of injection were

kept at 0.1-0.2 ml / 25 g BW of the animal. The end point was a generalized clonic seizure with loss of righting reflex within 60 minutes after injection of PTZ[6]. Eight mice per dose and five doses were used to establish ED_{50} of the EACA and AEDs to protect against PTZ by the method of Litchfield and Wilcoxon[8].

Determination of the median neurotoxic dose (TD_{50}) of EACA and AEDs (rotarod test)

The rotarod test was modified from the one previously described by Cuadrado et al carried out with a rod of 3.5 cm diameter, rotating at 18 rpm[9]. The end-point to evaluate the minimal neurotoxicity was the inability of the animal to maintain its equilibrium for at least 1 min on the rotating rod in each of three successive trials. Before the experiment, mice were placed on the rotating rod in a training session for 5 minutes. Untreated mice were able to maintain their balance on the rod for several minutes. The EACA, AEDs or combination were administered to each group of mice and they were tested again after a specific period of time. Eight mice per dose and five doses were used to determine the TD_{50} by the method of Litchfield and Wilcoxon[8].

Isobolographic analysis

Isobolographic analysis, the principal method applicable for understanding the real nature of drug interaction, was used to analyse the interactions between EACA and conventional AEDs (phenytoin, valproate, and gabapentin) in the PTZ test in mice. The ED_{50} value (with their 95% confidence limits) for each substance administered alone in the PTZ test was denoted directly from the respective drug-dose effect curve according to Litchfield and Wilcoxon[8]. The ED_{50} of each AEDs in the presence of EACA was also calculated in the same manner using three different dose pairs of equi-effective dose of EACA and respective AEDs[10]. In the present study, the mixtures of EACA with an AED were co-administered in a fixed-ratio combination of 1:1. This means that a combination was composed of $^1/_2$ of the ED_{50} of EACA and $^1/_2$ of the ED_{50} of AED resulting finally in the full ED_{50} of an EACA-AED combination[2]. Substances were delivered in such a way that they were at their time to peak effect during the assessment of effects on the dependent measure.

Isobologram was then constructed from the ED_{50} values of EACA and AEDs when each of them was given alone[11]. Straight line connecting between two ED_{50} values is the theoretical additive line representing dose pairs of EACA and AEDs that are additives in protecting 50 percent of the animals. Theoretical ED_{50} at the fix-ratio of 1:1 was then compared to the observed experimentally combined ED_{50} to estimate the nature of interaction. If the observed experimentally combined ED_{50} lies on the additivity line, then the dose pair having these coordinates is simply additive. On the other hand, the points lying below the line suggests synergistic interaction while the ones above the line would then suggest antagonistic nature of the combination[11,12].

In addition, various combinations of EACA and AEDs used to determine the observed experimentally combined ED_{50} mentioned above were subsequently used for the determination of TD_{50} by rotarod test.

Protective index (PI)

PI, a quantitative measure of the margin between doses producing anticonvulsant (protective) effect and motor toxicity, was calculated by dividing the TD_{50} value by the ED_{50} value. PI of EACA and AEDs in monotherapy and in combination were also calculated.

Data and statistical analysis

For the determination of the ED_{50} and TD_{50}, the dose response curve was plotted between doses (log scale) and probits, which were transformed from percentage of protection. Three to five different doses of each substance were used to construct the dose response curve. The linear regression method was used to fit the curve and the value with confidence limits for 95% probability was then calculated by the method of Litchfield and Wilcoxon[8].

In conjunction with isobolographic analysis which was used to estimate the nature of interaction between EACA and AEDs visually as mentioned above, Student's unpaired *t* test was used to determine statistical significant difference ($p < 0.05$) between the ED_{50} of AEDs in the presence and in the absence of EACA.

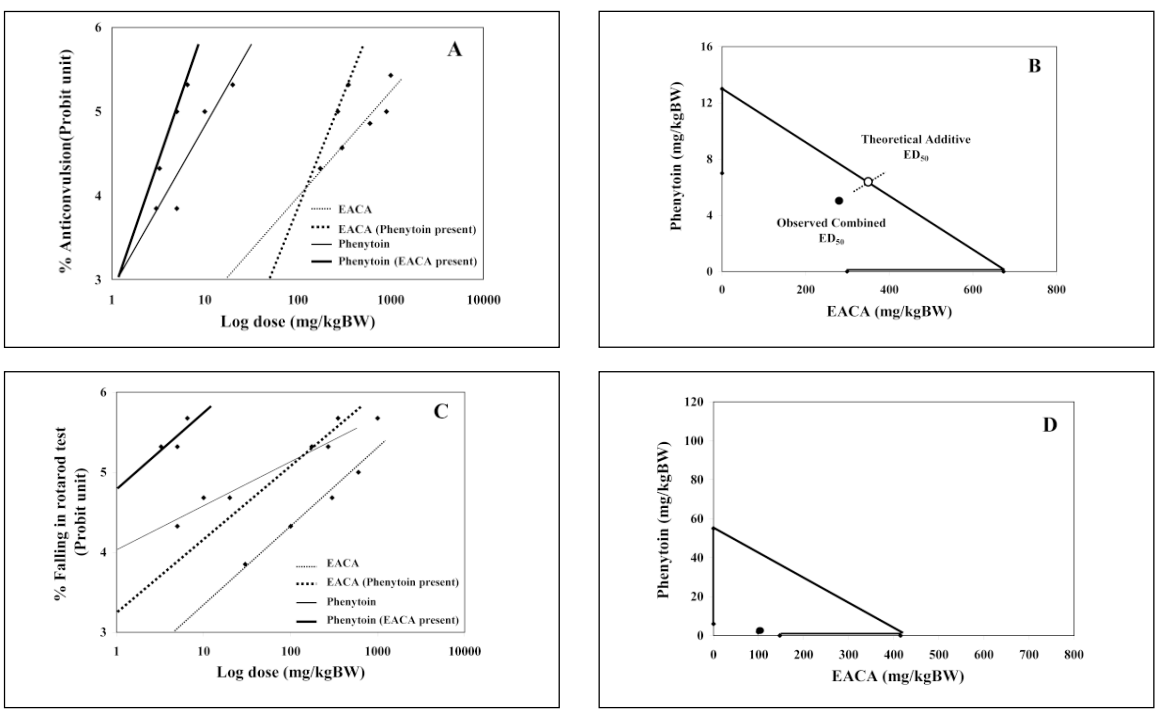

Fig. 1 Dose-response curves and isobolographic representation of EACA and phenytoin on ED_{50} and TD_{50} in the pentylenetetrazole model in mice. A, dose-response curves of anticonvulsant effect of EACA [ED_{50} = 673(299-1515);....], phenytoin [ED_{50} = 13(7-25);—], EACA in the presence of phenytoin [ED_{50} = 277(187-409);....] and phenytoin in the presence of EACA [ED_{50} = 5(3-8);—]. B, isobolographic representation of the interaction between EACA and phenytoin. In this graph the ED_{50} values of EACA and phenytoin are plotted as the x- and y-axis intercepts, respectively. The thicker lines directed from each ED_{50} value toward zero represent the lower 95% confidence limit of each ED_{50} value. The straight line connecting these two points is the theoretical additive line. The open circle that lies on the theoretical additive line represents the calculated theoretical ED_{50} value of the combination, were the interaction additive. The closed circle represents the experimentally observed ED_{50} value of the combination of EACA-phenytoin. In this experiment, the ED_{50} value of the combination of EACA-phenytoin fall below and inside the lower confidence limits of the theoretical additive, suggesting the interaction was synergy. Consistent with this, the experimental ED_{50} value was not significantly different from the theoretical additive ED_{50} value (Student's *t* test, p = 0.7015), indicating that the interaction was additive. C, dose-response curves of minimal neurotoxic effect of EACA [TD_{50} = 415(147-1169);....], phenytoin [TD_{50} = 55(6-491);—], EACA in the presence of phenytoin [TD_{50} = 99(11-854);....] and phenytoin in the presence of EACA [TD_{50} = 2(1-6);—]. D, isobolographic representation of the interaction between EACA and phenytoin. The experimental TD_{50} value was not significantly different from the theoretical additive TD_{50} value (Student's *t* test, p = 0.9203), indicating that the interaction was additive

Results

Anticonvulsant activity and neurotoxicity of CA extract

When four fractions (hexane, ethyl acetate, methanol and aqueous fraction) obtained from sequential extraction of CA were tested, only EACA (but not the other extract) demonstrated anticonvulsant activity. EACA (300, 600, 900 and 1000 mg/kg BW) given orally were able to protect the animals against PTZ-induced convulsion in a dose dependent manner exhibiting the ED_{50} of 673(299-1515) mg/kg BW at the pretreated time of 1 hour. Respective TD_{50} of EACA (30, 100, 300, 600 and 1000 mg/kg BW) assessed by rotarod test was found to be 415(147-1169) mg/kg BW. Subsequently, the EACA was further investigated for its interaction with currently available AEDs, namely phenytoin, valproate and gabapentin.

Isobolographic analysis of interaction between EACA and AEDs

• EACA and phenytoin

ED_{50} of intraperitoneally given phenytoin (3, 5, 10 and 20 mg/kg BW) was found to be 13(7-25) mg/kg BW when given alone and it was decreased to 5(3-8) mg/kg BW in the presence of orally given EACA (Fig. 1A and Table 1). The combination also decreased ED_{50} of EACA from 673(299-1515), when given alone, to 277(187-409) mg/kg BW (Fig.1A). The isobolographic representation of the interaction between EACA and phenytoin (Fig. 1B) illustrated that the observed combined ED_{50} value, constructed from the experimentally calculated ED_{50} of EACA (X axis) and phenytoin(Y axis), lay below the additive line. Thus, synergistic interaction of the combination was suggested. However, no statistical difference was noted between the ED_{50} values of phenytoin in the presence and in the absence of EACA (p= 0.7015). Therefore, the interaction of phenytoin and EACA was simply additive.

As illustrated in Fig. 1C, the combination also decreased TD_{50} of EACA from 415(147-1169), when given alone, to 99(11-854) mg/kg BW. The TD_{50} of phenytoin in the absence of EACA, 55(6-491) mg/kg BW, was not statistically different (p= 0.9203) from its corresponding value in the presence of EACA, 2(1-6) mg/kg BW. Taken together with the visual assessment of isobologram in Fig. 1D, the neurotoxicity of EACA and phenytoin was also additive in nature resulting in the PI of 0.4 for the combination (Table 1).

• EACA and valproate

Similarly, ED_{50} of intraperitoneally given valproate (70, 85, 100 and 150 mg/kg BW) was found to be 104(88-121) mg/kg BW when given alone and it was decreased to 29(21-40) mg/kg BW in the presence of orally given EACA (Fig. 2A and Table 1). The combination also decreased ED_{50} of EACA from 673(299-1515), when given alone, to 201(144-282) mg/kg BW (Fig. 2A). The isobolographic representation of the interaction between EACA and valproate (Fig. 2B) illustrated that the observed combined ED_{50} value lay below the additive line. Thus, synergistic interaction of the combination was likely. However, no statistical difference was noted between the ED_{50} values of valproate in the presence and in the

Table 1. The median effective doses (ED_{50}), median neurotoxic doses (TD_{50}) and protective indices (PI) of phenytoin, valproate and gabapentin given intraperitoneally either alone or in combination with ethyl acetate extract of *Centella asiatica* in mice

Groups	ED_{50} (mg/kg)	TD_{50} (mg/kg)	PI (TD_{50}/ED_{50})
EACA	673(299-1515)	415(147-1169)	0.62
Phenytoin	13(7-25)	55(6-491)	4.23
Phenytoin (with EACA)	5(3-8)	2(1-6)	0.4
Valproate	104(88-121)	247(107-568)	2.38
Valproate (with EACA)	29(21-40)	33(20-54)	1.14
Gabapentin	310(150-638)	719(141-3660)	2.32
Gabapentin (with EACA)	79(41-153)	622(89-4345)	7.87

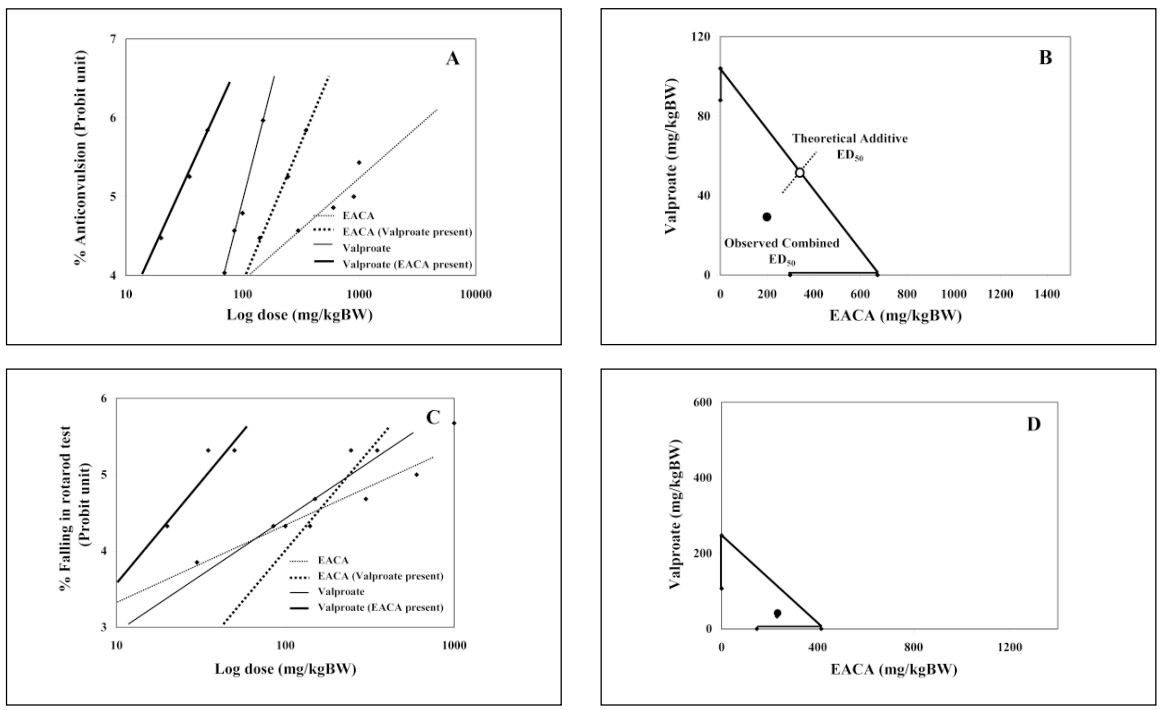

Fig. 2 Dose-response curves and isobolographic representation of EACA and valproate on ED_{50} and TD_{50} in the pentylenetetrazole model in mice. A, dose-response curves of anticonvulsant effect of EACA [ED_{50} = 673(299-1515);....], valproate [ED_{50} = 104(88-121);—], EACA in the presence of valproate [ED_{50} = 201(144-282);**....**] and valproate in the presence of EACA [ED_{50} = 29(21-40);**—**]. B, isobolographic representation of the interaction between EACA and valproate. The experimental ED_{50} value was not significantly different from the theoretical additive ED_{50} value (Student's *t* test, p = 0.3399), indicating that the interaction was additive. C, dose-response curves of minimal neurotoxic effect of EACA [TD_{50} = 415(147-1169);....], valproate [TD_{50} = 247(107-568);—], EACA in the presence of valproate [TD_{50} = 231(142-378);**....**] and valproate in the presence of EACA [TD_{50} = 33(20-54);**—**]. D, isobolographic representation of the interaction between EACA and valproate. The experimental TD_{50} value was not significantly different from the theoretical additive TD_{50} value (Student's *t* test, p = 0.5524), indicating that the interaction was additive

absence of EACA (p= 0.3399). Therefore, like the results of EACA and phenytoin previously mentioned, the interaction of valproate and EACA was also additive.

In the presence of valproate, the TD_{50} of EACA was decreased from 415(147-1169), when given alone, to 231(142-378) mg/kg BW (Fig. 2C). The TD_{50} of valproate in the absence of EACA, 247(107-568) mg/kg BW, was not statistically different (p= 0.5524) from its corresponding value in the presence of EACA, 33(20-54) mg/kg BW. Taken together with the visual assessment of isobologram in Fig. 2D, the neurotoxicity of EACA and valproate was also additive in nature resulting in the PI of 1.14 for the combination (Table 1).

• EACA and gabapentin

In line with the results of phenytoin and valproate, ED_{50} of intraperitoneally given gabapentin (100, 300, 700 and 1000 mg/kg BW) was found to be 310(150-638) mg/kg BW when given alone and it was decreased to 79(41-153) mg/kg BW in the presence of orally given EACA (Fig. 3A and Table 1). The combination also decreased ED_{50} of EACA from 673(299-1515), when given alone, to 183(94-354) mg/kg BW (Fig. 3A). The isobolographic representation of the interaction between EACA and gabapentin (Fig. 3B) illustrated that the observed combined ED_{50} value lay below the additive line. Thus, synergistic interaction of the combination was

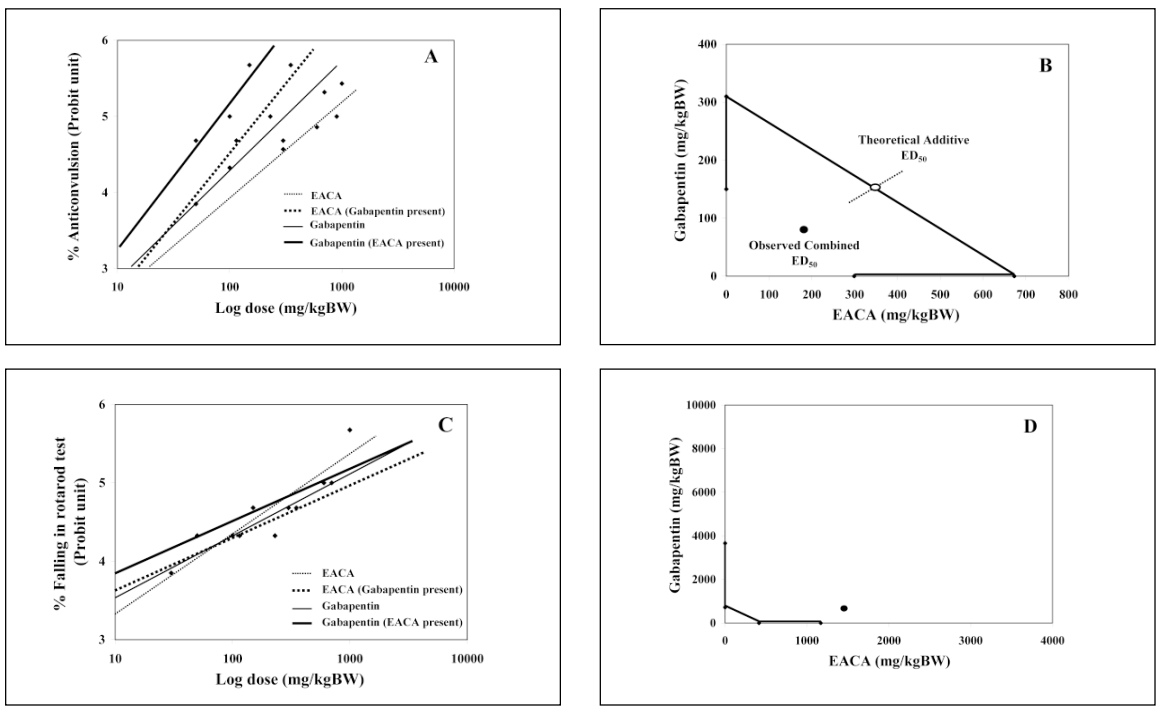

Fig. 3 Dose-response curves and isobolographic representation of EACA and gabapentin on ED_{50} and TD_{50} in the pentylenetetrazole model in mice. A, dose-response curves of anticonvulsant effect of EACA [ED_{50} = 673(299-1515);....], gabapentin [ED_{50} = 310(150-638);—], EACA in the presence of gabapentin [ED_{50} = 183(94-354);....] and gabapentin in the presence of EACA [ED_{50} = 79(41-153);—]. B, isobolographic representation of the interaction between EACA and gabapentin. The experimental ED_{50} value was not significantly different from the theoretical additive ED_{50} value (Student's *t* test, p = 0.6846), indicating that the interaction was additive. C, dose-response curves of minimal neurotoxic effect of EACA [TD_{50} = 415(147-1169);....], gabapentin [TD_{50} = 719(141-3660);—], EACA in the presence of gabapentin [TD_{50} = 1449(205-10198);....] and gabapentin in the presence of EACA [TD_{50} = 622(89-4345);—]. D, isobolographic representation of the interaction between EACA and gabapentin. The experimental TD_{50} value was not significantly different from the theoretical additive TD_{50} value (Student's *t* test, p = 0.9952), indicating that the interaction was additive

suggested. However, no statistical difference was noted between the ED_{50} values of gabapentin in the presence and in the absence of EACA (p= 0.6846). Therefore, the additive interaction between gabapentin and EACA was indicated.

In contrast to a decrease of TD_{50} of EACA when it was given in a combination with phenytoin or valproate, the combination between EACA and gabapentin increased TD_{50} of EACA from 415(147-1169), when given alone, to 1449 (205-10198) mg/kg BW (Fig. 3C). The TD_{50} of gabapentin in the absence of EACA, 719(141-3660) mg/kg BW, was not statistically different (p= 0.9952) from its corresponding value in the presence of EACA, 622(89-4345) mg/kg BW. Therefore, the antagonistic interaction of neurotoxicity between gabapentin and EACA which was visually suggested from isobologram in Fig. 3D was not accepted. The neurotoxicity of EACA and gabapentin could be just as additive as the other's. However, in contrast to the results of previously described combination, the combination between gabapentin and EACA increased protective index of gabapentin about 3 times from 2.32 in monotherapy to 7.87 in combination.

Discussion

Clinically combination of AEDs to control refractory epilepsy is advantageous if it fully controls the seizure and simultaneously causing no synergy

of adverse effects. There is increasing evidence suggesting that in addition to a consideration of mechanism of AED, animal experiments using isobolographic analysis could also be beneficial to predict clinical outcome[2].

Isobolographic analysis in the present study indicates that the combination of a herbal extract from CA can enhance anticonvulsant effect of all AEDs tested. A distinct additive effect was observed in all combinations; ED_{50} of phenytoin, valproate and gabapentin in combination with EACA were approximately 38%, 28% and 25% of their corresponding value, when being given alone.

The adverse effects of respective combination on motor coordination, estimated by rotarod test, were also increased as all the combined TD_{50} values were decreasing. However, interestingly, the protective index (PI) which is the ratio between the neurotoxic dose and effective dose of gabapentin in combination with EACA was markedly increased (7.87 vs 2.32), whereas respective values for phenytoin and valproate were decreased. Thus, a combination of gabapentin and EACA seemed to offer not only a higher protection of animals against PTZ induced convulsion but also a broader margin between anticonvulsant dose and neurotoxic dose as well. Though in the present study, gabapentin was given intraperitoneally, it can be anticipated that an addition of EACA into patients taking clinically available gabapentin tablets would result in better control of the seizure in parallel with a lesser degree of motor impairment than those exhibited by gabapentin alone. Additive effect of EACA was also demonstrated when it was combined with phenytoin or valproate. However, the advantage in these cases seemed to be offset by the finding that their respective protective indices were also decreased.

It is difficult to explain the underlying mechanism of the interaction observed. Firstly, this is the first evidence to demonstrate the additive anticonvulsant effect of currently available AEDs with CA's extract in which the active principles accounted for its anticonvulsant were not yet identified. Secondly, drug interaction of concurrently administered AEDs can occur by pharmacodynamic as well as pharmacokinetic mechanisms[13] and none of them could be ruled out by isobolographic analysis[11]. Though, different routes of administration of EACA and AEDs used in the present study make the interaction by enhancing absorption of AEDs unlikely, some other pharmacokinetic interaction should be further investigated. Furthermore, the fact that additivity of EACA was observed on phenytoin, valproate and gabapentin which are AEDs of different mechanisms of action and different pharmacokinetic profiles[14], thus, no clues on the possible mechanism of interaction can be anticipated.

Considering that CA is a traditional herbal medicine which is safe and easy to cultivate[3], results obtained in the present studies strongly support further investigation aiming to develop CA as an adjunctive medication in epileptic patients.

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การเสริมฤทธิ์การต้านชักระหว่างสารสกัดเอธิลอะซิเตทของบัวบกและยาต้านชัก

อนุสรา วัฒนจันทร์, Hiroshi Watanabe, มยุรี ตันติสิระ, บุญยงค์ ตันติสิระ

วัตถุประสงค์: *เพื่อศึกษาปฏิกิริยาระหว่างสารสกัดเอธิลอะซิเตทของบัวบกเมื่อให้โดยการป้อน กับยาต้านชัก ได้แก่ ฟีนีโทอิน วาลโปรเอท และกาบาเพนตินที่ให้โดยการฉีดเข้าทางช่องท้อง*

วัสดุและวิธีการ: *ใช้การวิเคราะห์โดยไอโซโบโลแกรม เพื่อแสดงชนิดของปฏิกิริยาระหว่างสารสกัดเอธิลอะซิเตทของบัวบกที่ให้ร่วมกับยาต้านชัก ในหนูถีบจักรที่ถูกเหนี่ยวนำให้ชักโดยสารเพนธีลีนเตตระโซล และใช้วิธี Rotarod test เพื่อศึกษาความเป็นพิษต่อระบบประสาทส่วนกลาง*

ผลการศึกษา: *เมื่อให้ยาต้านชัก ฟีนีโทอิน วาลโปรเอท และกาบาเพนติน จะให้ค่าในการต้านการชักได้จำนวนครึ่งหนึ่ง (ED_{50}) เท่ากับ 13, 104 และ 310 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัว ตามลำดับ ในขณะที่การให้ยาดังกล่าวร่วมกับสารสกัดเอธิลอะซิเตทของบัวบก ทำให้ค่า ED_{50} ของยาต้านชักทั้ง 3 ตัวลดลงเป็น 5, 29 และ 79 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัว และเมื่อวิเคราะห์โดยไอโซโบโลแกรม พบว่ามีการเสริมฤทธิ์กันระหว่างสารสกัดเอธิลอะซิเตทของบัวบกกับยาต้านชักแต่ละตัวที่ได้รับร่วมกัน เมื่อพิจารณาประกอบกับความเป็นพิษต่อระบบประสาทส่วนกลาง พบว่า เมื่อให้สารสกัดเอธิลอะซิเตทของบัวบกร่วมกับกาบาเพนติน จะทำให้ขอบเขตระหว่างขนาดของยาต้านชักที่ใช้ในการรักษากับขนาดที่เป็นพิษต่อระบบประสาทส่วนกลางกว้างขึ้น โดยไม่พบผลดังกล่าวเมื่อให้สารสกัดบัวบกร่วมกับ ฟีนีโทอิน หรือวาลโปรเอท*

สรุป: *ผลการทดลองที่ได้แสดงถึงศักยภาพของบัวบก ที่สามารถจะพัฒนาต่อไปเพื่อใช้เสริมฤทธิ์กับยาต้านชักในผู้ป่วยโรคลมชัก*