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Effects of hydroethanolic leaf extract of *Pseudospondias microcarpa* (A. Rich.) Engl. (Anacardiaceae) on the central nervous system in mice

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Abstract

Pseudospondias microcarpa (Anacardiaceae), the African grape tree, is used for various CNS disorders. The neuropharmacological activities of the hydroethanolic leaf extract of *Pseudospondias microcarpa* (PME) were screened in mice. In this study, the central nervous system (CNS) activity was tested in various animal models including Irwin test, pentobarbitone-induced sleeping time, spontaneous motor activity, rotarod test, pentylenetetrazole-induced convulsions and tail immersion test. The extract produced sedation and analgesia in the Irwin test with an LD₅₀ above 3000 mg kg⁻¹. PME potentiated pentobarbitone sleeping time and induced hepatic enzymes. It however, showed no effect on locomotor activity or motor coordination. Furthermore, the extract blocked convulsions induced by PTZ and showed analgesic activity in the tail withdrawal test. The present investigation suggests that PME may act as a sedative with analgesic and anticonvulsant activity, and thus supporting its use as a sedative and for the relief of various nervous system disorders.

Keywords: Analgesia, CNS depressant, Irwin test, Pentobarbitone (PBT), *Pseudospondias microcarpa* (PME).

Introduction

The World Health Organization estimates that about 75% of the world population—primarily those of developing countries—rely on traditional remedies (mainly herbs) for the health care of its people.¹ In Ghana, it is estimated that between 60 and 70% of people rely on traditional medical systems for their health needs.² Considering the great reliance on traditional medicinal plants for treatment of diseases and the potential for drug discovery, it becomes relevant to search for potent, effective and relatively safe plant medicines as well as to scientifically validate success claims about plants already in use by traditional medicine practitioners. As part of our continuing search for plants that act on the CNS, this work focuses on the screening of *Pseudospondias microcarpa* (A. Rich.) Engl. (Anacardiaceae).

Pseudospondias microcarpa is one of such plants used for managing various diseases including CNS disorders. In Ghana it is locally known as *katawani*³ literally meaning ‘close your eyes’ because the tree supposedly has a sedative effect on those who sit or sleep under it. The plant is therefore used in Ghana as a sedative and for treatment of general central nervous system disorders.⁴ Other medicinal uses of the bark and leaves are treatment of arthritis, rheumatism, eye problems, kidney disorders, naso-pharyngeal infections, stomach complaints, malaria and jaundice.⁴ Presence of saponins, phenols, terpenoids, flavonoids, cardiac glycosides and coumarines in both the methanol-methylene chloride and aqueous bark extracts of *P. microcarpa* have been reported.⁵ Also, the leaves contain alkaloids, tannins, terpenoids and steroids.⁶ The plant also possess antimicrobial properties⁷ potent antioxidant effect⁵ as well as anti-plasmodial properties⁸.

Despite the wide use of the plant, there are no data in the literature on its probable CNS activity. The core aim of this study was therefore to provide evidence of possible neuropharmacological properties in experimental animal models.

Materials and Methods

Collection of Plant Material

Fresh leaves of *P. microcarpa* were collected from the campus of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi near the Department of Agricultural Engineering (6° 40.626'N, 1° 34.041'W) during the month of August, 2010 and authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/HM1/2013/L005) was kept at the herbarium of the Faculty.

Plant Extraction

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 in 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 a week and kept in a refrigerator and used when required. The yield was 20.5 % (w/w). In this study the crude extract is subsequently referred to as PME or extract.

Animals

Male ICR mice (20-25 g) were purchased from the Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept in the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The animals were housed in groups of 5 in stainless steel cages (34 cm x 47 cm x 18 cm) with soft wood shavings as bedding and housing conditions controlled—temperature maintained at 24-25 °C, relative humidity 60-70 %, and 12 h light-dark cycle. They had free access to tap water and food (commercial pellet diet, GAFCO, Tema, Ghana). A period of at least one week for adaptation to the laboratory facilities was allowed. The studies 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.

Drugs and Chemicals

Caffeine (CFN), Diazepam (DZP), Pentobarbitone (PBT), Pentylentetrazole (PTZ) and Phenobarbitone (PHE) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Morphine hydrochloride (MOR) was obtained from Phyto-Riker Pharmaceuticals Limited, Accra, Ghana.

Phytochemical Screening

The extract was analyzed for phytochemical constituents for the detection of alkaloids, saponins, reducing sugars, flavonoids, terpenoids, tannins and steroids.⁹

General Pharmacological Observation (Irwin Test)

Male ICR mice were orally treated with the extract (30, 100, 300, 1000 and 3000 mg kg⁻¹) and placed in observation cages (20 × 40 × 22 cm) The mice were evaluated for general pharmacological and physiological behaviours as well as

mortality at 0, 15, 30, 60, 120, 180 min, up to 48 h after treatment as described by Irwin.¹⁰

Activity Meter Test

Locomotor activity of PME was evaluated with the Ugo Basile activity cage (model 7401, Comerio, VA, Italy). Mice were pre-treated orally with extract (as described above), diazepam (8 mg kg⁻¹), caffeine (16 mg kg⁻¹) or saline. After 1 h, the animals were individually placed in the activity meter cage and their activities scored every 5 min for 30 min. Diazepam and caffeine were used as CNS depressant and stimulant agents respectively.

Rotarod Test

Effect of PME on motor co-ordination was assessed with a rotarod apparatus (Ugo Basile, model 7600, Comerio, Milan, Italy). The rotarod consisted of a rotating rod (diameter: 3 cm) with individual compartments for each mouse. Mice were trained for 3 days before the test to stay on the rotating rod (speed 20 rpm) for at least 3 min. On the test day, mice were randomly divided into seven groups: saline-treated control group, PME group (30, 100, 300, 1000 or 3000 mg kg⁻¹, *p.o.*) and diazepam group (8 mg kg⁻¹, *p.o.*). After oral administration of the test compounds, mice were put on the rotarod and the latency until fall during a 3-min session was recorded at 0, 0.5, 1, 1.5 and 2 h.

Pentobarbitone-Induced Sleeping Time

Animals were randomly divided into eight groups (n=8): saline-treated control, PME (30, 100, 300, 1000 or 3000 mg kg⁻¹, *p.o.*), diazepam (8 mg kg⁻¹, *p.o.*) or caffeine (16 mg kg⁻¹, *p.o.*). Sodium pentobarbitone (50 mg kg⁻¹) was intraperitoneally administered 60 min after administration of test drugs. Two parameters were recorded: time elapsed since the application of pentobarbitone until the loss of the righting reflex (latency/onset of action) and the time elapsed from the loss to regaining of the righting reflex (duration of sleep).

Barbiturate Interaction Test

This was done to assess the influence of hepatic enzyme induction on pentobarbitone sleeping time. Mice were pre-treated with phenobarbitone (25 mg kg⁻¹, *i.p.*) for two consecutive days. On the third day, administration of test compounds was repeated as described above and sleeping time determined.

Convulsive Threshold Test (PTZ Seizure Test)

Mice were divided into seven groups (n=10) and received PME (30, 100, 300, 1000 or 3000 mg kg⁻¹, *p.o.*), vehicle or the standard drug diazepam (16 mg kg⁻¹, *p.o.*). One hour after administration of test compounds, animals were injected subcutaneously with a single dose of PTZ (100 mg kg⁻¹). Thereafter, mice were observed 60 min for both clonic and tonic seizures. Clonic seizures were characterized as appearance of facial myoclonus, forepaw myoclonus and forelimb clonus and tonic seizures were characterized as explosive clonic seizures with wild running and tonic forelimb and hind limb extension. The latency for the onset, frequency and duration of the convulsive episodes (clonic or tonic) were

recorded as indicators of pro- or anti-convulsive effect of compounds.

Tail Immersion Test

Tail immersion test, a measure of analgesia, was carried out as described by Janssen *et al.*, 1963, with modifications.¹¹ Male ICR mice were divided into 7 groups of 7 animals each: control, PME (30-3000 mg kg⁻¹, *p.o.*) and morphine (10 mg kg⁻¹, *i.p.*). The tail (up to 3.5 cm) was then dipped into a water bath maintained at 48±0.5 °C. The time in seconds to withdraw the tail out of the water was taken as the reaction time (T). A cutoff latency of 10 s was adopted to avoid tissue damage. Withdrawal latency was taken after 0.5, 1, 2, and 3 h intervals after 30 min (*i.p.*) or 1 h (*p.o.*) following administration of the test drugs.

The percentage maximal possible effect (% MPE) was calculated from the reaction times using the formula:

$$\%MPE = \frac{T_2 - T_1}{T_0 - T_1} \times 100\%$$

where T₁ and T₂ are the pre- and post- drug reaction times and T₀ is the cut-off time.

Statistical Analysis

In all experiments, a sample size of seven to ten (n=7-10) was used. Data are presented as mean ± SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls' test as *post hoc*. In some instances behavioural data were analyzed using two-way ANOVA followed by Bonferroni's test as *post hoc*. GraphPad® Prism for Windows 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. P<0.05 was considered statistically significant for all tests.

Results

Phytochemical Screening

Phytochemical analysis of the extract revealed the presence of alkaloids, saponins, reducing sugars, tannins, terpenoids and flavonoids. Steroids were, however absent.

Table 1: Phytochemical constituents of the ethanolic extract of the leaves of *P. Microcarpa*

Constituent	Results
Tannins	present
Alkaloids	present
Saponins	present
Reducing sugars	present
Terpenoids	present

Irwin Test

Treatment of mice with the extract produced sedation and analgesia at all doses used. No deaths were recorded over the 48 h observation period, indicating an LD₅₀ above 3000 mg kg⁻¹.

Table 2: Effects of *Pseudospondias microcarpa* hydroethanolic leaf extract (PME) in the primary observation test in mice

Dose (mg kg ⁻¹)	Mortality D/T	Effects
0	0/7	No change
30	0/7	Sedation, analgesia
100	0/7	Sedation, analgesia
300	0/7	Sedation, analgesia
1000	0/7	Sedation, analgesia
3000	0/7	Sedation, analgesia

D/T: Dead/Treated

Activity Meter Test

PME had no effect on locomotor activity at all the doses used (30-3000 mg kg⁻¹, *p.o.*). However, diazepam (8 mg kg⁻¹, *p.o.*), a CNS depressant significantly ($F_{2,15}=24.90$, $P<0.0001$) reduced activity in mice whereas caffeine (16 mg kg⁻¹, *p.o.*), a CNS stimulant, increased it.

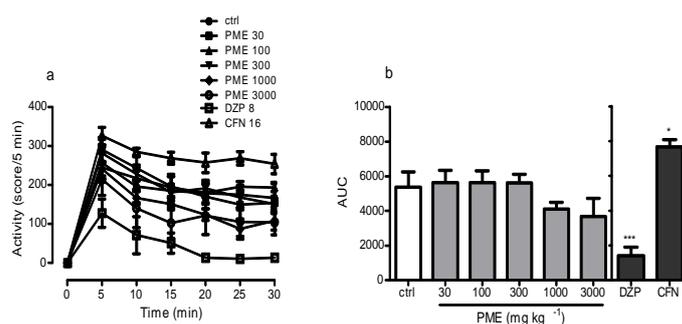


Figure 1: Effects of acute PME (30-3000 mg kg⁻¹, *p.o.*), diazepam (8 mg kg⁻¹, *p.o.*) and caffeine (16 mg kg⁻¹, *p.o.*) treatment in the activity meter test. Data are presented as group mean ± SEM. Analysis by one-way ANOVA followed by Newman-Keuls' *post hoc* test. *P<0.05, ***P<0.001.

Rotarod Test

The extract caused no significant effect on the latency to fall off the rotarod compared to the control at all the doses used ($P>0.05$ at 30-3000 mg kg⁻¹). However, at the dose used diazepam (8 mg kg⁻¹, *p.o.*), significantly decreased the latency to fall off the rotating rod ($P<0.01$).

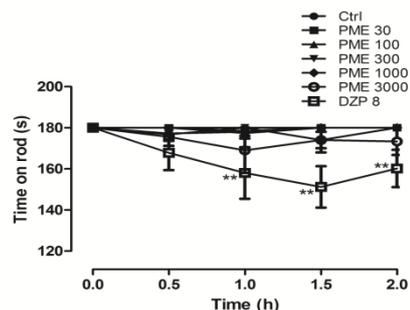


Figure 2: Effect of PME (30-3000 mg kg⁻¹, *p.o.*) and diazepam (8 mg kg⁻¹, *p.o.*) on the time course curve of the rotarod test in mice. Data are presented as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test).

Pentobarbitone-Induced Sleeping Time

Pre-treatment with PME (30-3000 mg kg⁻¹, *p.o.*) significantly decreased the latency to sleep ($F_{5,42}=4.090$, $P=0.0041$) and increased sleeping time ($F_{5,42}=4.86$, $P=0.0013$) induced by sodium pentobarbitone (50 mg kg⁻¹, *i.p.*). Similar to the extract, diazepam (8 mg kg⁻¹, *p.o.*), a CNS depressant, decreased latency to sleep ($F_{2,21}=12.69$, $P=0.0002$) and increased sleeping time ($F_{2,21}=55.15$, $P<0.0001$). In contrast to PME and diazepam, caffeine (16 mg kg⁻¹, *p.o.*), a CNS stimulant, delayed the onset and decreased duration of sleep (both at $P<0.05$).

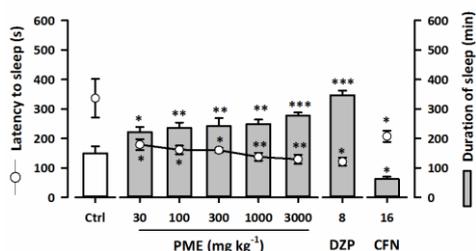


Figure 3: Effects of acute PME (30, 100, 300, 1000 and 3000 mg kg⁻¹, *p.o.*), diazepam (8 mg kg⁻¹, *p.o.*) and caffeine (16 mg kg⁻¹, *p.o.*) in the pentobarbitone-induced sleeping time. Data are presented as group mean ± SEM. Analysis was done by one-way analysis of variance followed by Newman-Keuls' *post hoc* test. Significantly different from control: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Barbiturate Interaction Test

In the phenobarbitone pretreated mice, PME, as revealed by ANOVA significantly increased duration of sleep ($F_{5,42}=3.251$, $P=0.014$). However, Newman-Keuls' *post hoc* analysis showed no statistical significance at all the doses used ($P>0.05$). Diazepam also increased duration of sleep in the phenobarbitone pre-treated mice ($P<0.01$). Caffeine had no effect.

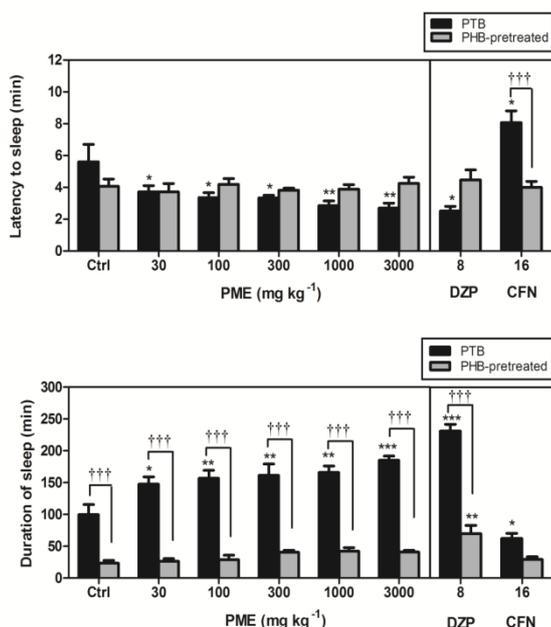


Figure 4: Effects of acute PME (30, 100, 300, 1000 and 3000 mg kg⁻¹, *p.o.*), diazepam (8 mg kg⁻¹, *p.o.*) and caffeine (16 mg kg⁻¹, *p.o.*) in the barbiturate interaction test. Data are presented as group mean ± SEM. Analysis was done by one-way analysis of variance followed by Newman-Keuls' *post hoc* test. Significantly different from control: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and two-way ANOVA followed by Bonferroni's test ††† $P<0.001$.

A two-way ANOVA showed a significant effect in the duration of sleep for PME when pentobarbitone treatment and phenobarbitone pretreatment were compared ($F_{5,84}=6.705$, $P<0.0001$). Similar results were obtained for diazepam ($F_{2,42}=59.54$, $P<0.0001$).

Convulsive Threshold Test (PTZ Seizure Test)

The extract showed significant anticonvulsant activity against PTZ-induced seizures. Pre-treatment of animals with PME (30-3000 mg kg⁻¹, *p.o.*) caused a significant delay in the latency to clonic ($F_{6,56}=67.88$, $P<0.0001$) and tonic ($F_{6,56}=3.636$, $P=0.0041$) convulsions. The frequencies of both clonic ($F_{6,56}=7.761$, $P<0.0001$) and tonic ($F_{6,56}=8.598$, $P<0.0001$) convulsions were also significantly reduced. ANOVA revealed that PME also significantly reduced the duration of both clonic ($F_{6,56}=7.534$, $P<0.0001$) and tonic ($F_{6,56}=6.247$, $P<0.0001$) convulsions. Furthermore, the extract provided 50% (at 30 and 100 mg kg⁻¹) and 60% (300, 1000 and 3000 mg kg⁻¹) protection against PTZ-induced tonic seizures in mice. The standard drug diazepam, at the dose used (16 mg kg⁻¹, *p.o.*) completely abolished convulsions.

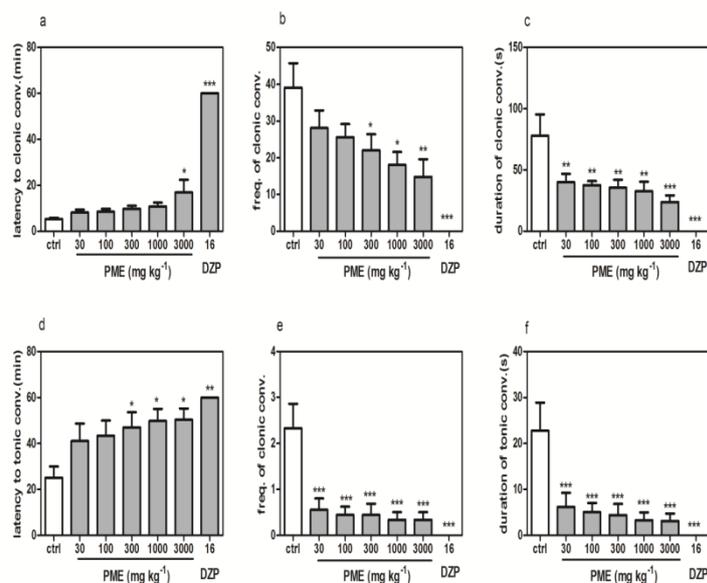


Figure 5: Effects of PME (30, 100, 300, 1000 and 3000 mg kg⁻¹, *p.o.*) and diazepam (16 mg kg⁻¹, *p.o.*) on the latency (a, d), frequency (b, e) and duration (c, f) of clonic and tonic seizures in mice. Data are presented as mean ± SEM. Analysis was done by one-way analysis of variance followed by Newman-Keuls' *post hoc* test. Significantly different from control: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Tail Immersion Test

From the time course curves in figure 6, two-way ANOVA (*treatment x time*) revealed a significant effect of drug treatments on the tail withdrawal latencies calculated as a percentage of the maximum possible effect (% MPE) ($F_{4,209}=14.71$, $P<0.0001$). PME (30-3000 mg kg⁻¹, *p.o.*) significantly increased tail withdrawal latency ($F_{6,42}=4.182$, $P=0.0022$), with a maximal effect at the dose of 1000 mg kg⁻¹. Morphine (10 mg kg⁻¹, *i.p.*), the standard analgesic drug used, also showed a significant increase in the withdrawal latency ($P<0.001$).

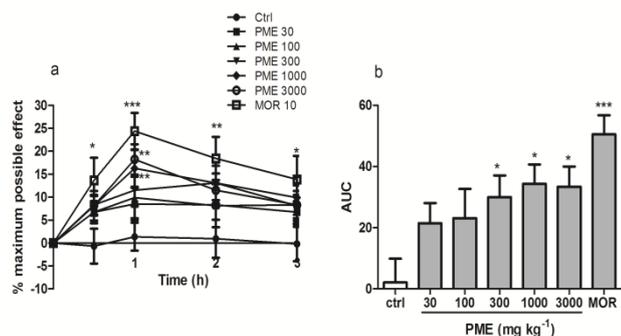


Figure 6: Effect of PME (30-3000 mg kg⁻¹, *p.o.*) and morphine (10 mg kg⁻¹, *i.p.*) on the time course curve (a) of the tail immersion test and the AUC (b) in mice. Data are presented as mean \pm S.E.M. **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test for time course curve or one-way ANOVA followed by Newman-Keuls *post hoc* test for AUC).

Discussion

Results of the present study show that a hydroethanolic leaf extract of the plant possesses CNS depressant, anticonvulsant and analgesic activity without affecting motor coordination in the animal models used. The extract is also metabolized by hepatic enzymes.

Before investigating a new substance in a specific test, substances are usually evaluated in the primary observation procedure originally described by Irwin.¹⁰ This test is used to estimate the general effects of a drug or drug candidate on central nervous system (CNS) activity, minimum lethal dose of a test substance and the primary effects on behaviour and physiological functions. Data from this test are also used to assess the safety pharmacology of drugs.^{10, 12, 13} In this study, mice treated with PME showed signs of sedation and analgesia, suggesting possible central depressant and analgesic effects. Furthermore, presence of sedation in the Irwin test suggests anxiolytic, antipsychotic, or anticonvulsant activity¹³ and thus confirming the traditional use of the plant. In drug discovery and evaluation, it is important to assess the toxicity (minimum lethal dose) of test compounds. In this experiment, the LD₅₀ of the plant extract, given orally, was found to be above 3000 mg kg⁻¹. At the relatively high doses used, the plant extract caused no mortality and appeared to cause no apparent toxicity. This suggests that PME is relatively non-toxic since substances with an LD₅₀ value of 1000 mg kg⁻¹ by the oral route are regarded as being safe or of low toxicity.¹⁴

Barbiturates are putative sedatives inducing sleep in human beings and animals by depressing the CNS.¹⁵ Pentobarbitone potentiates the effect of GABA, acting at the GABA receptor-ionophore complex.^{16, 17} Activation of GABA_A receptors depresses the CNS and favours sleep. Thus, increase or decrease in pentobarbitone-induced sleeping time can be a useful tool for examining influences on the GABAergic system.^{18, 19} In this test, a decrease in sleep latency and an increase in sleeping time are classically related to central nervous system (CNS) depressant drugs.²⁰⁻²² In the present study, it was demonstrated that PME, similar to diazepam, potentiated pentobarbitone-induced sleeping behaviour in mice indicative of a CNS depressant effect. This finding is consistent with the sedation observed in the Irwin test.

In addition to the pentobarbitone-induced sleeping time, pre-treatment with phenobarbitone for two consecutive days shortened the duration of sleep in PME treated mice. This reduction in sleeping time suggests that PME induced cytochrome P450 enzyme activity.^{23, 24} Drug metabolism through the cytochrome P450 enzymes has emerged as one important mechanism in the occurrence of herbal-drug or herbal-herbal interactions, which can result in drug or herbal toxicities.^{25, 26} Thus, the possibility of interactions between PME and other drugs may exist as it is an enzyme inducer.

Locomotor activity is required for many complex behavioural tasks and increases or decreases non-specifically affects performance in many behavioural tests.²⁷ The potential to impair psychomotor functions is one of the most common side effects of widely-used sedatives.²⁸ In this regard, the influence of *P. microcarpa* extract on locomotor activity was assessed in the activity meter test. Generally, CNS stimulation increases, and CNS depression decreases the amount of activity.²⁹⁻³² PME, in contrast to diazepam had no effect on spontaneous motor activity. This indicates that the sedative effect of PME does not impair psychomotor function, giving it an advantage over most of the clinically used sedatives.

Performance of mice on a rotarod is a sensitive, widely used method for assessing balance and coordination aspects of motor function.³³⁻³⁵ Thus, the fore and hind limb motor coordination and balance can be analyzed. This task requires an intact cerebellar function and motor coordination.³⁶ Mice with severe motor coordination problems will have difficulties to remain on the rotating rod. The extract did not alter the time of permanence on the bar in the rotarod test suggesting the absence of impaired motor function. Furthermore, this suggests a possible absence of neurotoxicity and that the inhibitory effect of the extract might be elicited via central mechanisms, not by peripheral neuromuscular blockade.^{37, 38} However, in contrast to PME, DZP decreased this parameter significantly indicating impaired motor coordination. Data in literature suggests that benzodiazepines, such as diazepam, act as anxiolytics (at low doses) and anticonvulsants, producing also a myorelaxant effect at higher doses.^{39, 40} This is therefore not surprising as diazepam produced a myorelaxant effect at the dose used.

The PTZ test is the most frequently used acute chemical experimental model employed in the search for new AEDs.⁴¹ PTZ blocks GABA-mediated Cl⁻ influx through an allosteric interaction in the Cl⁻ channel, thus leading to induction of convulsions in animals.^{42, 43} The GABAergic system has long been implicated in epilepsy. The enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsion respectively.⁴⁴⁻⁴⁶ Defects in GABA neurotransmission are linked to epilepsy in both experimental animal models and human syndromes.^{42, 47} The ability of an agent to prevent or delay the onset of clonic and tonic-clonic convulsions induced by PTZ in animals is an indication of anticonvulsant activity.^{48, 49} In this study, acute administration of PME and the benzodiazepine diazepam, exhibited anticonvulsant activity against PTZ-induced seizures by significantly and dose-dependently delaying the occurrence of seizures. In addition, they decreased the frequency and duration of both clonic and tonic convulsions in mice. The potent effect of diazepam as evident in the PTZ-induced

convulsions agrees with its enhancing effects in GABAergic neurotransmission.⁵⁰

In the tail immersion test, PME caused a prolonged latency period, indicating an increase in the nociceptive threshold. This test is able to differentiate between central opioid-like analgesics and peripheral analgesics.^{51, 52} The response to the tail-immersion test is a spinal reflex, but may also involve higher neural structures.^{53, 54} The antinociceptive effect of PME in this test is a further confirmation of analgesia observed in the Irwin test.

The presence of many biologically active phytochemicals such as flavonoids, triterpenes, alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties.⁵⁵⁻⁵⁸ Screening of PME showed the presence of flavonoids, tannins, saponins, glycosides, terpenoids and alkaloids. The presence of these phytochemicals could therefore be responsible for the observed pharmacological activities of the extract in the various animal models used. Previous research has showed that plants containing flavonoids, saponins and tannins are useful in many CNS disorders.⁵⁹⁻⁶¹

Conclusion

The present study concludes that the extract possesses CNS depressant, analgesic and anticonvulsant activity without affecting motor function.

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