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Pergularia daemia aqueous extract ameliorates seizures and prevents oxidative stress in mice model of epilepsy

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ABSTRACT

Pergularia daemia Forsk. (Choiv.) (Asclepiadaceae) is widely used in Cameroonian's folk medicine to treat epilepsy and infantile convulsions. In the present study, anticonvulsant effects of *Pergularia daemia* aqueous extract and possible antioxidant mechanisms were investigated on pentylenetetrazole (PTZ)-induced kindling model of epilepsy. All groups, excluding control group were kindled by 11 injections (i.e. 22 days) of PTZ (35 mg/kg, i.p.), one time every alternate day (48 ± 2 h), until the development of kindling (i.e. the manifestation of stage 5 of seizures for two successive trials in control animals). On the 26th day (i.e. the 12th injection), all mice were challenged with PTZ (75 mg/kg, i.p.). Following the completion of behavioral studies, hippocampi were removed and oxidative stress parameters were determined. *P. daemia* extract (24.5-49 mg/kg) significantly protected mice against myoclonic jerks and clonic seizures. The extract (12.3-49 mg/kg) significantly decreased the number of myoclonic jerks and development of PTZ kindling. PTZ-kindling induced significant oxidative stress alterations that were reversed by the extract. These results suggest that *P. daemia* has anticonvulsant effects facilitated in part by antioxidant activities. This clarifies consequently, its use in traditional medicine to treat epilepsy in Cameroon.

INTRODUCTION

Epilepsy is one of the major neurological complaints which involve nearly 1% of world population [1]. Epilepsy is due to abnormal recurrent and spontaneous electrical discharge of a group of neurons in the brain [2]. Despite the effective treatment of epilepsy with antiepileptics, approximately one third of patients with epilepsy are resistant to current pharmacological treatments and also have side effects [3]. In patients whose drug therapy is successful, existing antiepileptics are purely symptomatic and do not prevent the progression of the disease or the antecedents expected from epilepsy [4]. Therefore, it is necessary to develop treatments modifying the disease, stopping the progression of epilepsy. A cascade of biological procedures triggers the progression of epilepsy [5]. The generation of reactive oxygen species in the brain is measured as one of the main origins of persistent convulsions [5]. Oxidative stress and mitochondrial dysfunctions have long been recognized as major mechanisms in various neurological conditions. Therefore, recurrent seizures cause overproduction of mitochondrial superoxide radicals in rodent paradigms that can be converted into hydroxyl radicals [6]. The hydroxyl radical in the presence of Cu^{2+} and Fe^{2+} ions easily oxidizes proteins, lipids and DNA, resulting in altered protein function, membrane permeability and gene expression, respectively [7]. These events increase seizure threshold and increase in the neuronal excitability [7]. PTZ kindling is widely recognized as an experimental animal model to evaluate the efficacy of antiepileptic drugs [8]. Kindling is repetitive chemical or electrical stimulation of brain that initially does not induce seizures, but decrease the seizure threshold that ultimately leads to the incidence of seizures [9]. Kindled seizures have been shown to cause neuronal damage in limbic structures CA1, CA3, hippocampus, dentate gyrus of amygdala and entorhinal cortex [9]. According to WHO, about three-quarters of the world's population depends on medicinal plants [10]. Among these, the African and Asian tropical/subtropical plant *Pergularia daemia*

(Forsk.) Chiov. (Asclepiadaceae) (*P. daemia*) is used in African and Indian traditional medicine to treat leprosy, poisoning, asthma, anemia, seizures and mental disorders [11, 12]. In Northern Cameroon, traditional healers use decoctions of the roots of *P. daemia* to treat inflammatory disorders, malaria, mental disorders, febrile seizures, and epilepsy [13]. Whole plant extracts have been reported to have various pharmacological properties, including hepatoprotective, antidiabetic, analgesic, anti-inflammatory, antioxidant, antipyretic, analgesic and sedative activities [11, 12]. Phytochemically, the extract of *P. daemia* have been investigated for cardenolides, alkaloids, flavonoids, saponins, triterpenes, tannins and steroidal compounds [14]. These properties of *P. daemia* suggest its potential as a substance for the prevention of seizures that are more difficult to treat. Therefore, the objective of this study is to evaluate the effects of chronic administration of *P. daemia* on seizures and oxidative stress induced by PTZ-kindling.

MATERIALS AND METHODS

Plant material and extract preparation

The Fresh roots of *P. daemia* used were harvested in the department of Mayo-Tsanaga (Far-North Region of Cameroon), during the dry season (March 2014). The species were authenticated at the National Herbarium of Yaoundé (Cameroon), where a voucher was deposited (Sample Nº 7797/SRF/Cam). The roots were peeled-off, cut into fragments and allowed to dry at room temperature. The dried roots were then crushed into powder. The powder (5 g) was boiled in 75 ml of distilled water for 20 min at 70°C. After it cooled, the extract was collected and filtered with Whatman Nº1 filter paper. The filtrate, considered as the stock solution, was diluted in distilled water to obtain less concentrated solutions. The yield of the extraction was 7.34%. Thereafter, the dose of the stock solution was 49 mg/kg. Less concentrated solutions of the extract were obtained by dissolving 1/2, 1/4 and 1/10 of the stock solution in distilled water. Thus, the following doses were respectively obtained: 24.5, 12.3 and 4.9 mg/kg.

Chemicals

Vitamins C (VIC), PTZ and sodium valproate (SVA) were purchased from Sigma Chemical Co., St. Louis (USA). PTZ, VAS and VIC were injected intraperitoneally (i.p.), while *P. daemia* extract and distilled water were administered *per os* (*p.o.*) at a dose volume of 10 ml/kg.

Animals

The experiments were conducted on Swiss albino mice weighting 18-29 g of either sex were used in this study (Lanavet Garoua, Cameroon). All animals from our own breeding colony (Animal House-holding, University of Ngaoundere, Cameroon) were housed in a controlled environment, with free access to food and water available *ad libitum*. They were maintained on a 12 h/12 h day/night cycle (lights on at 7:00 a.m.). The investigation follows to the Guide for the Care and use of Laboratory Animal issued by the US National Institutes of Health (NIH, No. 85-23, revised 1996) and European Communities Council Directive (EEC, No. 86/609, 24 November 1986). The study was also conducted in accordance with the Cameroon National Ethical Committee (Ref No. FW-IRB00001954, 22 October 1987). Each animal was used once and handled according to regular protocols for the use of laboratory animals.

Kindling procedure

The mice were randomized into eight groups of seven mice each as follows: group I (control group) received distilled water; group II (PTZ group) received distilled water, groups III, IV, V and VI received *P. daemia* (4.9, 12.3, 24.5 and 49 mg/kg), groups VII and VIII received SVA (300 mg/kg) or VIC (250 mg/kg). Then all mice, except control group, were kindled by a total of 11 period injection of PTZ (35 mg/kg), on alternate day (48 ± 2 h) up to day 22 (i.e. the 11th injection). The animals were considered to be kindled after having received 11th PTZ injections and having reached at least two consecutive stage 5 seizures (control animals used as reference). Treatments were also administered on alternate days, 30 min before PTZ administration. On the 26th day (i.e. 12th injection), mice (including control group) were challenged with PTZ (75 mg/kg, i.p.). The challenge dose injection of PTZ produced seizures (clonic and tonic). Clonic seizures are characterized by periodic contractions of forelimbs and/or hind limbs. A tonic seizure comprised of a inflexible extension of the front and/or hind limbs with or without loss of posture. Mice were placed in insulated cages and the displayed stages of seizure (0-5) were observed for 30 min after PTZ injection and classified using the following scale [15]. The scale presents five stages as follows [16] : stage 0: no response; stage 1: hyperactivity, vibrissae twitching; stage 2: head nodding, head clonus and myoclonic jerks; stage 3: unilateral forelimb clonus; stage 4: rearing with bilateral forelimb clonus; stage 5: generalized tonic-clonic seizure with loss of righting reflex. Animals were observed for 24 h mortality.

Biochemical tests

After the behavioral tests, mice were killed by cervical dislocation under diethyl ether (8%, v/v) anesthesia. After decapitation, the brain of each mice was rapidly dissected out and cleaned with ice-cold saline (0.9%, w/v) to remove hippocampi [17]. The hippocampi were weighed and maintained at −43°C until analyzed.

Tissue preparation

For all test procedures, 10% (w/v) homogenate was processed, with ice-cold (0.9%) 0.1 M phosphate buffer (pH 7.4). The homogenate was then centrifuged at 10 000 rpm for 15 min at 0°C. The aliquots of the supernatant were seperated and used for biochemical evaluations.

Determination of malondialdehyde level (MDA)

The method of Wilbur et al [18] was used for MDA determination. Briefly, in the control vial, 250 µl of distilled water were introduced. In the test vials, 20 µl of homogenate, 250 µl of Tris-HCl buffer (50 mM, pH 7.4), 500 µl of trichloroacetic acid (20%) and 1000 µl of thiobarbituric acid (0.67%) were added. The mixture was heated in a water-bath (90°C for 10 min). After cooling to room temperature, the tubes were centrifuged at 3000 rpm for 15 min. The absorbance of the pink colored supernatant was detremined at 530 nm against the blank. The MDA concentration was calculated using an extinction coefficient of 1.56×105 $mmol^{-1}cm^{-1}$. MDA level was expressed in term of µmol/g of protein in the hippocampus tissue.

Estimation of reduced glutathione (GH)

GH was measured according to the method of Ellman [18]. In 100 µl of homogenate (test vial) or 100 µl of Tris-HCl buffer (50 mM, pH 7.4) (blank vial), 1500 µl of 5′5-dithiobis-2-nitrobenzoic acid and 500 µl of Tris-HCl buffer (50 mM, pH 7.4) were added. The mixture was incubated for 1h, and the optical density was read at 412 nm against the blank. The GH concentration was

calculated using an extinction coefficient of 13600 $mol^{-1}cm^{-1}$. The concentration of GH was expressed as µmol/g of protein in the hippocampus tissue.

Data analysis

Data were expressed as mean ± standard error of the mean (S.E.M.) per group. Statistical differences between control and treated groups were tested by the one-way measures analysis of variance (ANOVA). When the difference exists, control groups were compared to the treated groups by Student-Newman-Keul's (SNK) as *post-hoc* test. The percentages of protection against seizures were measured and Fisher's exact test (two-tailed) was used to compare percentages of protection. Analyses were performed using Graph Pad Prism version 5.01 for Window (Graph Pad Software, San Diego California, USA) and XLStat, 2007. The differences were considered significant at $p < 0.05$.

RESULTS

Effect of *P. daemia* on the development of PTZ kindling

Repeated administration of PTZ in mice treated with distilled water induced a significant ($p < 0.001$) decrease in the latency up to 14 ± 0.00 days and 17.71 ± 0.92 days, respectively for clonic seizures and generalized tonic-clonic seizures compared to control group (Figure 1). The extract of *P. daemia* resulted in a moderate ($p < 0.05$) increase in the clonic seizure latency. This time increased from 14 ± 0.02 days in the PTZ group to 18.66 ± 0.44 days (24.97%) in dose 49 mg/kg. Similarly, the latency time of generalized tonic-clonic seizures, increased up to 22 ± 0.00 days (36.30%) ($p < 0.05$) at the same dose (Figure 1). Conversely, the dose 24.5 prevented ($p < 0.05$) development of clonic and generalized tonic-clonic seizures. Sodium valproate and vitamin C induced a non-significant increase in the latency time of clonic seizures and generalized tonic-clonic seizures (Figure 1).

Effects of *P. daemia* on the incidence of seizures in PTZ-kindled and -challenged mice

Administration of distilled water to PTZ group didn't protect mice against seizures induced by PTZ compared to control group (Figure 2). The extract of *P. daemia* protected 100% ($p < 0.001$) and 71.43% ($p < 0.01$) of mice against clonic seizures, at respective doses of 24.5 and 49 mg/kg (Figure 2). *P. daemia* protected 100% of mice ($p < 0.001$) against generalized tonic-clonic seizures at the dose 24.5 and 49 mg/kg (Figure 2). Sodium valproate protected 57.14 ($p < 0.05$) and 100% ($p < 0.001$) of mice against clonic seizures and generalized tonic-clonic seizures, respectively. Vitamin C protected 85.71% ($p < 0.01$) of mice against generalized tonic-clonic seizures (Figure 2). The extract induced non significant protection against seizures induced by PTZ challenge in all treated groups compared to control (Figure 2).

Effects of *P. daemia* on the number of seizures in PTZ kindled mice

Chronic administration of PTZ in distilled water-treated mice resulted in a significant ($p < 0.001$) increase in the number of myoclonic jerks up to 11.57 ± 0.65 compared to control group [$F(7, 56) = 74.87$, $p < 0.0015$] (Figure 3). The extract of *P. daemia* induced a significant ($p < 0.001$) decrease in the number of myoclonic jerks up to 5.71 ± 0.58 ($p < 0.01$) and 4 ± 0.39 ($p < 0.01$), respectively at doses 12.3 and 24.5 mg/kg. The dose 49 mg/kg of the extract decreased the number of myoclonic jerks up to 2 ± 0,34 ($p < 0.001$) (Figure 3). Sodium valproate significantly ($p < 0.01$) decreased the number of myoclonic jerks up to 4.75 ± 0.36. Vitamin C also significantly ($p < 0.01$) decreased the

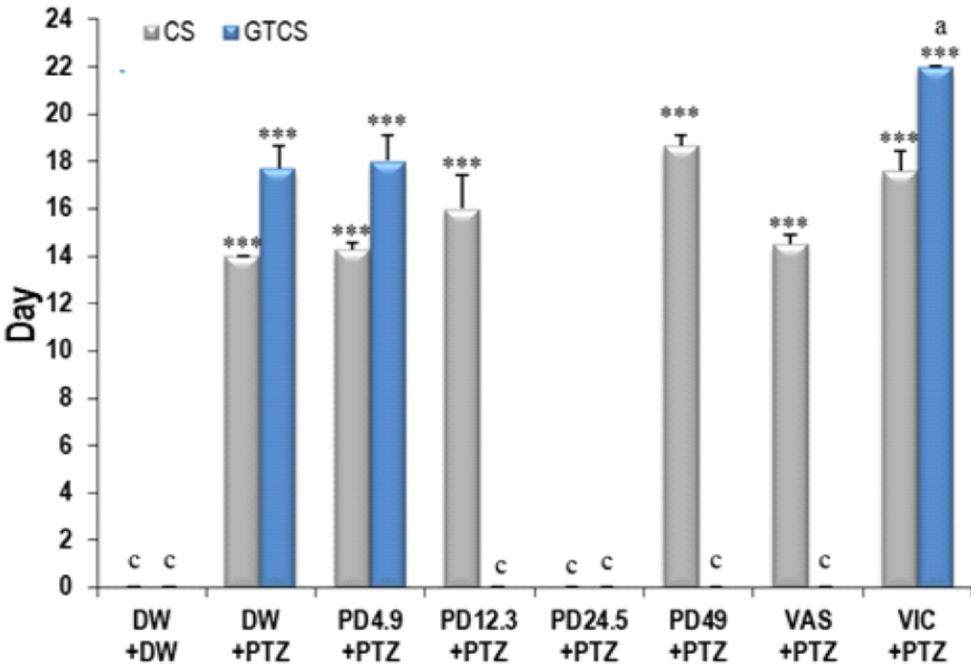

Fig 1 : Effects of *P. daemia* on the development of PTZ kindling. Data are mean ± SEM, N = 7 per group. Newman-Keuls *post hoc* test: (*i*) vs. PTZ control group (DW+PTZ): $^{a}p < 0.05$, $^{c}p < 0.001$ (*ii*) vs. Control animals receiving distilled water (DW+DW): $^{***}p < 0.001$. **DW**: distilled water; **PD**: *Pergularia daemia*; **PTZ**: pentylenetetrazole; **VIC**: vitamin C; **VAS**: sodium valproate; **CS**: clonic seizures; **GTCS**: generalized tonic-clonic seizures.

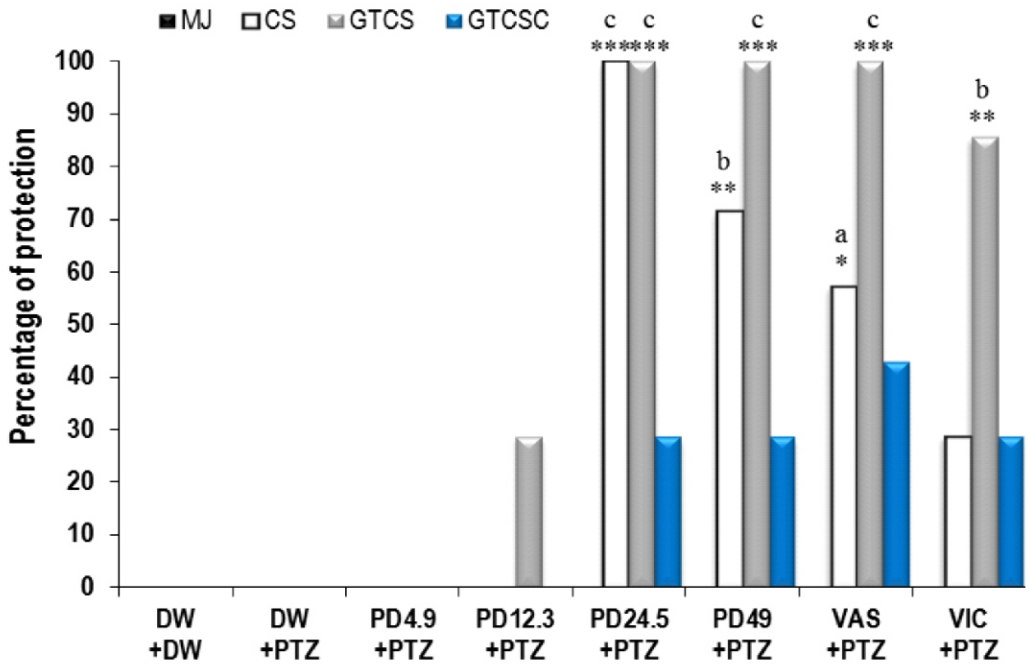

Fig 2 : Effects of *P. daemia* on the incidence of seizures in PTZ- kindled and -challenged mice. Data are mean ± SEM, N = 7 per group. Newman-Keuls *post hoc* test: (*i*) vs. PTZ group (DW+PTZ group): $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ (*ii*) vs. control group (DW+DW group): $^{***}p < 0.001$. **DW**: distilled water; **PD**: *Pergularia daemia*; **PTZ**: pentylenetetrazole; **VIC**: vitamin C; **VAS**: sodium valproate; **MJ**: myoclonic jerks; **CS**: clonic seizures; **GTCS**:; GTCSC: generalized tonic-clonic seizures induced by the challenge challenge.

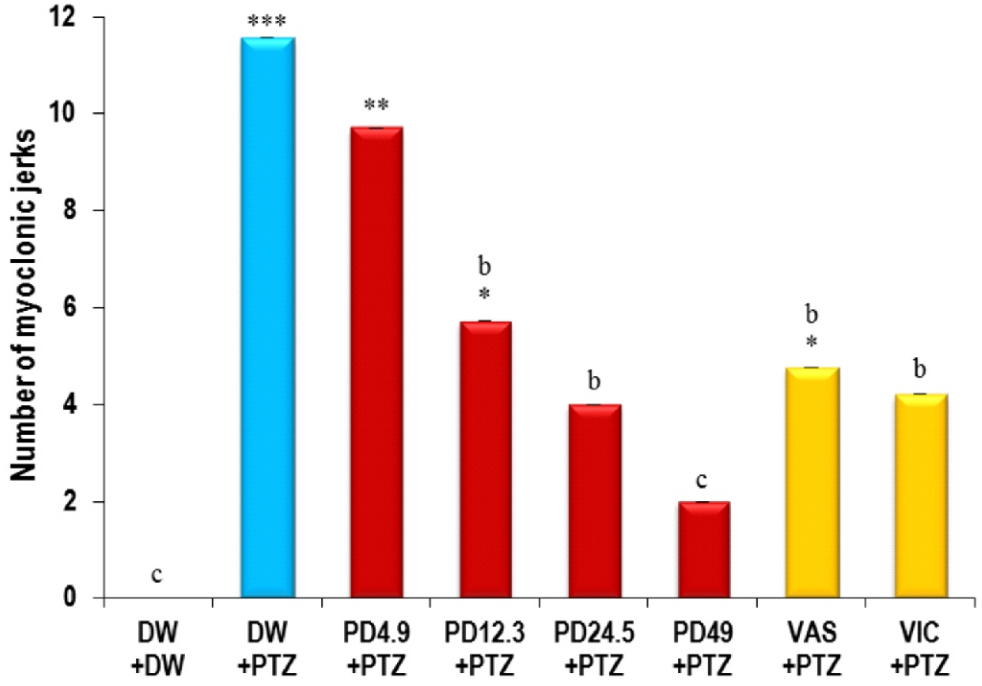

Fig 3 : Effects of *P. daemia* on the number of myoclonic jerks in PTZ kindled mice. Data are mean ± SEM, N = 7 per group. Newman-Keuls *post hoc* test: (*i*) vs. PTZ group (DW+PTZ group): $^{b}p < 0.01$, $^{c}p < 0.001$ (*ii*) vs. control group (DW+DW group): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **DW**: distilled water; **PD**: *Pergularia daemia*; **PTZ**: pentylenetetrazole; **VIC**: vitamin C; **VAS**: sodium valproate.

Table 1 : Effects of *P. daemia* on oxidative stress parameters in PTZ-kindled mice

Treatment	Dose (mg/kg)	Oxidative stress parameters	
		MDA (µmol/g)	GH (µmol/g)
DW + DW	– + –	1.49 ± 0,12b	13.69 ± 0.05b
DW + PTZ	– + 35	3.63 ± 0.03**	7.92 ± 0.01**
PD + PTZ	4.9 + 35	1.99 ± 0.04b	12.30 ± 0.15a
PD + PTZ	12.3 + 35	1.83 ± 0.03b	13.99 ± 0.19b
PD + PTZ	24.5 + 35	1.81 ± 0.04b	16.81 ± 0.17*c
PD + PTZ	49 + 35	0.91 ± 0.07c	13.92 ± 0.11b
VAS + PTZ	300 + 35	2.63 ± 0.03*a	15.04 ± 0.14b
VIC + PTZ	250 + 35	1.28 ± 0.05c	15.59 ± 0.22b

Results are expressed as mean ± S.E.M for the oxidative stress in the hippocampus, N = 7 per dose. Newman-Keuls *post hoc* test: (*i*) vs. PTZ group (DW+PTZ group): $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ (*ii*) vs. control group (DW+DW group): $^{*}p < 0.05$, $^{**}p < 0.01$. **DW**: distilled water; **PD**: *Pergularia daemia*; **PTZ**: pentylenetetrazole; **VIC**: vitamin C; **VAS**: sodium valproate; MDA: malondialdehyde; GH: reduced glutathione.

number of myoclonic jerks up to 4.20 ± 0.17 (Figure 3).

Effects of *P. daemia* on oxidative stress induced by PTZ kindling

Table 1 shows that alternate administration of PTZ in mice treated with distilled water resulted in a significant [F (7, 56) = 213, $p < 0.0001$] increase of the MDA level up to 3.63 ± 0.03 µmol/g ($p < 0.01$) compared to control group. *P. daemia* at all doses resulted in reduced lipid peroxidation induced by PTZ (Table 1). Thus, the most effective doses (24.5 and 49 mg/kg), respectively reduced the MDA level up to 1.81 ± 0.04 µmol/g (49.58%) ($p < 0.01$) and 0.91 ± 0.07 µmol/g (74.93%) ($p < 0.001$). Sodium valproate and vitamin C respectively decreased the level of MDA up to 2.63 ± 0.03 µmol/g (27.54%) ($p < 0.05$) and 1.28 ± 0.05 µmol/g (64.74%) ($p < 0.001$) (Table 1).

Table 1 shows that repeated administration of PTZ in mice treated with distilled water caused a significant [F (7, 56) = 221.2, $p < 0.0001$] decrease in the level of GH. This level decreased from 13.69 ± 0.05 µmol/g ($p < 0.01$) in the control group to 7.92 ± 0.01 µmol/g in the PTZ group. *P. daemia* induced significant increase in the level of GH. The most effective dose of the extract (24.5 mg/kg) significantly ($p < 0.001$) increased the GH level up to 16.81 ± 0.17 µmol/g (52.89%) (Table 1). Sodium valproate and vitamin C respectively increased the GH level up to 15.04 ± 0.14 µmol/g (47.34%)($p < 0.01$) and 15.59 ± 0.22 µmol/g (49.19%)($p < 0.01$) (Table 1).

DISCUSSION

In the present study, administration of PTZ on alternate days induced severe seizures and rapid development of kindling in distilled water-treated mice. These results are consistent with those of Mehla et al. [8]. Administration of *P. daemia* prevented these effects. In deed it significantly inhibited the development of kindling, reduced the number of myoclonic jerks and protected mice against seizures. These effects were more marked that those of sodium valproate an antiepileptic drug. It is well-known that PTZ-kindling seizures induces a long-lasting decrease of the gamma amino butyric acid (GABA) function in the brain [9]. Since, PTZ exerts its action via blockade of $GABA_A$ receptor complex [19]. It is also known that N-methyl-D-aspartate plays a role in the PTZ-induced kindling [20]. In addition, the protection provided by sodium valproate on PTZ-induced kindling is well established [21, 22]. Previous studies demonstrated that sodium valproate increases the brain GABA level via several mechanisms including, the inhibition of GABA-transaminase (enzyme that break down GABA) activity, the stimulation of glutamate decarboxylase (enzyme that enhances GABA release) activity, etc. [23]. Altogether, these results suggest that *P. daemia* has anticonvulsant effects mediated via GABAergic or glutamatergic neurotransmission. Furthermore the bioactive molecules from the roots could account for these effects. In fact, saponins, alkaloids, flavonoids, kaempferol are recognized to induce anticonvulsant effects via different interactions comprising, the potentiation of $GABA_A$ receptor complex, the increase of GABA concentration, etc [11, 12]. Further studies need to be done in order to determine mechanisms involved in the realization of these properties.

All current antiepileptic drugs act only on the symptoms of epilepsy, rather than underlying natural causes [4]. Thereby, there is a need to develop drugs that target the underlying mechanisms of seizures. The present findings revealed that the PTZ challenge induced more severe seizures in distilled water-treated mice when compared to control group. This observation demonstrates that PTZ-kindling makes animals more prone to seizures, because of structural and biochemical alterations (decrease in seizure threshold) [8]. The PTZ challenge is used to screen drugs that interfere with natural causes of seizures (pharmacoresistant drugs) [8]. Thus this test differentiates drugs acting on symptoms, rather than those acting on the causes of seizures. In this experiment, *P. daemia* extract induced a non significative protection against PTZ challenge-induced seizures. These effects were more effective than vitaminc C and valproate sodium (alters epileptogenesis) [8]. May be these effects are the consequence of synergetic action of bioactives molecules of the extract. These results suggest that *P. daemia* act partially on underlying

mechanisms of seizures [24]. Another experiments need to be done in order to confirm this results

Among causal mechanisms of seizures, oxidative stress contributes partially to the development and pathogenesis of seizures [25, 26]. PTZ is known to induce oxidative stress which may be partially responsible for seizures [27]. In this study, PTZ-kindling increased the MDA level (end product of free radical generation) in distilled water-treated mice [28]. *P. daemia* however prevented the rise in the MDA level. The significant decrease in the MDA level indicates an attenuation of lipid peroxidation. A significant decrease in the GH level was also observed in PTZ group. GH (free radical scavenger) plays an important role in protecting cells against oxidative damage as a free radical scavenger [29]. Therefore, this result shows that free radicals triggered by PTZ kindling have probably depleted the GH level [29]. Treatment with *P. daemia* caused a significant increase in the GH level. These data suggest that *P. daemia* protects cells against harmful effects of free radicals and reactive oxygen species. These findings are in accordance with a recent study indicating free radical scavenging and antioxidant capacity of the ethanolic extract from the roots of *P. daemia*. Indeed, *P. daemia* inhibited lipid peroxidation and oxidative DNA damage, and increased GH level [14]. Previous phytochemical studies of *P. daemia* revealed the presence of polyphenolic compounds [11, 12]. Given that polyphenolic compounds have antioxidant properties, the antioxidant effects of *P. daemia* may be related to the presence of polyphenolic compounds [30].

CONCLUSIONS

In conclusion, the present study demonstrates that *P. daemia* extract ameliorated PTZ-induced seizures and prevented oxidative stress in mice, thereby showing the promise of *P. daemia* as a possible therapeutic agent. This study thus suggests the potential of *P. daemia* as an adjuvant to antiepileptic drugs in patients with temporal lobe epilepsy or drug resistant epilepsy.

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Competing interest

The authors have no conflicting interests.

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