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Neuropharmacological effects of an ethanolic fruit extract of *Xylopia aethiopica* and xylopic acid, a kaurene diterpene isolate, in mice.

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ABSTRACT

Background: Even though the central analgesic effects of *Xylopia aethiopica* (XAE) and xylopic acid (XA) have been reported, XAE and XA have however not been evaluated for their effects on other neurological functions.

Objectives: To determine the effects of XAE and XA on spontaneous activity, neuromuscular function, convulsive threshold and sedation as well as their interaction with hepatic enzymes

Methods: The activity meter, rotarod, PTZ-induced convulsion and pentobarbitone-induced sleep tests were used to evaluate spontaneous activity, neuromuscular function, convulsive threshold and sedation respectively in mice. Effects of hepatic enzyme inhibition and induction were estimated using duration of pentobarbitone-induced sleep.

Results: XAE and XA showed significant central nervous system depressant effects in pentobarbitone-induced hypnosis and spontaneous activity test. Both XAE and XA showed neuromuscular coordination impairment tendency above 300 mg/kg. Whereas XAE significantly increased seizure threshold at all doses tested, XA had no effect on PTZ-induced convulsion. XAE may induce hepatic enzymes at lower doses whereas XA showed a bidirectional effect by inhibiting hepatic enzymes at lower doses and inducing hepatic enzymes at higher doses. Both XAE and XA may however be metabolized by hepatic enzymes.

Conclusion: Xylopic acid and the fruit extract of *Xylopia aethiopica* have significant central nervous system depressant effects in mice

Key words: CNS depressant, anticonvulsant, kaurene diterpenes, mice

Effets neuropharmacologiques d'un extrait de fruit éthanolique de Xylopia aethiopica et acide xylopic, kaurene diterpénique isoler, chez la souris.

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RÉSUMÉ

Contexte: Même si les effets analgésiques centraux de Xylopia aethiopica (XAE) et de l'acide xylopic (XA) ont été rapportés, XAE et XA n'ont cependant pas été évaluées pour leurs effets sur d'autres fonctions neurologiques.

Objectifs: déterminer les effets de XAE et XA sur l'activité spontanée, la fonction neuromusculaire, le seuil convulsif et sédation ainsi que leur interaction avec des enzymes hépatiques

Méthodes: Les tests du sommeil induits Le compteur d'activité, rotarod, convulsions et pentobarbital induit PTZ ont été utilisés pour évaluer l'activité spontanée, la fonction neuromusculaire, le seuil convulsif et sédation respectivement chez la souris. Effets de l'inhibition des enzymes hépatiques et de l'induction ont été estimées en utilisant la durée du sommeil pentobarbitoneinduced.

Résultats: XAE et XA ont montré des effets dépresseurs du système nerveux central importants dans l'hypnose induite par le pentobarbital et test d'activité spontanée. Les deux XAE et XA ont montré une insuffisance neuromusculaire coordination tendance au-dessus de 300 mg / kg. Considérant que XAE augmenté de manière significative le seuil de saisie à toutes les doses testées, XA n'a eu aucun effet sur les convulsions PTZ-induite. XAE peut induire des enzymes hépatiques à des doses plus faibles que XA a montré un effet bidirectionnel en inhibant les enzymes hépatiques à des doses inférieures et induire des enzymes hépatiques à des doses plus élevées. Les deux XAE et XA peuvent cependant être métabolisés par les enzymes hépatiques.

Conclusion: Xylopic acide et l'extrait de fruit de Xylopia aethiopica ont des effets dépresseurs du système nerveux central importantes chez les souris

Mots clés: CNS dépresseurs, anti-convulsivants, diterpènes de kaurene, souris

INTRODUCTION

Xylopia aethiopica (Dunal) A. Rich is a slim, tall, aromatic tree with a straight crown or buttressed stems. It is a member of the custard apple family Annonaceae, widely distributed in Ghana, Democratic Republic of Congo, Ethiopia, Kenya, Mozambique, Nigeria, Senegal, Tanzania and Uganda and commonly used as condiment[1]. It is referred to as 'Hwentia' in the Akan dialect. The plant has attracted several investigations thus revealing several documented activities. It has been shown to possess antibacterial and antifungal[2, 3], antihelminthtic[4], analgesic[5] and cytotoxicity activity against pancreatic and leukemic cells[6] among others.

Several compounds including the diterpene kaurene derivatives have been isolated and characterized[7] from the plant. Diterpenes are isoprenoid molecules commonly found in plants and fungi and biosynthesized from mevalonic acid.[8, 9] Kaurenes represent a very important group of tetracyclic diterpenes which serve as important intermediates in synthesis of important plant hormones like gibberlins.[8, 10] An array of biological activities have been attributed to them including antimicrobial, cytotoxic, anti-parasitic, insect antifeedant, anti-HIV, anti- inflammatory and neuroprotective effects.[11-14] Kaurene diterpenes isolated from *Xylopia aethiopica* include xylopic acid which has antiplasmodial,[15] analgesic,[5] cardiovascular and diuretic effects.[16] Others include kaurenoic acid which has antitrypanosomic, analgesic and anti-inflammatory effects[17, 18], acetylgrandifloric acid reported to have antibacterial effect[19] and ent-15-oxokaur-16 en-19-oic acid (EKOA) which is antiproliferative[20]

Several diterpenes have known effects on the central nervous system[11, 13, 21, 22] The central analgesic effects of *Xylopia aethiopica* and xylopic acid have recently been reported.[5] However *Xylopia aethiopica* and xylopic acid have not been evaluated for their neuropharmacological effects. We have used a two pronged approach to evaluate the neurotoxic and neurotrophic effects of *Xylopia aethiopica* and xylopic acid using the International Commission on Harmonization (ICH) S7A Guideline for Safety Pharmacology.[23]

MATERIALS AND METHODS

Plant collection and extraction

Fresh unripe fruits were collected from the botanical garden of Kwame Nkrumah University of Science and Technology (KNUST) (06° 41'6.39" N; 01° 33' 45.35" W) in December 2012. Its authenticity was confirmed by Dr Kofi Annan of the Department of Pharmacognosy, College of Health Sciences, KNUST and subsequently compared to a voucher specimen (No FP/09/77) at the Department's herbarium. The fruits were shade-dried until they were brittle to break (two weeks) before pulverizing to a powder with a hammer mill. One kilogram (1 kg) of the powered material was exhaustively extracted with 70 % v/v ethanol for two consecutive seventy-two (72) hour periods by cold maceration. The extract was concentrated using a rotary evaporator at 60 °C to yield a semi-solid mass of *Xylopia aethiopica* extract (XAE) representing a 32.9 % w/w yield.

Isolation and purification of Xylopic acid

Xylopic acid was isolated and purified as previously described.[5, 24] One kilogram (1 kg) of the powdered fruits was exhaustively extracted with petroleum ether for two consecutive seventy-two hour periods by cold maceration. The extract was concentrated with a rotary evaporator at 60 °C. The concentrate deposited crude crystals after three (3) days which was purified by recrystalization with a reflux condenser to yield 13.62 g (1.36 % w/w) of xylopic acid. Purity was confirmed by TLC, melting point determination and HPLC.

Animals

Male ICR mice (20-25 g) were obtained from Noguchi Memorial Institute for Medical Research, Accra, Ghana and housed at the vivarium of the Department of Pharmacology, KNUST, Ghana. They were grouped in stainless steel cages (34 × 47 × 18 cm^3) with soft wood shavings as bedding and fed *ad libitum* with commercial pellet diet (GAFCO, Tema, Ghana) and water. All animals used were naïve and used only once. All procedures employed were in accordance with the National Institute of Health Guidelines for Care and Use of laboratory animals and were approved by the Departmental Ethics Committee.

Drugs and chemicals

Caffeine, diazepam, d-tubocurarine, ketoconazole, phenobarbitone, pentobarbitone, pentylenetetrazole and morphine were purchased from Sigma Aldrich Inc., St Louis, MO, USA.

Irwin test

The qualitative effects of *Xylopia aethiopica* (XAE) and xylopic acid (XA) on behaviour and physiological function were investigated using the original procedure described by Irwine.[25] Male ICR mice (20 -25 g) were

randomly distributed to ten groups (n=7) and left to acclimatize for 24 hours. Animals were fasted overnight but had free access to water. They were treated with XAE in oral doses of 30-1000 mg/kg and XA 10-1000 mg/kg while animals in the control group received distilled water 10 mL/kg *p.o*. The animals in the respective groups were observed for death as well as changes in behavioural and physiological function at 0 to 15, 30, 60, 120, 180 minutes and at 24 hours post-treatment.

Spontaneous locomotion test

Effect of XAE and XA on spontaneous locomotion was evaluated using an activity cage (Ugo Basile model 7401, Comerio, VA, Italy). Mice were randomly assigned to twelve groups (n=7) and treated with XAE (30-1000 mg/kg), XA (10-1000 mg/kg), diazepam (4 mg/kg), caffeine (16 mg/kg) or distilled water (10mL/kg) *p. o.* Animals were then placed individually in the activity cage and their activity scored every 5 min for 30 min.

Rotarod test

To elucidate the neuromuscular coordination impairment tendency of XAE and XA, mice were grouped randomly into twelve groups (n=7) and trained to walk over a three day period on a rotating rod (Ugo Basile model 7600, Comerio, VA, Italy) rotating at a constant speed of 25 revolutions/min for 180 s[26] Twenty-four hours after the last training session, the animals received XAE (30-1000 mg/kg), XA (10-1000 mg/kg), diazepam (8 mg/kg), d-tubocurarine (0.1 mg/kg) or distilled water (10 mL/kg) *p.o.* and were placed on the rod to walk. Latency to fall off the rotating rod within a maximum time of 180 s was determined at 0, 1, 1.5 and 2 h post treatment.

Pentobarbitone-induced sleep test

The effect of XAE and XA on pentobarbitone-induced sleeping time was investigated. Mice were assigned randomly to twelve groups (n=7) and received either XAE (30-1000 mg/kg), XA (10-1000 mg/kg), diazepam (8 mg/kg), caffeine (16 mg/kg) or distilled water (10 mL/kg) *p.o.* Sodium pentobarbitone (50 mg/kg) was administered i.p. one (1) hour after respective drug treatments. Latency to sleep (time between pentobarbitone injection and loss of righting reflex) and duration of sleep (time between loss of and regaining of righting reflex) were recorded with a stopwatch.

Interaction with hepatic enzymes

To determine the effect of XAE and XA on hepatic enzymes, mice were assigned to thirteen groups (n=7), I-XIII. Groups I-III and IV-VI were pretreated with XAE (30-300 mg/kg) and XA (10-100 mg/kg) respectively for five (5) days. Twenty-four hours after the last pretreatment, the animals received sodium pentobarbitone (50 mg/kg) *i.p.* Groups VII-IX and X-XII (naïve) received respectively a single dose of XAE (30-300 mg/kg) and XA (10-100 mg/kg) and 1 hour later were given sodium pentobarbitone (50 mg/kg) *i.p.* Group XIII received only sodium pentobarbitone (50 mg/kg) *i.p.* Duration of sleep were recorded as earlier *described (vide supra).*

In a separate experiment, the effect of liver enzyme induction on XAE and XA was studied. Twelve groups of animals (n=7) were pretreated with phenobarbitone (25 mg/kg daily i.p) for two (2) days. Twenty-four hours after the last pretreatment, the animals received XAE (30-1000 mg/kg), XA (10-1000 mg/kg), diazepam (8 mg/kg), caffeine (16 mg/kg) or distilled water (10 mL/kg) *p.o* They then received sodium pentobarbitone (50 mg/kg) i.p one (1) h after drug treatment. Duration of sleep was recorded.

In another experiment, the effect of liver enzyme inhibition was also studied. Twelve groups of animals (n=7) were pretreated with ketoconazole (100 mg/kg *p.o*) for seven (7) consecutive days. Twenty-four hours after the last pretreatment, the animals received XAE (30-1000 mg/kg), XA (10-1000 mg/kg), diazepam (8 mg/kg), caffeine (16 mg/kg) or distilled water (10 mL/kg) *p. o.* They then received sodium pentobarbitone (50 mg/kg) i.p. one (1) h post drug treatment. Duration of sleep was recorded.

Convulsive threshold test

Male ICR mice randomly assigned to twelve (12) groups received either XAE (30-1000 mg/kg), XA (10-1000 mg/kg), diazepam (8 mg/kg) or distilled water (10 mL/kg) *p.o.* One hour after drug treatment, seizure was induced by subcutaneous administration of pentylenetetrazole (85 mg/kg) and each animal was placed in plastic observational cages. Latency to, frequency and duration of convulsions were recorded with a video camera for 30 minutes and quantified with the open access behavioural analysis software, JWatcher version 1.0.

Analysis of data

All results are presented as mean ± SEM. Data was analyzed using one-way analysis of variance (ANOVA). When ANOVA was significant, multiple comparisons between treatments was done using Holm-Sidak *post hoc* test. GraphPad Prism for Windows Version 6 (GraphPad Software, San Diego, USA) was used for all statistical analyses.

RESULTS

Irwin's test

XAE (30-1000 mg/kg) and XA (10-1000 mg/kg) did not have any lethal effects over the 24 hour period of observation. Both XAE and XA produced decrease reactivity to touch, analgesia and sedation at all doses spanning 30 to 180 minutes.

Spontaneous locomotion test

Xylopic acid reduced spontaneous locomotion significantly at 30-1000 mg/kg ($F_{7, 48}$=6.320 p<0.0001) whereas XAE reduced activity significantly ($F_{6, 42}$=6.078 p<0.0001) only at 300 and 1000 mg/kg as did diazepam 8 mg/kg. Caffeine 16 mg/kg increased activity significantly (Fig 1).

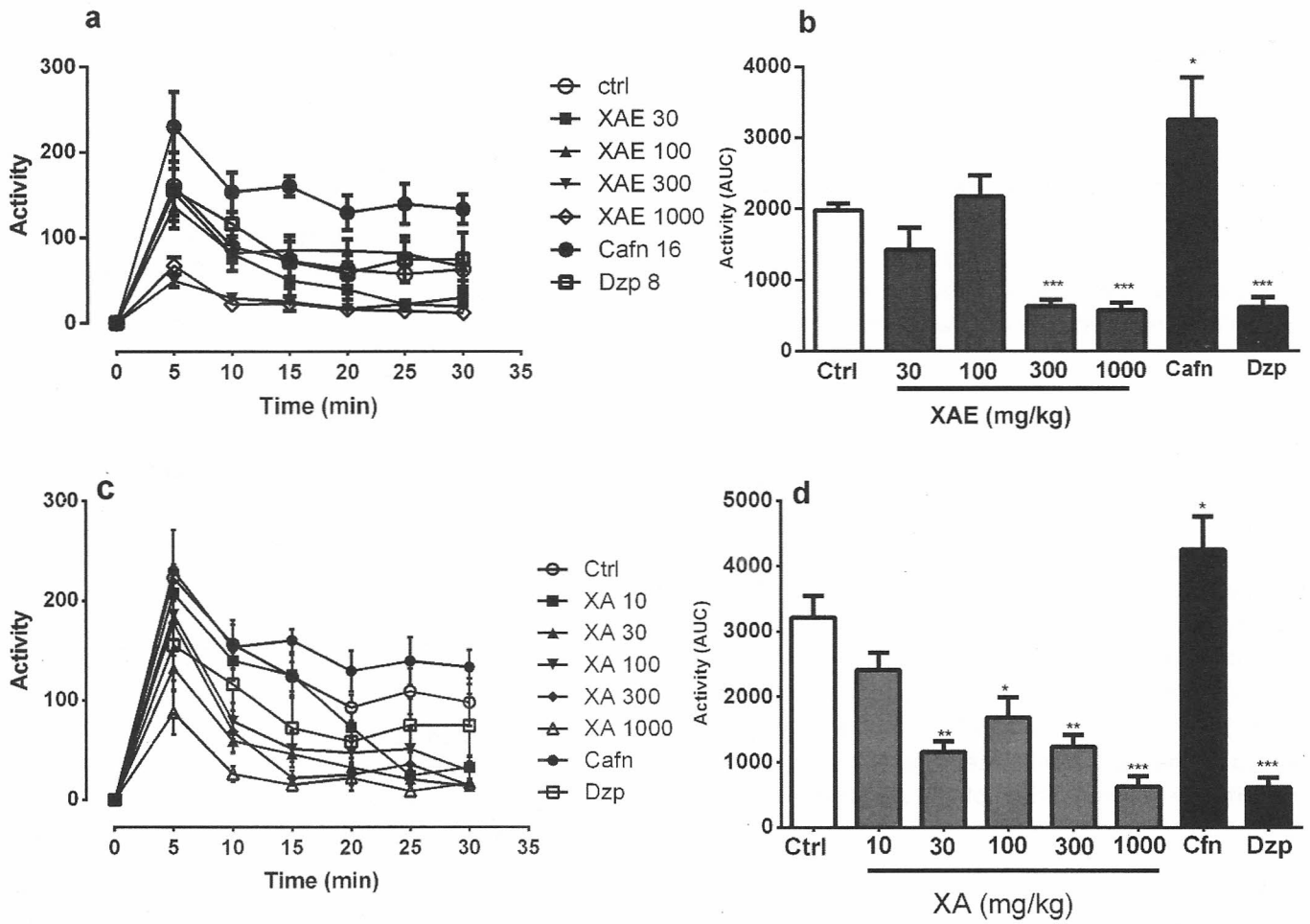

Figure 1 Effect of *Xylopia aethiopica* extract (XAE), xylopic acid (XA), diazepam (Dzp) and caffeine (Cfn) on spontaneous activity in mice. Data are mean ± SEM (n=7), ***p<0.001, **p<0.01 and *p<0.05 compared to control.

Rotarod test

XA significantly ($F_{7, 48}$=16.85 p<0.0001) reduced time spent on the rod only at doses 300-1000 mg/kg with similar observations in respect of XAE ($F_{6, 42}$=21.96 p<0.0001). Both reference muscle relaxants, diazepam and d-tubocurarine, also significantly reduced time spent on the rod.

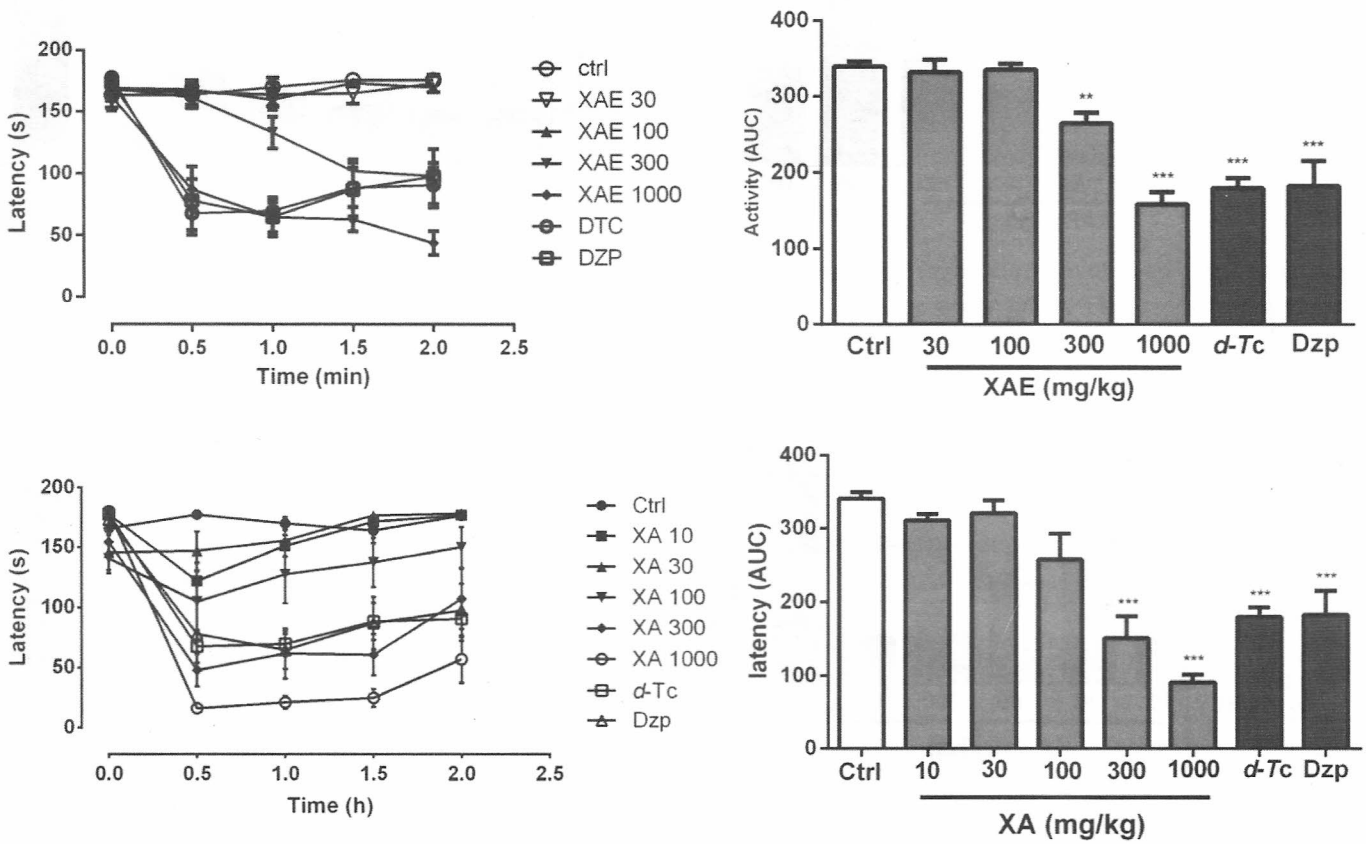

Figure 2 Effect of *Xylopia aethiopica* extract (XAE), xylopic acid (XA), diazepam (Dzp) and *d*-tubocurarine (*d*-Tc) on neuromuscular coordination in mice in the rotarod test. Data are Mean ± SEM (n=7), ***p<0.001 and **p<0.01 compared to control.

Pentobarbitone-induced sleep test

XA exhibited sedative effect as seen in Fig 3c and d. It showed a significant ($F_{7, 48}$=9.476 p<0.0001) and dose-dependent decrease on onset of sleep from doses 100-1000 mg/kg but not at lower doses (Fig 3c). Sleep duration was prolonged significantly ($F_{7, 48}$=52.49 p<0.0001) only at 300 and 1000 mg/kg (Fig 3b). Although XAE did not significantly affect the onset of sleep, it significantly ($F_{6, 42}$=133.0 p<0.0001) prolonged sleep duration (Fig 3d) in a dose-dependent manner. Diazepam and caffeine, the reference CNS depressant and stimulant respectively produced significant increase and decrease in sleep duration, respectively as expected.

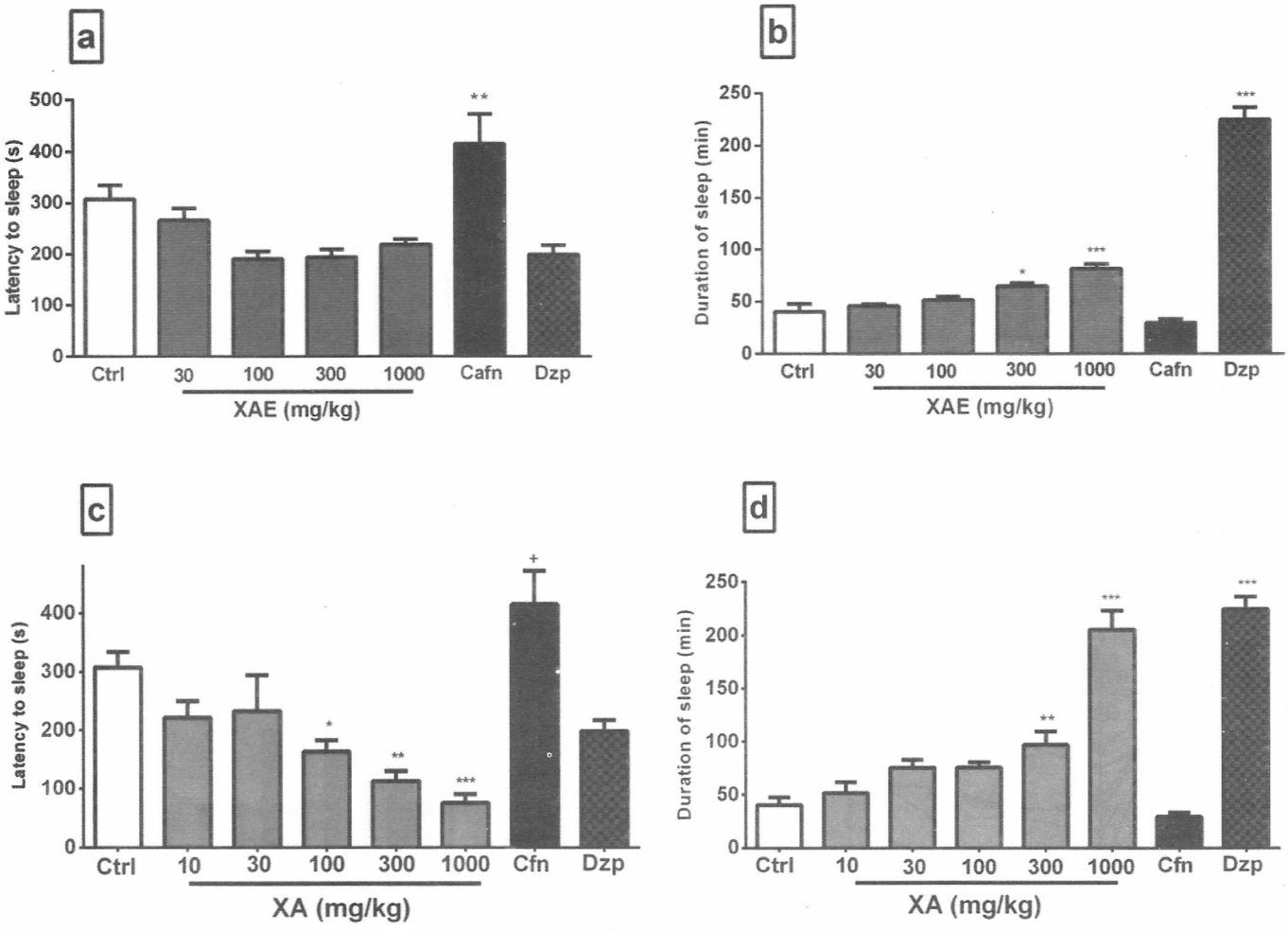

Figure 3: Effect of *Xylopia aethiopica* extract (XAE), Xylopic acid (XA), Diazepam (Dzp) and Caffeine (Cfn) on latency to sleep (a and c) and duration of sleep (b and d) in pentobarbitone-induced sleep test. Data are mean ± SEM, (n=7), ***p<0.001, **p<0.01 and *p<0.05 compared to control.

Interaction with hepatic enzymes

Pentobarbitone-induced sleep duration was significantly ($F_{1,36}$=117.9 p<0.0001) reduced at all doses after XAE pretreatment as compared to vehicle treated animals (*v.i)*. XA pretreatment however exhibited a biphasic effect on sleep duration (Fig 4c and d). Whereas a lower dose (10 mg/kg) prolonged sleep, high dose (100 mg/kg) significantly decreased duration of sleep as compared to the control group. No significant effect was observed between the pretreated and untreated groups at 30 mg/kg.

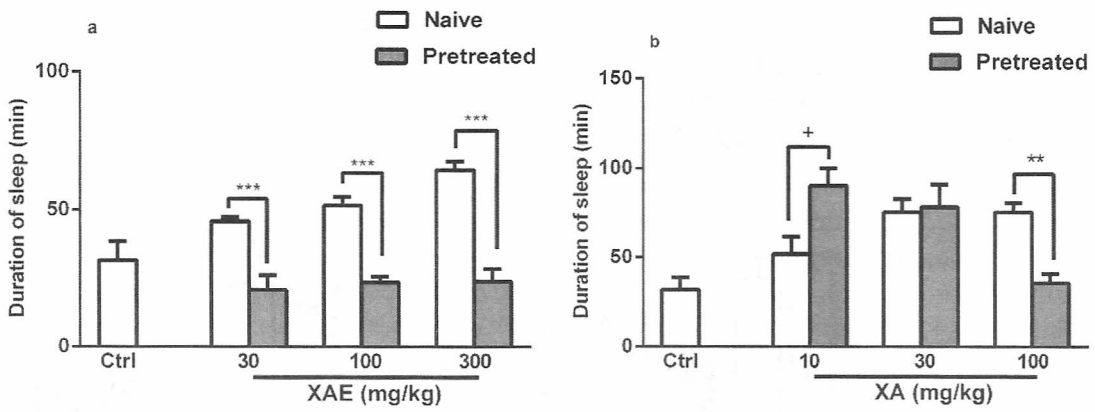

Figure 4: Effect of pretreatment with *Xylopia aethiopica* extract (a) and Xylopic acid (b) on duration of sleep of mice. Data are mean ± SEM (n=7), ** *P*<0.0001; comparison between dose and treatment (Two-way ANOVA followed by Holm-Sidak's test).**

Hepatic enzymes induction by phenobarbitone significantly (XAE; $F_{1, 84}$=197.4 p<0.0001, XA $F_{1, 12}$=263.0 p<0.0001) shortened duration of sleep at all dose levels in XAE and XA treated mice as well as the diazepam treated animals (Fig. 5a and c). Enzymes inhibition by ketoconazole rather produced a paradoxical decrease in sleep duration in XA (Fig 6 b and d) treated mice in contrast to XAE, diazepam and caffeine treated groups in which sleep was prolonged.

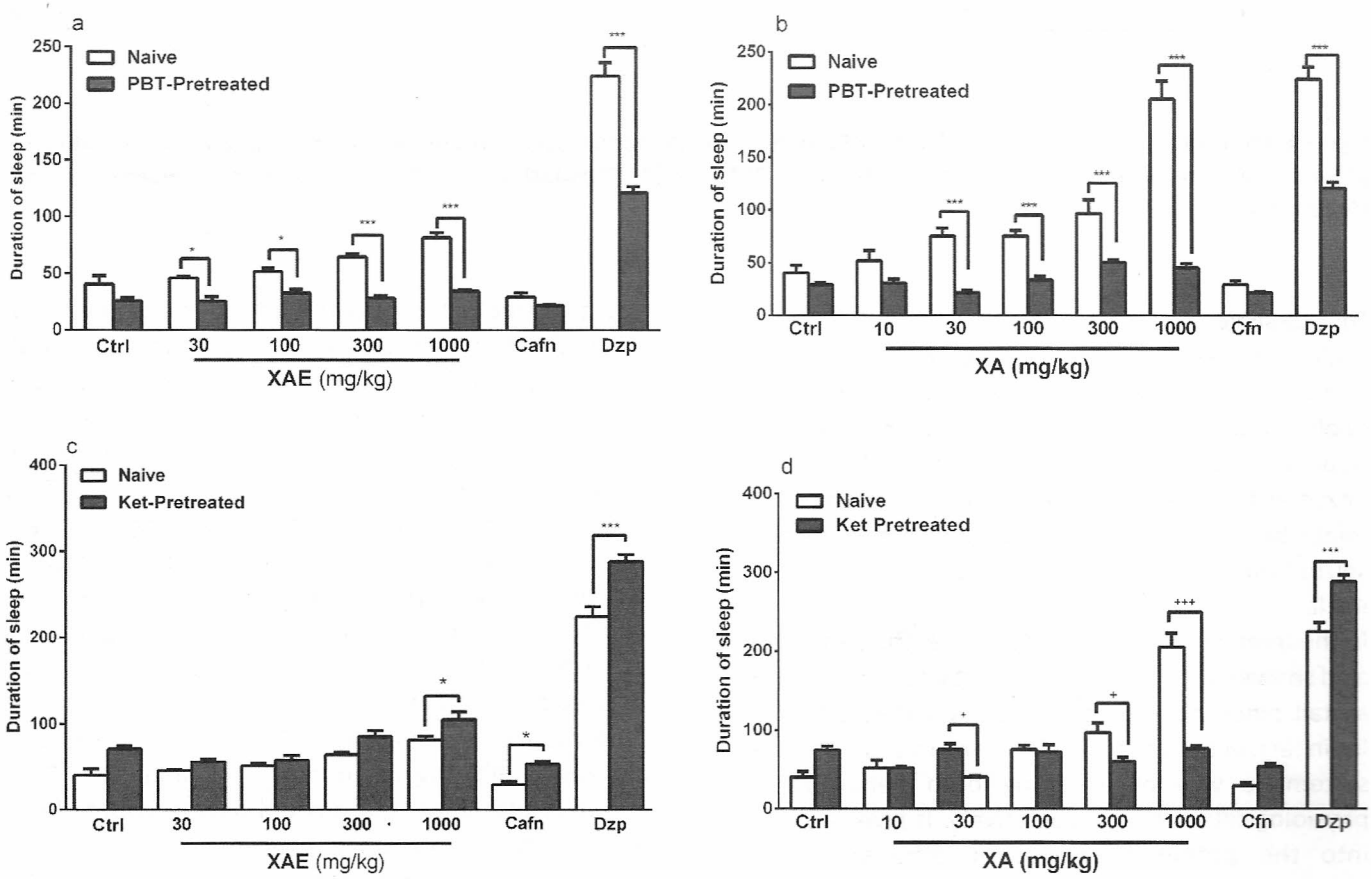

Figure 5: Effect of enzyme induction (a and c) and enzyme inhibition (b and d) on duration of sleep of *Xylopia aethiopica* extract (XAE), xylopic acid (XA), diazepam (Dzp) and caffeine (Cfn) treated mice. Data are mean ± SEM (n=7),*p<0.001, **p<0.01 and *p<0.05; comparison between dose and treatment (Two-way ANOVA followed by Holm-Sidak's test).**

Convulsive threshold test

Although XA delayed onset of PTZ-induced convulsions, it neither reduced the frequency nor duration of convulsions. XAE on the other hand significantly delayed onset of convulsions as well as decreased the frequency and duration of convulsions. Diazepam, the reference anticonvulsant, also significantly reduced frequency and duration of convulsions.

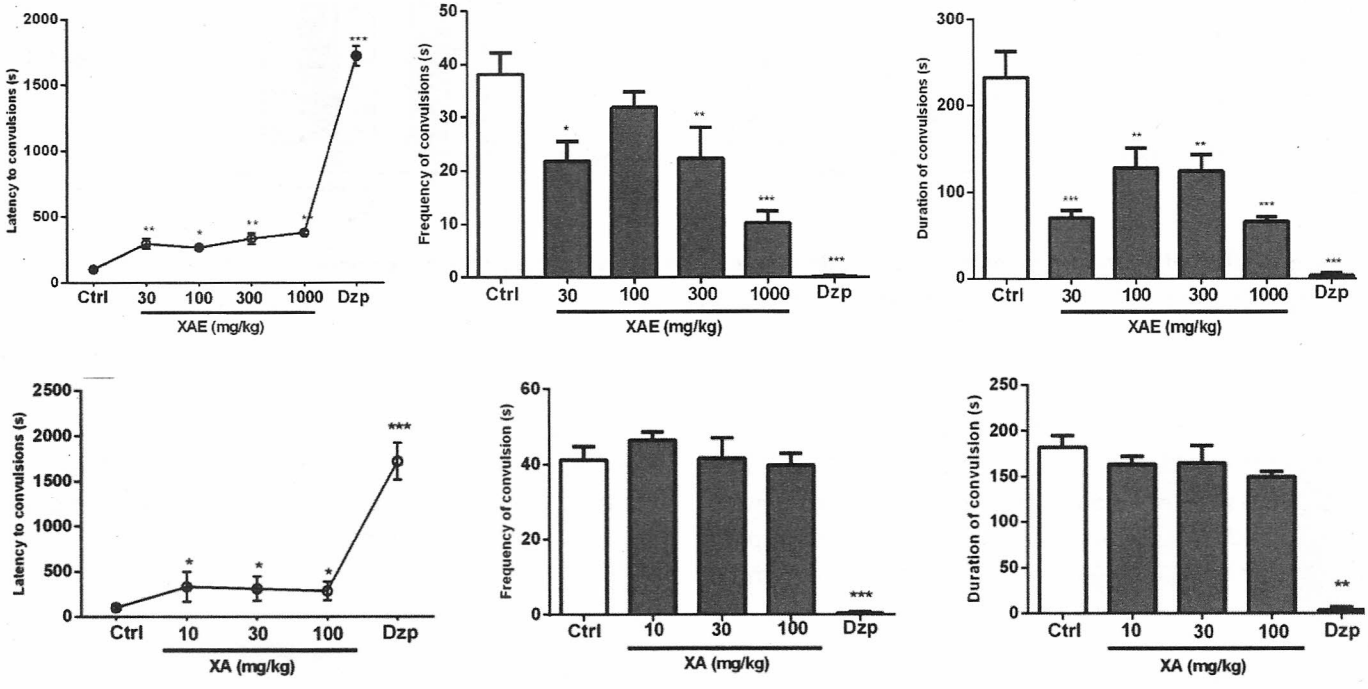

Figure 6: Effect of *Xylopia aethiopica* extract (XAE), xylopic acid (XA) and diazepam (Dzp) on latency, frequency and duration of ptz-induced convulsion. Data are mean ± SEM (n=7), ***p<0.001, **p<0.01 and *p<0.05; comparison between dose and treatment.

DISCUSSION

Preliminary assessment of *Xylopia aethiopica* extract and xylopic acid has shown their CNS depressant and analgesic effect. Xylopic acid has a biphasic effect on hepatic enzymes, might be metabolized by hepatic enzymes but has little effect on seizure threshold. XAE might be metabolized by hepatic enzymes, increases seizure threshold but has no effect on hepatic enzymes itself.

In the Irwin test, *Xylopia aethiopica* extract and xylopic acid showed reduced activity, reactivity to touch as well as tail pinch pointing towards a sedative effect. The Irwin test, initially described by Irwin in 1968, provides a systematic way of assessing both behavioral and physiological function qualitatively. It gives an insight into the potential toxicity or otherwise of the investigational drug and may also lead to novel therapeutic agents discovery.[25, 27, 28] Mortality after 24 hours was zero suggesting the LD_{50} is above 1000 mg/kg for both XAE and XA.

The activity meter test quantified the decreased activity observed in the Irwin test and assessed the effects of *Xylopia aethiopica* extract and xylopic acid on spontaneous locomotion. Decreased spontaneous locomotion is predictive of sedation although neuromuscular impairment may confound it.[29, 30] The ability of XAE and xylopic acid to significantly decrease activity suggests possible effectiveness in CNS hyper-excitability states such as epilepsy and anxiety. Because a compromise in motor function can lead to a significant decrease in spontaneous locomotion, the rotarod test was used to elucidate the cause of decreased activity.[29, 30] The rotarod challenge revealed motor impairment only above 300 mg/kg in both XAE and XA. The neuromuscular impairment could have accounted for the increased sedation at higher doses.

Pentobarbitone potentiates GABA-mediated post-synaptic inhibitions at the GABA receptor to cause hypnosis.[31, 32] Interaction of substances with pentobarbitone has the potential of unmasking the

sleep-enhancing or stimulant effects of drugs which may not be observed when the agents are giving alone even at higher doses.[32] The results indicate that XAE and xylopic acid have sedative effect which was unmasked by pentobarbitone. Potentiation of pentobarbitone-induced hypnosis is an indication of central depressant activity[33] giving further credence to the fact that XAE and xylopic acid have CNS depressant effect.

The cytochrome P450 system is involved in the metabolism of several drugs accounting for over 90 % of all human drug oxidations.[34] Again most psychotherapeutic drugs are metabolized by CYP2C19 and CYP2D6 isoenzymes of the cytochrome P450 enzymes.[34] Several neurological disease states require co-administration of psychotherapeutic drugs to reach optimum efficacy.[34] For example, 30 % of epileptic cases are refractory and may require the use of more than one antiepileptic drug.[35] Again, after unsuccessful attempt to treat epilepsy with two individual drugs sequentially, polytherapy may be most beneficial in managing such patients.[36,37] Enzymes inhibitors may potentiate effect of other co-administered drugs thus enhancing their neurotoxic effects while inducers reduce their effects.[34] Drug metabolism may produce metabolites that may affect the therapeutic value or produce side effects of the drug.[38] Drugs that are extensively metabolized by hepatic enzymes may require dose adjustment to meet desirable therapeutic concentrations. In this regard XAE and XA were evaluated for their effects on hepatic enzymes.

XAE may induce hepatic enzymes at lower doses. Xylopic acid however exerted a biphasic effect on hepatic enzymes. At a lower dose of 10 mg/kg, XAE increased duration of sleep whilst decreasing onset of sleep pointing towards the fact that hepatic enzymes has been inhibited. High dose of XA (100 mg/kg) however exhibited an opposite effect, reduction in duration of sleep, which is suggestive of enzyme induction. The bidirectional effect on barbital hypnosis suggests a biphasic effect on hepatic enzymes.[39] The biphasic effect may possibly be due biotransformation to different metabolites responsible for varying effects.

Pretreatment of mice with phenobarbitone induces hepatic enzymes.[40] Pentobarbitone is extensively metabolized by hepatic enzymes.[41, 42] Thus, that the duration of pentobarbitone-induced hypnosis decreased considerably after phenobarbitone pretreatment is a strong argument that XAE and xylopic acid may be metabolized by hepatic enzymes.

Ketoconazole administration inhibits hepatic enzymes.[43] Enzyme inhibition did not increase duration of sleep in XA treated mice at all doses as observed in XAE, diazepam, caffeine and control treated groups. It is possible that xylopic acid requires hepatic enzyme biotransformation into an active metabolite that is responsible for its sedative effect and thus inhibiting hepatic enzymes would result in a decreased effect.

The extract showed significant reduction in seizure threshold. This is not surprising considering the fact that several kaurene diterpenes have demonstrated neuroprotective effects.[12, 21] Paradoxically, even though XA has demonstrated significant CNS depressant activity it was ineffective as an anticonvulsant. This suggests that the anticonvulsant effect of XAE may be due to constituents other than the major kaurene diterpenes, xylopic acid.

CONCLUSION

We have demonstrated the significant central nervous system depressant effect of *Xylopia aethiopica* and Xylopic acid in murine models.

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