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Anticonvulsant activity of *Pseudospondias microcarpa* (A. Rich) Engl. hydroethanolic leaf extract in mice: The role of excitatory/inhibitory neurotransmission and nitric oxide pathway

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Picrotoxin (PubChem CID: 5311359)
Isoniazid (PubChem CID: 3767)
Strychnine (PubChem CID: 441079)
4-AP (PubChem CID: 1727)
L-NAME (PubChem CID: 39836)
L-Arginine (PubChem CID: 6322)
Methylene blue (PubChem CID: 6099)
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ABSTRACT

Ethnopharmacological relevance: *Pseudospondias microcarpa* (A. Rich) Engl. is a plant used for managing various diseases including central nervous system disorders.
Aim of the study: This study explored the anticonvulsant activity of *P. microcarpa* hydroethanolic leaf extract (PME) as well as possible mechanism(s) of action in animal models.
Methods: Effects of PME was assessed in electroconvulsive (the maximal electroshock and 6-Hz seizures) and chemoconvulsive (pentylenetetrazole-, picrotoxin-, isoniazid-, 4-aminopyridine-, and strychnine-induced seizures) models of epilepsy. In addition, effect of the extract on the nitric oxide pathway and $GABA_A$ receptor complex was evaluated.
Results: The extract (30, 100 and 300 mg kg^{-1}, *p.o.*) significantly delayed the onset as well as decreased the duration and frequency of pentylenetetrazole-, picrotoxin- and strychnine-induced seizures. In addition, PME pre-treatment significantly improved survival in the 4-aminopyridine- and isoniazid-induced seizure tests. Furthermore, the extract protected against 6-Hz psychomotor seizures but had no effect in the maximal electroshock test. The anticonvulsant effect of PME (100 mg kg^{-1}, *p.o.*) was also reversed by pre-treatment with flumazenil, L-arginine or sildenafil. However, L-NAME or methylene blue (MB) augmented its effect.
Conclusion: Results show that PME has anticonvulsant activity and may probably be affecting GABAergic, glycinergic, NMDA, K^+ channels and nitric oxide-cGMP pathways to exert its effect.

1. Introduction

Epilepsy, a brain disorder, is characterized by occurrence of more than one seizure with a persistent predisposition to generate subsequent epileptic seizures. This is associated with neurobiological, cognitive, psychological, and social disturbances (Raol and Brooks-Kayal, 2012). Epilepsy is the second most common neurological disorder, with 0.5% prevalence, and a 2–3% life time risk of being diagnosed of it (Browne and Holmes, 2001; Löscher, 2002a). More than 80% of people with epilepsy live in developing countries, where

Abbreviations: AED, Antiepileptic drug; CNS, Central Nervous System; PME, Pseudospondias microcarpa extract; CBZ, Carbamazepine; FMZ, Flumazenil; VPA, Valproic acid; KNUST, Kwame Nkrumah University of Science and Technology; PTZ, Pentylenetetrazole; PTX, Picrotoxin; Hz, Hertz; GABA, Gamma amino butyric acid; NO, Nitric oxide; 4-AP, 4-aminopyridine; STN, Strychnine; INH, Isoniazid; cGMP, cyclic GMP; cAMP, cyclic AMP; sGC, soluble guanylyl cyclase; PDE5, Phosphodiesterase V; L-NAME, L-Nitro Arginine Methyl Ester; MB, Methylene Blue; DZP, Diazepam; NOS, Nitric oxide synthase; ANOVA, Analysis of variance; GAD, Glutamic acid decarboxylase; L-ARG, L-Arginine; BZD, Benzodiazepine; NMDA, N-Methyl D-Aspartate; MES, Maximal electroshock seizure

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the condition remains largely undiagnosed, poorly untreated and hence results in a poor prognosis (de Boer et al., 2008).

Despite the availability of many antiepileptic drugs (AEDs), nearly one in three patients with epilepsy who have access to current AEDs show less than satisfactory prognosis and a similar proportion experience unacceptable AED-related adverse effects (Brodie, 2005; Löscher, 2002b). Thus, the need to source for clinically efficacious and safer AEDs with improved clinical profiles is still valid. Plant extracts are attractive sources of new drugs, and have shown to produce promising results for the treatment of epilepsy. Examples of plants that have shown promise as a source of good pharmacological properties include: *Passiflora incarnate* (LINN.), *Berberis vulgaris* (LINN.), *Butea monosperma* (Lam.) Taub. and *Cymbopogon winterianus* (Jowitt.) (Bhutada et al., 2010; Kasture et al., 2002; Nassiri-Asl et al., 2007; Quintans-Junior et al., 2008).

Pseudospondias microcarpa has been extensively used in Ghana and other parts of Africa as medication for different diseases. The plant is suspected of having a sedative effect on those who sit or sleep under it, hence the Ghanaian name *katawani*, literally meaning "close your eyes". It is therefore used traditionally as a sedative and for treating general central nervous system (CNS) disorders (Burkill, 1985). Preliminary studies from our laboratory showed that the hydroethanolic leaf extract of *P. microcarpa* (PME) possesses sedative effects (Adongo et al., 2014), confirming the traditional use of the plant. Moreover, in this study, we indicated the presence of some phytochemical constituents which were reported earlier (Yondo et al., 2009). The antioxidant (Yondo et al., 2009), antimicrobial (Kisangau et al., 2008), cytotoxic and antiplasmodial (Malebo et al., 2009) effects of the plant have also been reported. We also showed in our preliminary studies that PME protected against convulsions induced by pentylenetetrazole (PTZ) (Adongo et al., 2014). Therefore, this study further explored the anticonvulsant activity of PME and possible mechanism(s) in mice models.

2. Materials and methods

2.1. Collection of plant material and extraction

Fresh leaves of *P. microcarpa* were collected from the campus of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi (6° 40.626′N, 1° 34.041′W). The plant was authenticated at the Department of Herbal Medicine, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/HM1/2013/L005) was subsequently kept at the herbarium of the Faculty. Leaves of the plant were room-dried for seven days and pulverised into fine powder. The powder was extracted by cold percolation with 70% (v/v) ethanol in water over a period of 72 h and the resulting extract concentrated into a syrupy mass under reduced pressure at 60 °C in a rotary evaporator. It was further dried in a hot air oven at 50 °C for 7 days and kept in a refrigerator for use with a yield of 20.5% (w/w). The crude extract is subsequently referred to as (*Pseudospondias microcarpa* extract*)* PME or simply, extract.

2.2. FT-IR analysis of crude extract

To identify the possible functional groups that may be present in the sample, a triplicate FT-IR (PerkinElmer UATR Two) spectra was generated and baseline corrected. The spectra between 400 and 1400 cm^{-1} is usually considered as the unique region for every compound/compound mixtures and hence can be used for identification and quality control.

2.3. Animals

Male ICR mice (20–25 g) were purchased from the Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept in the vivarium of the Department of Pharmacology, KNUST. The animals were housed in groups of five (5) in stainless steel cages (34 cm×47 cm×18 cm) with soft wood shavings as bedding. Housing conditions of mice were as follows: controlled–temperature maintained at 24–25 °C, relative humidity 60–70%, and 12 h light-dark cycle. All mice had free access to food and water ad libitum. A period of at least one week for acclimatization to the laboratory environment was allowed. All laboratory procedures were conducted in accordance with accepted principles for laboratory animal use and care (NRC, 2010). Approval for this study was obtained from the Faculty Ethics Committee.

2.4. Drugs and chemicals

Pentylenetetrazole (PTZ), picrotoxin (PTX), 4-aminopyridine (4-AP), strychnine (STN), isoniazid (INH), N-nitro-L-arginine methyl ester (L-NAME), L-arginine (L-arg) and methylene blue (MB) (Sigma-Aldrich Inc., St. Louis, MO, USA); diazepam, DZP (INTAS, Gujarat, India); sildenafil, SIL (Pfizer, U.S.A); carbamazepine, CBZ (Tegretol®, Novartis, Basel, Switzerland); flumazenil, FMZ (Anexate®, Roche products Ltd., Herts, England); sodium valproate, VPA (Epilim®, Sonofi-Synthelabo Ltd-UK).

2.5. Pentylenetetrazole-induced seizures

Clonic convulsions was induced using Pentylenetetrazole (60 mg kg^{-1}, s.c.) according to methods described by Oliveira et al. (2001). Mice were divided into 7 groups (n=8) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or vehicle (normal saline; 10 mL kg^{-1} i.p.) 30 min (i.p.) or 1 h (*p.o.*) before subcutaneous injection of pentylenetetrazole (PTZ), respectively. Immediately after subcutaneous injection of PTZ, animals were placed in Perspex-walled testing chambers (15 cm×15 cm ×15 cm) with a mirror angled at 45° below the floor of the chamber to allow a complete view of convulsive events, if present. The convulsive behaviour was captured with a camcorder placed at a favourable distance directly opposite to the mirror. Video outputs of each 30 min session was later scored using JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.) for behavioural parameters including: latency, frequency and duration of clonic convulsions. The observed clonic seizures were characterized for the appearance of facial myoclonus, forepaw myoclonus and forelimb clonus. The ability of a drug/extract to reduce or prevent the seizures or delay/prolong the latency or onset of the clonic convulsions was considered as an indication of anticonvulsant activity.

2.6. Picrotoxin-induced seizures

Anticonvulsant testing method by Leewanich et al. (1996) was modified and adopted for this test. Briefly, mice were divided into 7 groups (n=8) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or vehicle (normal saline; 10 mL kg^{-1} i.p.) 30 min (i.p.) or 1 h (*p.o.*) before the injection of picrotoxin (3 mg kg^{-1}, i.p.) respectively. The latency to, frequency and duration of clonic convulsions were recorded for 30 min.

2.7. Isoniazid-induced seizures

Mice were divided into seven groups (n=10) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), vehicle or the standard drug diazepam (0.1, 0.3 or 1.0 mg kg^{-1}, i.p.). One hour (*p.o.*) or 30 min (i.p.) after administration of test compounds, animals were injected with isoniazid (300 mg kg^{-1}, s.c.). Thereafter, mice were observed for 120 min for characteristic behavioural signs, such as intermittent forelimb extension, clonic seizures, tonic seizures and death. The latencies to the

onset of the convulsive episode (clonic or tonic) and death were recorded as indicators of pro- or anticonvulsive effect of compounds.

2.8. Strychnine-induced seizures

The method described by Porter et al. (1984) was employed. Mice received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or vehicle (normal saline; 10 mL kg^{-1} i.p.) 30 min (i.p.) or 1 h (*p.o.*) before the injection of STN (0.5 mg kg^{-1}, i.p.), respectively. Latency, frequency and duration of clonic convulsions were assessed for 30 min.

2.9. 4-Aminopyridine-induced seizures

The method as described by Rogawski and Porter (1990) was adopted for this test.. Mice received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), vehicle or the standard drug carbamazepine (30, 100 or 300 mg kg^{-1}, *p.o.*). One hour after administration of test compounds, animals were treated with a single injection of 4-AP (12 mg kg^{-1}, i.p.). Thereafter, mice were observed 60 min for both clonic and tonic seizures. Clonic seizures were characterized as described in Section 2.5 and tonic seizures were characterized as explosive clonic seizures with wild running and tonic forelimb and hind limb extension. Latencies for the onset of convulsive episodes (clonic or tonic) and death were recorded as indicators of pro- or anticonvulsive effect of compounds.

2.10. Maximal electroshock seizure test

Electroconvulsions were produced by application of electric current (60 Hz, 50 mA, 0.2 s) delivered via ear-clip electrodes with an ECT Unit 7801 (Ugo Basile, Comerio, Italy). This current intensity elicited complete tonic extension of the hind limbs in saline treated mice. Mice received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), carbamazepine (3, 10 or 30 mg kg^{-1}, *p.o.*) or vehicle (normal saline; 10 mL kg^{-1}, *p.o.*) 60 min before tonic hind limb convulsions were induced. Protection against tonic hind limb seizures was determined. An animal was considered to be protected if the characteristic electroshock convulsive seizure pattern was absent.

2.11. 6 Hz seizure test

The 6-Hz seizure model was performed according to methods previously described (Brown et al., 1953; Luszczki et al., 2012). Psychomotor (limbic) seizures were induced via trans auricular stimulation (6 Hz, 0.2 ms rectangular pulse width, 32 mA, 3 s duration) delivered by an ECT Unit 5780 (Ugo Basile, Comerio, Italy). Normal saline (0.9%) was used to wet the electrodes immediately before testing to ensure good electrical contact. Animals were manually restrained and released immediately after the stimulation and recorded the presence or absence of psychomotor seizure activity. Immediately following stimulation, mice were placed separately in Plexiglas cages (25 cm×15 cm ×10 cm) for behavioural observation. After stimulation, the animals exhibited behavioural signs of psychomotor seizures—behavioural arrest, forelimb clonus, twitching of the vibrissae, and Straub tail—that lasted for 60–120 s in untreated animals. Animals resumed normal exploratory behaviour after seizure induction. The experimental endpoint was protection against the seizure: an animal was considered to be protected if it resumed its normal exploratory behaviour within 10 s after stimulation. Protection in the 6 Hz model was defined as the absence of a seizure. Mice not experiencing seizures exhibited normal exploratory behaviour when placed in the cages. Mice were divided into 8 groups (n=10) and received PME (30, 100, 300 or 1000 mg kg^{-1}, *p.o.*), sodium valproate (100, 200 or 400 mg kg^{-1}, *p.o.*) or vehicle (normal saline; 10 mL kg^{-1}, *p.o.*) 60 min before psychomotor seizures were induced.

2.12. Effect of PME on $GABA_A$

To investigate the possible involvement of γ-aminobutyric acid $(GABA)_A$ receptors in the anticonvulsant activity of PME, mice were pre-treated with flumazenil (2 mg kg^{-1}, i.p.), a selective benzodiazepine receptor antagonist or vehicle 15 min before PME (100 mg kg^{-1}, *p.o.*) or diazepam (0.3 mg kg^{-1}, i.p.) administration. After 45 min, mice were challenged subcutaneously with PTZ (60 mg kg^{-1}, s.c.) and assessed 30 min for latency, frequency and duration of clonic convulsions.

2.13. Effect of PME on L-arginine-nitric oxide-cGMP pathway

Doses of the modulators were chosen based on pilot experiments and previous reports (Akula et al., 2008; Bahremand et al., 2010). To investigate the possible involvement of the L-arginine-NO-cGMP pathway in the anticonvulsant action of PME, mice were pre-treated with sub-effective doses of L-arginine [150 mg kg^{-1}, i.p., a precursor of nitric oxide (NO)], L-NAME [30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase (NOS) inhibitor], methylene blue [1 mg kg^{-1}, i.p., an inhibitor of NO synthase and an inhibitor of soluble guanylyl cyclase (sGC)], sildenafil [5 mg kg^{-1}, i.p., a phosphodiesterase 5 (PDE 5) inhibitor] or vehicle 15 min before PME (100 mg kg^{-1}, *p.o*) administration. After 45 min, mice were challenged subcutaneously with PTZ (60 mg kg^{-1}) and assessed 30 min for latency, frequency and duration of clonic convulsions.

2.14. Grip-strength test

The effects of PME and diazepam on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer et al. (1979). The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 cm×8 cm) connected to an isometric force transducer. Mice were randomly divided into eight groups (n=6): saline-treated control group; diazepam group (0.1, 0.3, 1 and 3 mg kg^{-1}, i.p.) and PME group (30, 100 and 300 mg kg^{-1}, *p.o.*). Thirty minutes after i.p. and 1 h after oral administration of the test compounds, mice were lifted by the tails so that their forepaws could grasp the grid. Mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 4 measurements for each animal was calculated and subsequently, the mean maximal force was determined. Skeletal muscular strength in mice was expressed in Newton (N).

2.15. Statistical analysis

In all experiments, a sample size of six to ten (n=6–10) was used. Data are presented as mean ± SEM. To compare differences between groups, one-way analysis of variance (ANOVA) was performed with Newman-Keuls' test as *post hoc*. In analysing the possible role of GABAergic and nitric oxide mechanisms in the anticonvulsant effect of the extract, two-way ANOVA with the Bonferroni's *post hoc* test (*treatment* × *dose*) was performed. In the 4-aminopyridine and isoniazid seizure tests, the Kaplan-Meier method was used in estimating survival relative to time and survival differences were analysed with the log-rank test. GraphPad® Prism Version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. $P < 0.05$ was considered statistically significant for all test.

3. Results

3.1. IR analysis

FT-IR spectroscopy was used for the distinct functional group identification and run under IR region between the ranges of 400 and 4000 cm^{-1}. The characteristic spectra in the region from 400 to 1400 cm^{-1} was as a fingerprint region for subsequent comparison of

future extracts. Baseline corrected Infrared spectra and peak values, with labels, is provided in the Appendix A.

3.2. Pentylenetetrazole-induced seizures

The extract significantly and dose-dependently delayed the onset of clonic convulsions ($F_{3,28}$=3.009, P=0.0469; Fig. 1a) with statistical significance at 300 mg kg^{-1} ($P < 0.05$). One-way ANOVA revealed that PME also significantly reduced the frequency of clonic convulsions ($F_{3,28}$=6.947, P=0.0012; Fig. 1a) at all tested doses ($P < 0.05$ at 30 mg kg^{-1}; $P < 0.01$ at 100 and 300 mg kg^{-1}). Newman-Keuls' *post hoc* test indicated a statistical significant reduction in the duration of clonic convulsions by the extract at all the doses used ($P < 0.05$ at 30 mg kg^{-1}; $P < 0.001$ at 100 and 300 mg kg^{-1}). The reference anticonvulsant, diazepam, delayed the onset of clonic convulsions ($F_{3,36}$=24.76, $P < 0.001$; Fig. 1c) with statistical significance at 0.3 and 1.0 mg kg^{-1} (both $P < 0.001$). Also, diazepam caused significant and dose-dependent reduction in the frequency ($F_{3,36}$=38.01, $P < 0.001$; Fig. 1c) and duration of clonic convulsions ($F_{3,36}$=49.60, $P < 0.0001$; Fig. 1d).

3.3. Picrotoxin-induced seizures

As shown in Fig. 2, the extract significantly and dose-dependently reduced the frequency ($F_{3,36}$=5.071, P=0.0063; Fig. 2a) and duration ($F_{3,36}$=6.117, P=0.0025; Fig. 2b) of clonic convulsions induced by picrotoxin. In addition, PME significantly delayed the onset of clonic convulsions ($F_{3,28}$=6.117, P=0.0028) with statistical significance observed at 300 mg kg^{-1} ($P < 0.01$). Diazepam produced effects similar to that of the extract. It significantly delayed the onset of convulsions ($F_{3,36}$=9.7118, $P < 0.0001$; Fig. 2c) and reduced the frequency ($F_{3,36}$=9.131, $P < 0.0001$; Fig. 2c) and duration ($F_{3,36}$=15.63, $P < 0.0001$; Fig. 2d) of convulsions.

3.4. Isoniazid-induced seizures

Isoniazid (300 mg kg^{-1}, s.c.) elicited clonic convulsions followed by tonic hind limb extensions and mortality in mice. Fig. 3 indicates that treatment of mice with PME (30–300 mg kg^{-1}, *p.o.*) significantly delayed the onset to both clonic ($F_{3,36}$=12.90, $P < 0.0001$) and tonic ($F_{3,36}$=15.63, $P < 0.0001$) convulsions as compared to vehicle-treated mice. Furthermore, in Fig. 4, PME significantly (P=0.0005, $\chi 2$ (df =3) =17.65) improved survival of the animals after induction of convulsions. As compared to vehicle-control group, mice treated with diazepam (0.1–1.0 mg kg^{-1}, i.p.) showed significant protection against INH-induced mortality (P=0.003, $\chi 2$ (df=3) =13.90). In addition, it significantly delayed onset of convulsions [clonic ($F_{3,36}$=19.69, $P < 0.0001$) and tonic ($F_{3,36}$=25.46, $P < 0.0001$)].

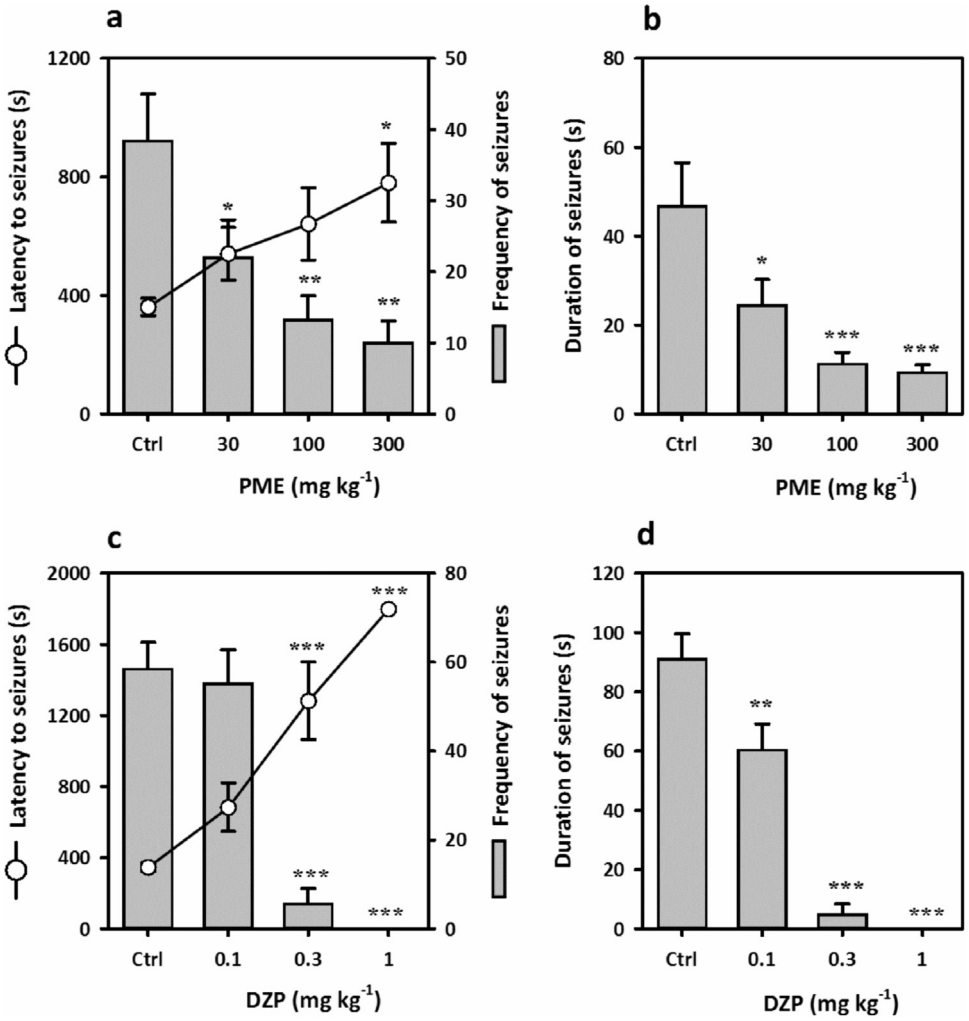

Fig. 1. Effect of PME (30–300 mg kg^{-1}) and diazepam (0.1–1.0 mg kg^{-1}) on frequency (a, c), latency (a, c) and duration (b, d) of PTZ-induced clonic seizures in mice. Data are expressed as mean ± SEM. Each group consisted of 8 mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

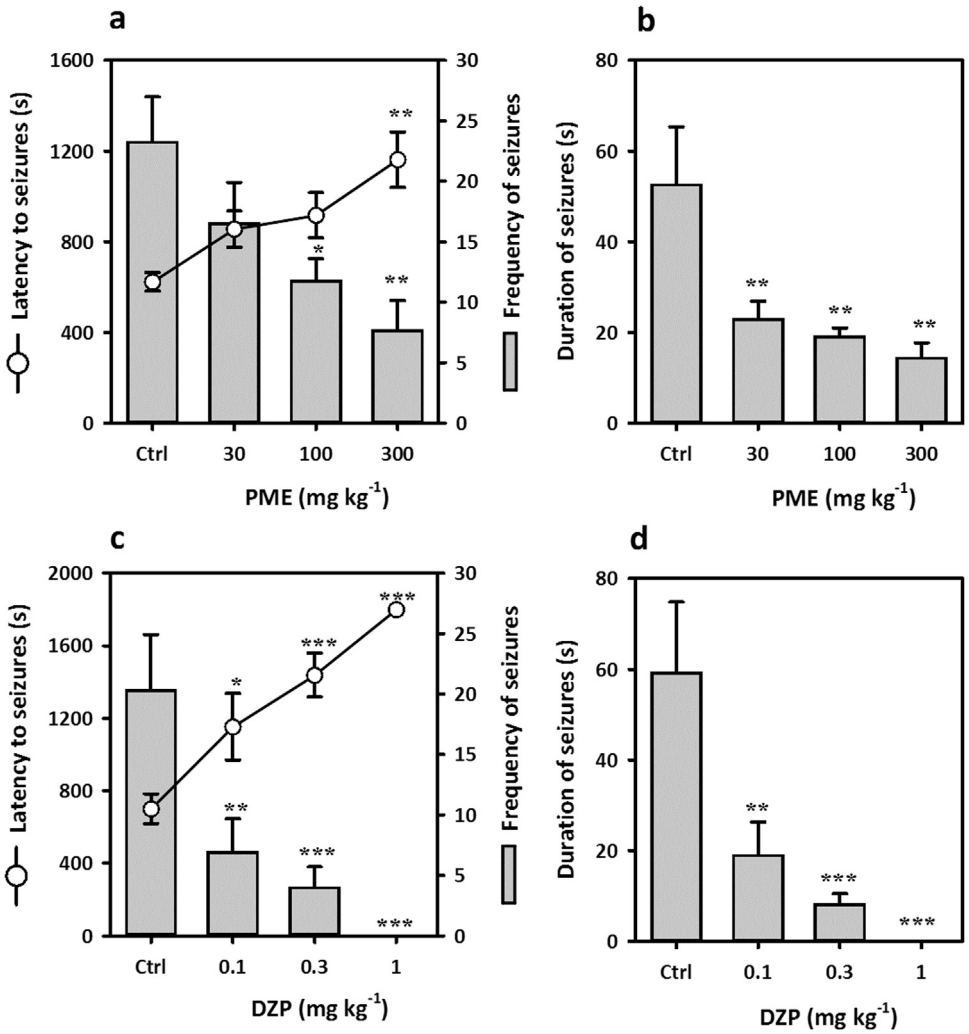

Fig. 2. Effect of PME (30–300 mg kg^{-1}) and diazepam (0.1–1.0 mg kg^{-1}) on frequency (a, c), latency (a, c) and duration (b, d) of picrotoxin-induced clonic seizures in mice. Data are expressed as mean ± SEM (n=8). *P < 0.05; **P < 0.01; ***P < 0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

3.5. Strychnine-induced seizures

Fig. 5 shows the effects of PME (30–300 mg kg^{-1}, *p.o.*) and diazepam (0.1–1 mg kg^{-1}, i.p.) on latency, frequency and duration of clonic convulsions induced by strychnine in mice. One-way ANOVA revealed that the extract exhibited a dose-dependent effect against strychnine-induced clonic seizures by significantly increasing latency to convulsions ($F_{3,24}$=4.208, P=0.0159) and reducing the frequency ($F_{3,24}$=7.569, P=0.0010). In addition, PME provided 29% (at 30 mg kg^{-1}), 43% (at 100 mg kg^{-1}) and 57% (at 300 mg kg^{-1}) protec-

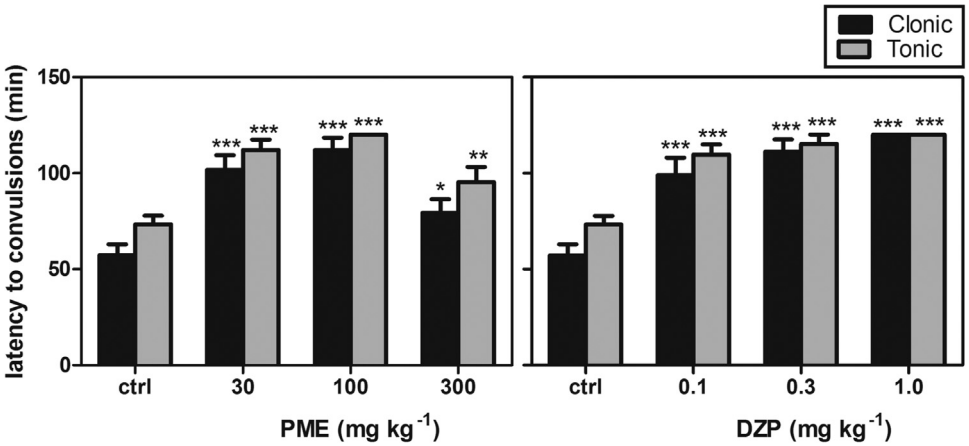

Fig. 3. Effect of PME (30–300 mg kg^{-1}) and diazepam (0.1–1.0 mg kg^{-1}) on latency to isoniazid-induced seizures in mice. Data are expressed as mean ± SEM (n=10). *P < 0.05; **P < 0.01; ***P < 0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

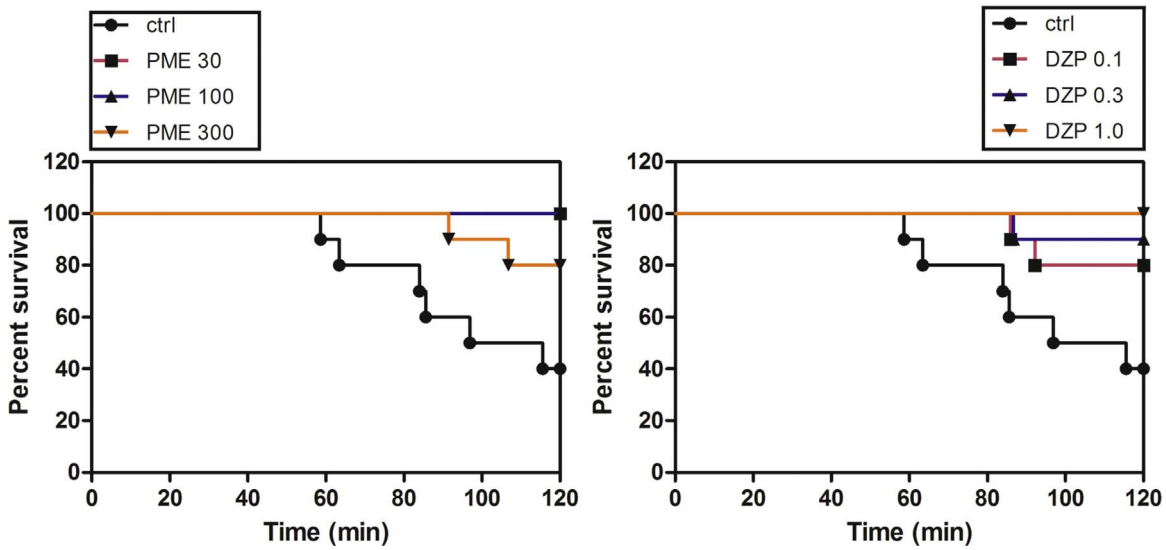

Fig. 4. Kaplan–Meier estimates of overall survival of animals treated with PME (30, 100 and 300 mg kg^{-1}) and diazepam, DZP (0.1, 0.3 and 1 mg kg^{-1}) in the isoniazid-induced seizure test over a 2 h observation period (n=10).

tion against strychnine-induced seizures. Duration of clonic seizures was also reduced by the extract even though this was not statistically significant as compared to the control ($F_{3,24}$=2.593, P=0.0761). Diazepam significantly delayed the onset of convulsions ($F_{3,24}$=13.32, $P < 0.0001$) and reduced the frequency ($F_{3,24}$=7.768, P=0.0009) and duration ($F_{3,24}$=6.721, P=0.0019) of convulsions. Acute treatment with

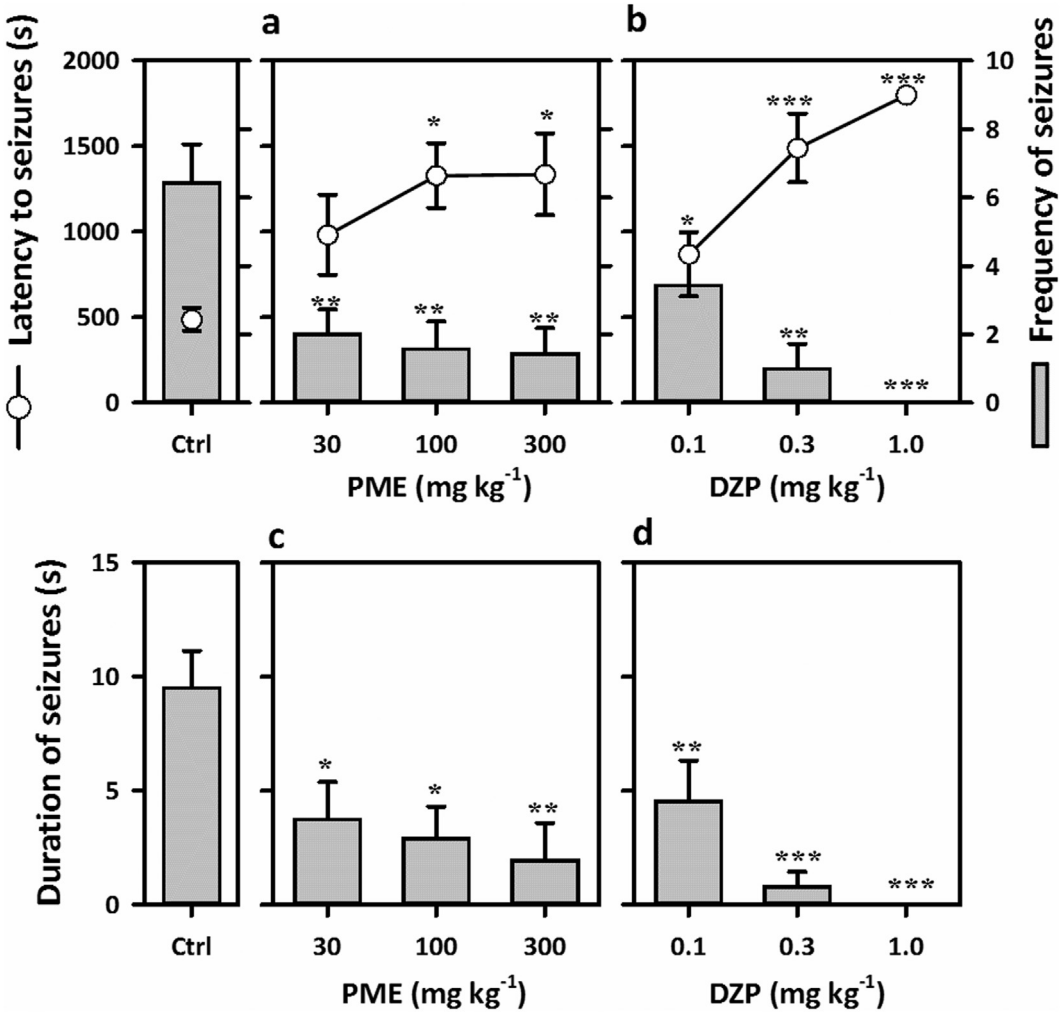

Fig. 5. Effect of PME (30–300 mg kg^{-1}) and diazepam (0.1–1.0 mg kg^{-1}) on frequency (a and b), latency (a and b) and duration (c and d) of strychnine-induced clonic seizures in mice. Data are expressed as mean ± SEM (n=8). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

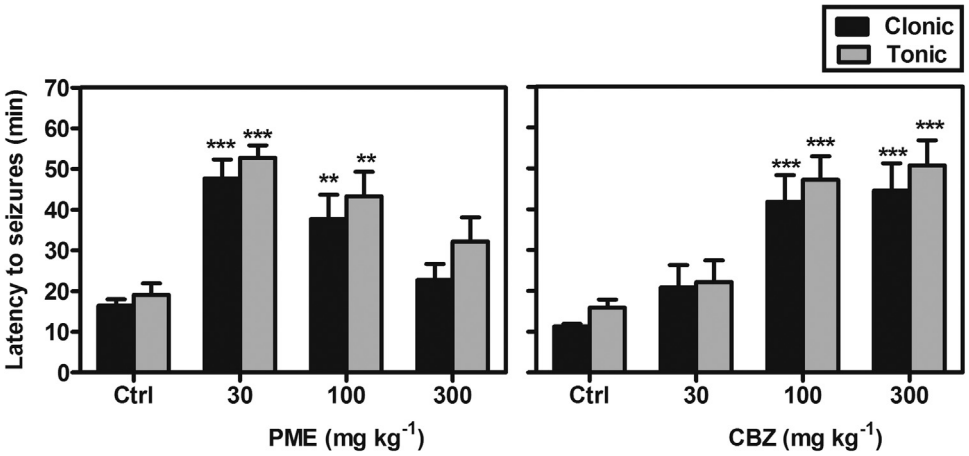

Fig. 6. Effect of PME (30–300 mg kg^{-1}) and carbamazepine (30–300 mg kg^{-1}) on latency of 4-AP-induced seizures in mice. PME and carbamazepine were administered *p.o.* 60 min before behavioural assessments for 1 h. Data are expressed as mean ± SEM (n=10). $^{**}P < 0.01$; $^{***}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

diazepam provided 29% (at 0.1 mg kg^{-1}), 71% (at 0.3 mg kg^{-1}) and 100% (at 1 mg kg^{-1}) protection against strychnine-induced clonic seizures.

3.6. 4-Aminopyridine-induced seizures

The effects of PME and carbamazepine on 4-AP-induced seizures are shown in Figs. 6 and 7. A single administration of 4-AP (12 mg kg^{-1}, i.p.) caused clonic and tonic seizures as well as death in all saline-treated mice. In contrast, pre-treatment of animals with PME (30–300 mg kg^{-1}, *p.o.*) caused a significant delay in the latency to both clonic ($F_{3,36}$=10.81, $P < 0.0001$) and tonic ($F_{3,36}$=9.513, $P < 0.0001$) seizures. The effects of the extract on 4-AP-induced seizures decreased with increasing dose. PME provided 40% (at 30 mg kg^{-1}), 30% (at 100 mg kg^{-1}) and 0% (at 300 mg kg^{-1}) protection against 4-AP-induced clonic seizures. Furthermore, the extract provided 50% (at 30 mg kg^{-1}), 50% (at 100 mg kg^{-1}) and 20% (at 300 mg kg^{-1}) protection against 4-AP-induced tonic seizures in mice. Carbamazepine (CBZ) produced effects analogous to the extract in the 4AP-induced seizure test but the effects increased with increasing dose. It caused a significant delay in the latency of clonic ($F_{3,36}$=9.040, $P < 0.0001$) and tonic seizures ($F_{3,36}$=12.06, $P < 0.0001$). Carbamazepine provided 10% (at 30 mg kg^{-1}), 50% (at 100 mg kg^{-1}) and 60% (at 300 mg kg^{-1}) protection against 4-AP-induced clonic seizures. Furthermore, it provided 10% (at 30 mg kg^{-1}), 60% (at 100 mg kg^{-1}) and 80% (at 300 mg kg^{-1}) protection against 4-AP-induced tonic seizures in mice. The extract significantly ($P < 0.0001$, χ^2 (*df*=3) =30.27) improved survival of the animals after induction of convulsions. Carbamazepine also produced similar effects on survival ($P < 0.0001$, χ^2 (*df*=3) =36.61).

3.7. Effect on maximal electroshock seizures

Electrical stimulation produced tonic hind limb extensions (HLEs) in all saline-control mice (Table 1). The extract did not protect against tonic hind limb extensions. In contrast to PME, carbamazepine at the dose of 30 mg kg^{-1}, completely protected mice against tonic hind limb extensions. In addition, no deaths were recorded at 10 and 30 mg kg^{-1}.

Data shows the percentage of mice (n=10) that produced tonic convulsions and deaths.

3.8. Effect on psychomotor seizures

In Table 2, it was observed that all control mice exhibited psychomotor seizures (0% protection) after electrical stimulation that lasted 60–120 s. However, acute treatment with PME protected against

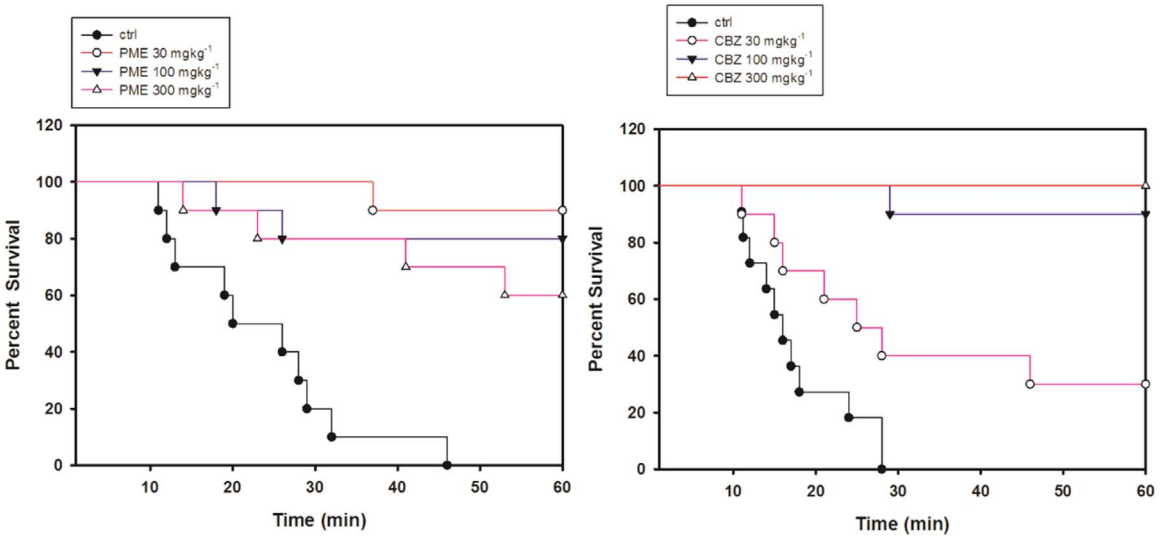

Fig. 7. Kaplan–Meier estimates of overall survival of animals treated with PME (30, 100 and 300 mg kg^{-1}) and carbamazepine (30, 100 and 300 mg kg^{-1}) in the 4-aminopyridine seizure test over a one hour observation period (n=10).

Table 1
Effects of PME and carbamazepine (CBZ) on maximal electroshock (MES)-induced seizures in mice.

Group	Dose (mg kg^{-1})	Incidence of tonic extensions (%)	Death (%)
Control		100	33.3
PME	30	100	33.3
	100	100	50
	300	100	33.3
	1000	100	33.3
CBZ	3	100	16.7
	10	83.3	0
	30	0	0

Table 2
Effects of PME and sodium valproate (VPA) in the mouse 6 Hz-induced limbic seizure model.

Group	Dose (mg kg^{-1})	% protection
control		0
PME	30	30
	100	40
	300	60
	1000	90
VPA	100	20
	200	60
	400	90

Data indicates the percentage of mice (n=10) that were protected.

6 Hz-induced seizures. It provided 20% (at 30 mg kg^{-1}), 40% (at 100 mg kg^{-1}), 60% (at 300 mg kg^{-1}) and 90% (at 1000 mg kg^{-1}) protection against 6 Hz-induced seizures. Sodium valproate (VPA), the reference anticonvulsant, produced effects similar to the extract as it provided 10% (at 100 mg kg^{-1}), 60% (at 200 mg kg^{-1}) and 90% (at 400 mg kg^{-1}) protection against 6 Hz-induced seizures.

3.9. Effect on $GABA_A$

As shown in Fig. 8, PME (100 mg kg^{-1}, *p.o*) significantly increased latency and decreased both frequency and duration of clonic convulsions. Administration of flumazenil (2 mg kg^{-1}, i.p.) had no effects on latency, duration and frequency of convulsions as compared with saline (vehicle)-treated animals. However, pre-treatment with flumazenil significantly reversed the effect of PME (100 mg kg^{-1}, *p.o*) by decreasing latency ($F_{1,36}$=36.48, $P < 0.0001$) as well as increasing duration ($F_{1,36}$=24.04, $P < 0.0001$) and frequency ($F_{1,36}$=23.10, $P < 0.0001$) of clonic seizures induced by PTZ. Similar results were obtained for diazepam.

3.10. Effect of PME on L-arginine-NO-cGMP pathway

Figs. 9 and 10 show the effects of L-NAME, methylene blue, L-arginine or sildenafil on the actions of PME in the PTZ-induced seizure test. Acute PME (100 mg kg^{-1}, *p.o*) treatment significantly increased latency and decreased both frequency and duration of clonic convulsions. Administration of L-arginine (150 mg kg^{-1}, i.p.) had no effect on latency, frequency and duration of convulsions compared with saline (vehicle)-treated animals. However, pre-treatment with L-arginine significantly inhibited PME action by decreasing latency ($P < 0.01$) and increasing duration ($P < 0.05$) of clonic seizures as revealed by *post hoc* analysis. Pre-treatment with either L-NAME (30 mg kg^{-1}, i.p.) or methylene blue (1 mg kg^{-1}, i.p.) significantly potentiated PME action as evident from the delayed expression of clonic convulsions [L-NAME ($F_{1,24}$=4.474, $P < 0.05$); MB ($F_{1,24}$=6.005, $P < 0.05$)] induced by PTZ.

Fig. 10 shows the effect of pre-treatment of sildenafil, a phosphodiesterase 5 inhibitor on PTZ-induced clonic seizures. Concomitant administration with sildenafil (5 mg kg^{-1}, i.p.) significantly reversed the effect of PME. This is observed as a decrease in the onset ($P < 0.05$) and increase in the duration of clonic ($P < 0.05$) convulsions induced by PTZ.

3.11. Grip-strength test

Fig. 11 shows the results of the effect of PME and diazepam on skeletal muscle strength in the grip-strength test. One-way ANOVA revealed that pretreatment of mice with PME (30–300 mg kg^{-1}, *p.o.*) did not significantly affect the grip-strength in mice ($P > 0.05$). In contrast to PME, diazepam (0.1–3 mg kg^{-1}, i.p.) significantly and dose-dependently decreased ($F_{3,16}$=4.308, P=0.0113) the grip-strength in mice with Newman-Keuls' *post hoc* analysis revealing a significant effect at the dose of 3 mg kg^{-1} ($P < 0.05$).

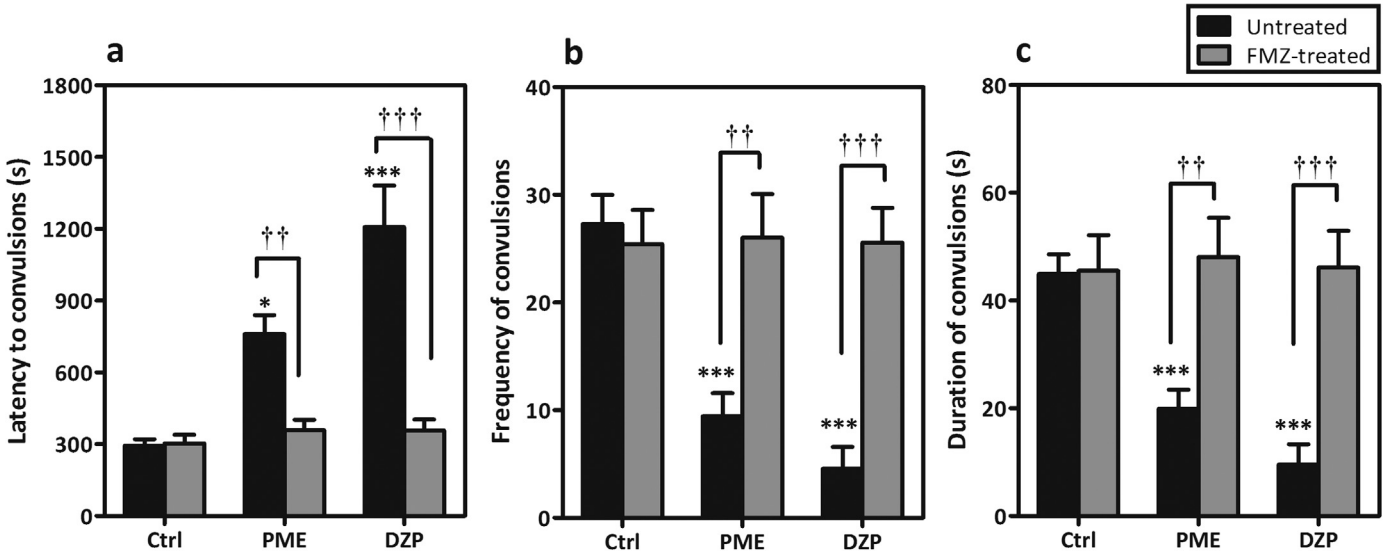

Fig. 8. Effect of flumazenil on the latency (a), frequency (b), and duration of seizures (c) of PME (100 mg kg^{-1}, *p.o.*) and diazepam (0.3 mg kg^{-1}, i.p.) in PTZ-induced seizures. Data are presented as mean ± SEM (n=7). *?? < 0.05 and ***?? < 0.001 compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* test). ††?? < 0.01 and †††?? < 0.001 (two-way ANOVA followed by Bonferroni's test).

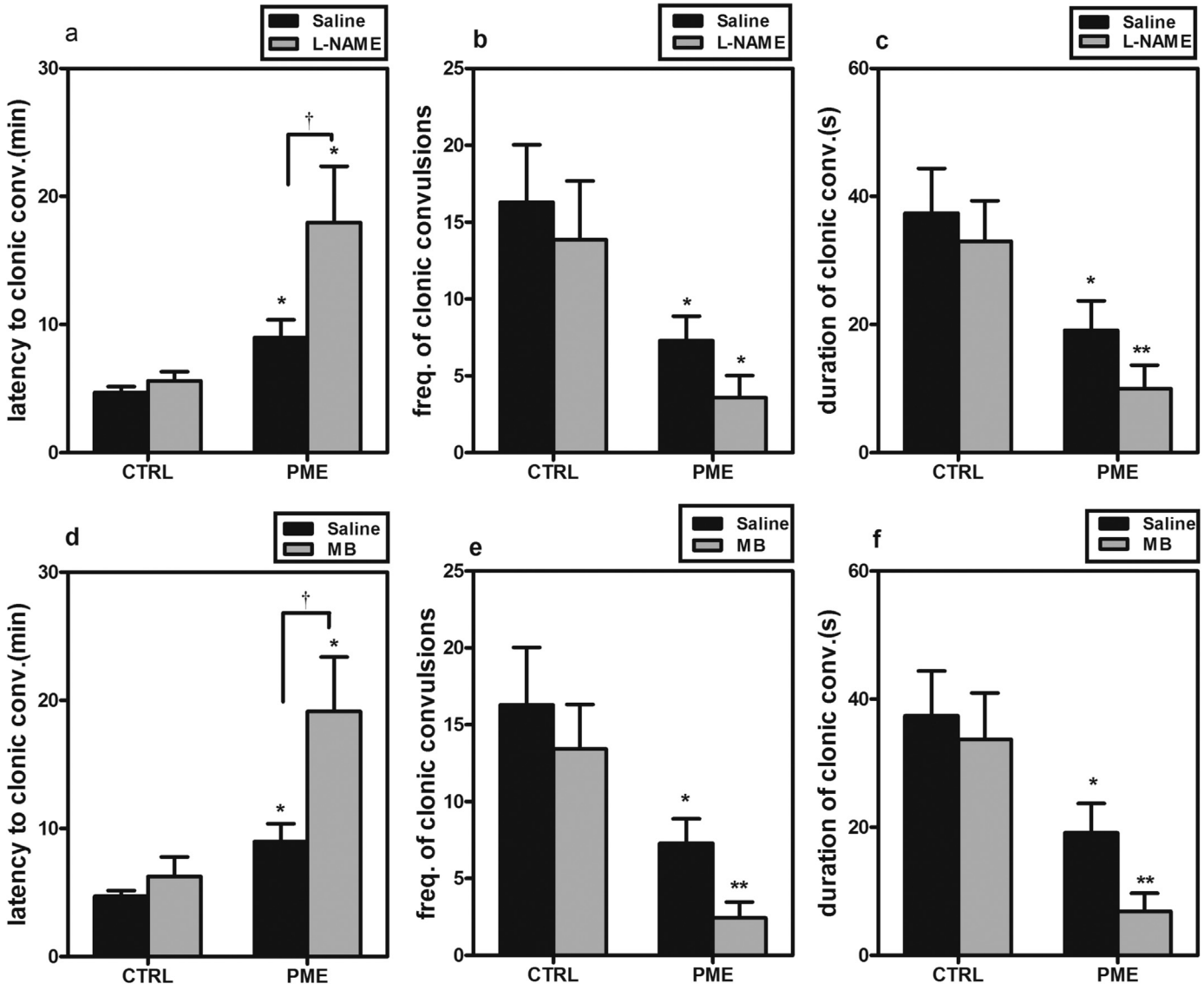

Fig. 9. Effects of pre-treatment of L-NAME [30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase (NOS) inhibitor] and methylene blue [1 mg kg^{-1}, i.p., an inhibitor of NO synthase and an inhibitor of soluble guanylyl cyclase (sGC)] on the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o.*) in the PTZ-induced seizure test. L-NAME, MB or saline were administered 15 min before administration of PME and 45 min before determination of PTZ-induced seizures. Data are presented as group mean ± SEM (n=7). $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated animals (One-way ANOVA followed by Newman Keuls' test). Significant difference between treatments: $^{\dagger}P < 0.05$ (Two-way ANOVA followed by Bonferroni's test).

4. Discussion

Results of the present study provide evidence that a hydroethanolic leaf extract of the *Pseudospondias microcarpa* possess anticonvulsant activity in pharmacologically validated experimental animal models.

The GABAergic system is implicated in epilepsy since enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsions respectively (Quintans-Junior et al., 2008). Pentylenetetrazole (PTZ), a $GABA_A$ blocker, is the most frequently used epileptogenic agent employed in the search for new antiepileptic drugs (AEDs) (Löscher, 2011). It blocks GABA-mediated chloride ion influx through an allosteric interaction in the Cl^- channel, thus leading to induction of convulsions in animals (Kubova, 2009; Velíšek, 2006). Accordingly, drugs such as benzodiazepines and phenobarbitone which enhance $GABA_A$ receptor-mediated inhibitory neurotransmission can block PTZ-induced clonic seizures (Macdonald and Kelly, 1995). In this study, acute administration of PME and the benzodiazepine diazepam, exhibited anticonvulsant activity against PTZ-induced seizures. Ability of an agent to prevent or delay the onset of convulsions induced by PTZ in animals is an indication of anticonvulsant activity. Thus, it could be said that the extract exhibits anticonvulsant effect against PTZ-induced clonic seizures probably due to interference with GABAergic mechanism(s). The significant effect of diazepam as evident in the PTZ-induced convulsions agrees with its enhancing effects in GABAergic neurotransmission (Patil et al., 2011). It has also been suggested that PTZ-induced clonic seizures model myoclonic seizures (Kubova, 2009). Thus, the extract could possibly protect against myoclonic seizures.

Similar to PTZ, picrotoxin exerts its convulsant action via blockade of the $GABA_A$ receptor-linked chloride ion channel, which normally opens to allow increased chloride ion conductance following the activation of $GABA_A$ receptors by γ-aminobutyric acid (GABA) (Nicoll, 2001; Velíšek, 2006). Data from this study shows that PME and diazepam exhibited anticonvulsant activity against picrotoxin-induced seizures. Attenuation of picrotoxin-induced convulsions by PME further indicates possible GABAergic neurotransmission in its anticonvulsant action. In addition, similar to diazepam, PME significantly delayed convulsions (clonic and tonic) and reduced mortality against INH-induced convulsions. Isoniazid induces convulsions by inhibiting GABA synthesis (Costa et al., 1975). It inhibits glutamic acid decarboxylase (GAD) activity (enzyme involved in GABA synthesis),

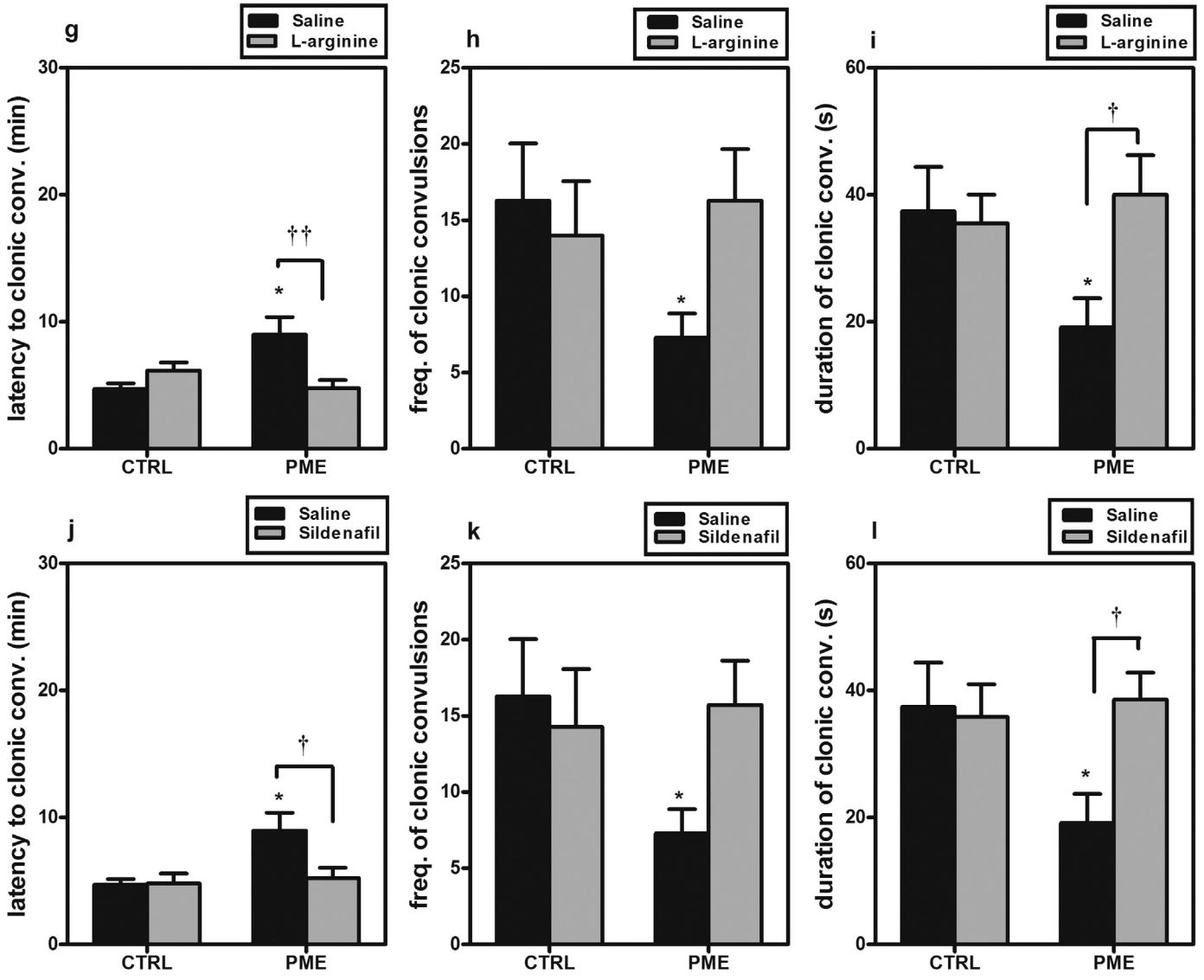

Fig. 10. Effects of pre-treatment of L-arginine [150 mg kg^{-1}, i.p., a precursor of nitric oxide (NO)] and sildenafil [5 mg kg^{-1}, i.p., a phosphodiesterase 5 (PDE 5) inhibitor] on the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o.*) in the PTZ-induced seizure test. L-arginine, sildenafil or saline were administered 15 min before administration of PME and 45 min before determination of PTZ-induced seizures. Data are presented as group mean ± SEM. *$P < 0.05$ versus vehicle-treated animals (One-way ANOVA followed by Newman Keuls' test). Significant difference between treatments: †$P < 0.05$, ††$P < 0.01$ (Two-way ANOVA followed by Bonferroni's test).

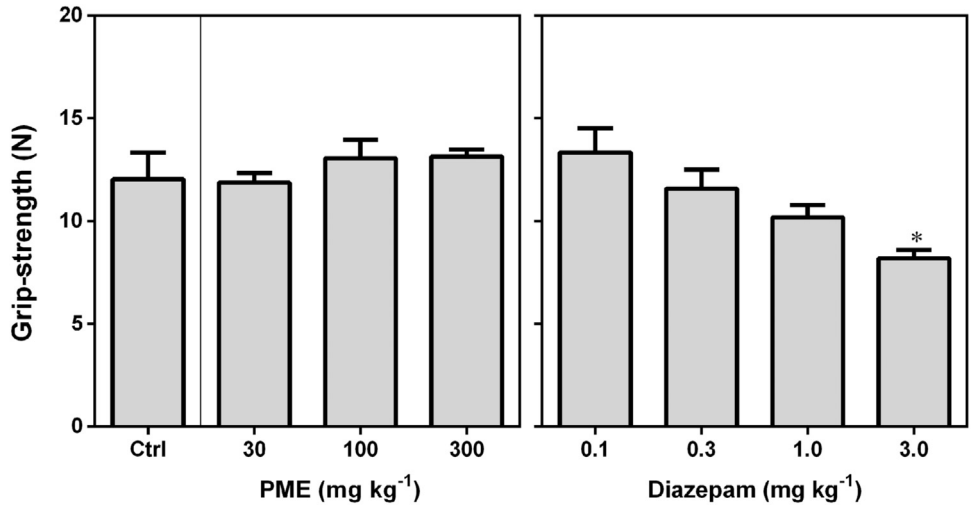

Fig. 11. Behavioural effects of PME and DZP on muscle relaxant activity in the grip-strength test in mice. Data are expressed as group mean ± SEM (n=6). *$P < 0.05$ compared to control group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

resulting in decreased levels of GABA (Raygude et al., 2012; Vergnes et al., 2000). Anticonvulsant effect against INH-induced convulsions further confirm the GABA-enhancing activity of the plant extract.

Additionally, the anticonvulsant effect of the extract was blocked by flumazenil, a specific antagonist of the benzodiazepine site in the $GABA_A$-Benzodiazepine receptor complex (Brogden and Goa, 1991; File and Pellow, 1986). The extract, like diazepam, may therefore be acting via direct activation of benzodiazepine site of the $GABA_A$ receptor complex. This further gives confirmation to the hypothesis that PME may be affecting GABAergic mechanism(s) to exert its anticonvulsant activity.

Activation of glycine receptors results in an influx of chloride ions into the neuron, which is then hyperpolarized and inhibited. Strychnine acts as a selective competitive antagonist that blocks inhibitory effect of the chloride channel associated with glycine at all glycine receptors (Curtis et al., 1971; Patil et al., 2011), resulting in seizures. The extract exhibited anticonvulsant activity against seizures induced by strychnine by decreasing frequency and duration of convulsions. In addition, latency to convulsions was significantly delayed. Thus, the observed protection of the extract against strychnine-induced seizures is mediated through the glycinergic pathway.

Pre-treatment of PME and carbamazepine before administration of 4-AP significantly increased the latency to seizures and reduced the incidence of mortality indicating anticonvulsant activity against 4-AP-induced seizures. Action of 4-AP occurs through a K^+-channel blockade at the presynaptic neuronal level (Brito et al., 2009). As a result, efflux of intracellular K^+ is suppressed and calcium influx is enhanced, leading to an increase in neurotransmitter release (Molgo et al., 1985). At the neuronal level, K^+ channels are involved in neuronal excitability (Pongs, 1999). Thus, direct activation of voltage dependent K^+ channels hyperpolarizes the neuronal membrane and limits the firing of an action potential (Porter and Rogawski, 1992). Accordingly, K^+ channel activators possess anticonvulsant effects in some seizure models (Rostock et al., 1996). Therefore, the extract being able to protect against 4-AP-induced seizures may probably be due to direct activation of K^+ channels and could therefore contribute to membrane hyperpolarization (Herrero et al., 2002). Moreover, since GABAergic receptor activation can result in enhancement of potassium conductance, it is therefore possible that PME may be acting indirectly to enhance potassium ion conductance. Currently, retigabine is the only approved antiepileptic drug which activates potassium currents (Hecht, 2012; Rundfeldt, 1997). The present study therefore gives PME a distinct profile than the widely used antiepileptic agents, since it may have the potential to activate potassium channels and also be a source of anticonvulsant compounds that will enhance potassium conductance.

Furthermore, the convulsant effect of 4-AP is due to the release of excitatory neurotransmitters such as glutamate and this results in over activation of mainly the *N*-methyl-D-aspartate (NMDA)-type receptors (Morales-Villagrán et al., 1996). Indeed, an enhancement in the glutamatergic neurotransmission has been linked to the 4-AP convulsant action (Tapia et al., 1999), since the administration of NMDA receptor antagonists protects against 4-AP induced seizures (Fragoso-Veloz and Tapia, 1992). Anticonvulsant activity of PME against seizures induced by 4-AP may also possibly be due to its inhibition of the glutamate signal pathway: NMDA receptors and could therefore protect against neuronal excitotoxicity.

As a well-validated preclinical model, the maximal electroshock seizure (MES) test predicts anticonvulsant drug efficacy against generalized tonic-clonic (grand mal) seizures (Holmes, 2007; Löscher, 1998). In addition, it permits the evaluation of the ability of a substance to prevent seizure spread through neural tissue (Castel-Branco et al., 2009). Since the extract produced no anticonvulsant effect against MES-induced tonic seizures in mice, it therefore indicates its inability to protect against generalized tonic-clonic seizures as well as prevent seizure spread.

In contrast to the MES test, the extract protected against seizures in the 6-Hz seizure test. This test uses a low-frequency, long-duration stimulation paradigm to induce psychomotor seizures that involve forelimb clonic convulsions and stereotyped behaviours similar to those seen in complex partial epilepsy (Giardina and Gasior, 2001). Protection of PME against 6 Hz-induced psychomotor seizures therefore suggests anticonvulsant activity against complex partial seizures. In addition, it has been suggested that the 6-Hz seizure test might identify anticonvulsant compounds with novel mechanisms of action and serve as a test of human drug-resistant epilepsy (Barton et al., 2001; Duncan and Kohn, 2005). Thus, like levetiracetam (Surges et al., 2008), PME could as well possess a distinct profile of activity from the commonly used antiepileptic drugs, implying possible efficacy in pharmacoresistant epilepsies.

Various preclinical studies suggest nitric oxide (NO) as a modulator of seizure activity with both anticonvulsant (Noh et al., 2006) and proconvulsant (Royes et al., 2007) effects. These effects have been shown to be based on the type of seizure, route of administration, source of NO and other neurotransmitter systems involved (Riazi et al., 2006). Osonoe et al. (1994), demonstrated that decreased NO levels results in suppression of convulsions, and inhibition of nitric oxide synthase (NOS) activity shows anticonvulsant property against pentylenetetrazole-induced seizures in rats. Studies have therefore shown that L-NAME, inhibitor of both endothelial and neuronal NOS activity (Rees et al., 1990; Talarek and Fidecka, 2003), inhibits pentylenetetrazole and strychnine-induced seizures in mice (Kaputlu and Uzbay, 1997). In the present study, a sub-effective dose of L-NAME given alone did not influence pentylenetetrazole-induced seizures. However, it potentiated the anticonvulsant effect of PME by delaying latency as well as decreasing frequency and duration of PTZ-induced clonic seizures. This potentiating effect has been observed for anticonvulsants that act via the GABAergic pathway such as the benzodiazepines (Talarek and Fidecka, 2003). This shows that PME also elicits its anticonvulsant effect in the pentylenetetrazole seizure model probably by interacting with the nitric oxide pathway.

L-arginine administered at high doses enhances seizure susceptibility through excessive release of nitric oxide in chemical seizure models induced by GABA antagonists, likely due to hyperexcitability (Riazi et al., 2006). Results showed that L-arginine [nitric oxide synthase (NOS) substrate] at the dose used attenuated the anticonvulsant activity of PME by significantly decreasing latency and increasing duration of convulsions. This is consistent with various reports where L-arginine decreased the antiepileptic effect of compounds in the pentylenetetrazole model of epilepsy (Bahremand et al., 2010; Gholipour et al., 2008). This further confirms the possible involvement of the nitric oxide pathway in the anticonvulsant effect of PME.

Nitric oxide stimulates cGMP generation via soluble guanylyl cyclase, which plays a major role in seizure (Snyder and Bredt, 1991). Methylene blue inhibits soluble guanylyl cyclase (Meller and Gebhart, 1993), and it has widely been applied in experiments to determine the contribution of the cGMP pathway in the effects of nitricoxidergic system (Talarek and Fidecka, 2003). Although methylene blue was not effective in antagonizing clonic seizures induced by PTZ when administered alone, it potentiated the anticonvulsant effect of PME. This confirms the role of the nitric oxide pathway in the anticonvulsant effect of PME. The potentiating effect of methylene blue has also been demonstrated in the anticonvulsant effect of diazepam and clonazepam in PTZ-induced seizures in mice (Talarek and Fidecka, 2003).

Intra-cellular concentrations of cGMP are also regulated not only by soluble guanylate cyclase (sGC), but also by Phosphodiesterase V (PDE 5) enzyme, which catalyses the hydrolysis of the second messengers cAMP and cGMP (Akula et al., 2008). Sildenafil is a PDE 5 inhibitor and enhance the NO-mediated effects by inhibiting cGMP degradation in target tissues (Gholipour et al., 2009; Jackson et al., 1999). Sildenafil

inhibited the anticonvulsant effect of PME by significantly delaying latency and increasing duration of clonic convulsions. This is in agreement with reports establishing the proconvulsant effect of sildenafil (Akula et al., 2008; Riazi et al., 2006). Results of this study therefore suggest possible contribution of the cGMP pathway in the anticonvulsant effect of the extract.

Several studies have demonstrated potential levels of interaction between GABA and the NO system in the regulation of seizure susceptibility (Paul, 2003; Paul and Subramanian, 2002). For instance, increased synthesis of NO can decrease GABA-stimulated chloride ion influx by inhibiting $GABA_A$ receptor function (Gholipour et al., 2008; Zarri et al., 1994). In addition, release of endogenous NO participates in the excitatory transmission through NMDA receptors (Riazi et al., 2006), where activation of NMDA-type glutamate receptors causes a reduction in the effect of GABA (Talarek and Fidecka, 2003). Moreover, NMDA receptor blockade or suppression has been shown to decrease susceptibility to seizure development (Ahmed et al., 2005; Ghasemi et al., 2010). Just like the benzodiazepines, PME has shown to possess anticonvulsant activity against convulsions induced by GABA antagonists probably by interacting with $GABA_A$ receptors. The extract has also shown to elicit its anticonvulsant effect probably via an interaction with the NMDA receptor complex. Therefore, the influence of NO modulators in the anticonvulsant activity of PME could possibly be through its interaction with GABA and/or NMDA receptors.

In this study, the neuromuscular tone of animals was evaluated by use of the grip-strength test. This test is a widely-used non-invasive method designed to evaluate mouse limb strength and has been used to investigate the effects of neuromuscular disorders and drugs. It is based on the natural tendency of the mouse to grasp a grid when it is suspended by the tail. PME had no significant impairment effect on skeletal muscular strength. In contrast, diazepam at the highest dose (3 mg kg^{-1}) impaired neuromuscular strength by decreasing the force (N). This effect is in agreement with various reports in which some classical and second generation antiepileptic drugs—diazepam, carbamazepine, valproate, clonazepam, phenytoin, phenobarbital, lamotrigine, oxcarbazepine and topiramate—produced impairment of skeletal muscular strength in mice in a dose-dependent manner (Łuszczki et al., 2008; Zadrozniak et al., 2009).

5. Conclusion

Findings of the present study suggests that administration of *Pseudospondias microcarpa* hydroethanolic leaf extract has anticonvulsant activity and may probably be affecting GABAergic, glycinergic, NMDA, K^+ channels and nitric oxide-cyclic GMP pathways to exert its effect.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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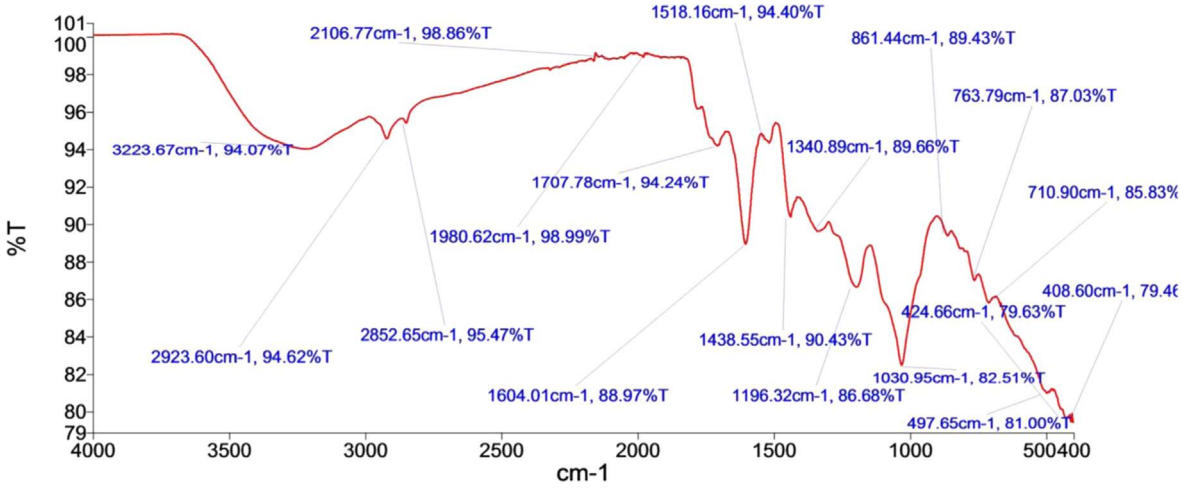
Appendix A



Infrared spectra of the hydroethanolic leaf extract of *P. microcarpa* (PME).

Peak table for IR spectra of the hydroethanolic leaf extract of *P. microcarpa* (PME).

Peak	X (cm^{-1})	Y (%T)	Peak	X (cm^{-1})	Y (%T)	Peak	X (cm^{-1})	Y (%T)	Peak	X (cm^{-1})	Y (%T)
1	3223.67	94.07	**2**	2923.60	94.62	**3**	2852.65	95.47	**4**	2323.74	98.30
5	2162.45	98.77	**6**	2106.77	98.86	**7**	1980.62	98.99	**8**	1780.04	96.23
9	1707.78	94.24	**10**	1604.01	88.97	**11**	1518.16	94.40	**12**	1438.55	90.43
13	1340.89	89.66	**14**	1196.32	86.68	**15**	1030.95	82.51			

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