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Full Length Research Paper

Anticonvulsant and antioxidant effect of hydro-alcoholic extract of *Cyperus rotundus rhizome* on pentylentetrazole-induced kindling model in male mice

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Regarding high incidence of epilepsy in human society and with respect to insufficient therapies, in the present study, anticonvulsant effect of *Cyperus rotundus* extract was experimentally examined. Sixty male mice were randomly selected and divided into 6 groups; 1. Control, 2. Pentylentetrazole (PTZ)-kindled mice, 3. positive control group which received valproate (100 mg/kg) as anticonvulsant drug, and 4 to 6 which received *C. rotundus rhizome* extract at three doses of 100, 200 and 400 mg/kg; i.p). All groups except for control group were kindled by 11 injections of PTZ (35 mg/kg; i.p) with an interval of 48 h. In the 12th injection, all groups except for control group were tested for PTZ challenge dose (75 mg/kg). The exhibited phases of seizure (0-6) were observed and noted for 30 min after PTZ injection. At last, all brains of mice were removed and then malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO) levels of brain tissues were determined. Data analysis showed that the hydro-alcoholic extract of *C. rotundus* could reduce intensity and duration of seizure. Also, the extract could increase the level of SOD and NO and decrease MDA level in mice brain. It is concluded that *C. rotundus rhzome* extract, probably via its antioxidant properties could have exerted a potent antiepileptic effect.

Key words: *Cyperus rotundus,* epilepsy, pentylentetrazole (PTZ), superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO).

INTRODUCTION

Epilepsy is one of the most prevalent neurological disorders which engage about 0.5 to 1% of world population (Michael-Titus et al., 2007). Epilepsy is due to abnormal recurrent and spontaneous electrical discharge of a group of neurons in the brain and exhibits itself as a seizure occurrence in the patients (Chawla et al., 2002). Glutamate and γ-aminobutyric acid (GABA) are two important excitatory and inhibitory neurotransmitters in the epilepsy (Bernard et al., 2003; Krivoshein and Hess, 2006). In spite of generally good treatment of epilepsy by anticonvulsant drugs, about one-third of this population suffers from un-prevented neurological changes induced by epileptic seizure and also exhibits some accompanied side effects. The long time seizure-induced neuronal activity might result in neurological changes and finally is ended by neuronal death (Ilhan et al., 2005). Oxidative stress and free radicals production are of the most important mechanisms by which neurological disorders such as epileptic seizure occurs (Oliver et al., 1990; Rauca et al., 1999). Nitric oxide (NO) is known as a neurotransmitter in the brain that has shown paradoxical role in the seizure modulation, as an inhibitor (Buisson et al., 1993; Theard et al., 1995) or promoter (Osonoe et al., 1994; Nidhi et al., 1999) in different cases. The final product of lipid peroxidation is MDA and MDA level could be considered as an index of lipid peroxidation. Increased level of MDA as an index of lipid peroxidation in the PTZ

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mice may lead to conclude that free fatty acids and free radicals are made from membrane phospholipid metabolism. SOD is an intracellular antioxidant enzyme that catalyses converting of peroxidase to hydrogen peroxide (H_2O_2) to protect the cell from superoxide radical and oxidative stress.

Cyperus rotundus (coco-grass, red nut sedge) is a species of sedge (Cyperaceae), a perennial plant which is distributed throughout Iran (Zargari, 1991). Roots and rhizomes of this plant are used in different diseases like nausea, fever and inflammation, for pain reduction, for muscle relaxation and many other disorders (Dilipkumar et al., 2009). The main active substances which have been identified in *C. rotundus* include: α-cyperone, β-selinene, cyperene, cyperotundone, patchoulenone, sugeonol, kobusone, and isokobusone that may scientifically explain its folk- and alternative-medicine uses (Lawal and Oyedeji, 2009). In Iranian traditional medicine, *C. rotundus rhizome* has been used for nausea, inflammation and epilepsy (Zargari, 1991). Experimental reports have shown the potent antioxidant and free radical scavenger activity of the plant (Yazdanparast and Ardestani 2007; Kilani-Jaziri et al., 2009). Therefore, in the present study we have investigated the antiepileptic effect of *C. rotundus* extract via assessing the anticonvulsant property of the plant. We have also tried to consider the antioxidant effect of the plant via assessment of MDA, NO and SOD in a kindling model. We have also compared anticonvulsant and antioxidant effect of *C. rotundus* with valproate as a standard drug for epilepsy.

MATERIALS AND METHODS

Animals

In this experimental research, a total of 60 male Albino mice weighing 20 to 25 g (Razi Institue, Iran) were randomly divided into six groups including 1. control, 2. PTZ, 3. positive control (PTZ and valproate 100 mg /kg; i.p. as an anticonvulsant drug), and 4-6-treatment groups which received the extract in three doses of 75, 150 and 300 mg/kg; i.p respectively. Ten mice were housed in each cage at a temperature of 21 ± 2°C and on 12 h light-dark cycles. The mice had free access to standard food and tap water *ad libitum*. The experimental protocol was approved by the Ethic Committee of Shahed University.

Kindling procedure

All animals except for control group (group 1) were kindled by a total of 11 period injection of PTZ (35 mg/kg; i.p). Each administration was carried out every second day and for a period of 22 days. The challenge dose of 75 mg/kg PTZ was injected to kindled mice on day 26 (test day). The challenge dose injection of PTZ produced convulsions (clonic and tonic) and lethality. All kindled mice were tested for PTZ challenge dose (75 mg/kg)-induced seizures and status. However, the exhibited phases of seizure (0-6) were observed and categorized using following scale (Atilla et al., 2006) for 30 min after PTZ injection. The scale introduces six phases as follows:

I. 0: no response.
II. 1: ear and facial twitching.
III. 2: convulsive waves axially through the body.
IV. 3: myoclonic body jerks.
V. 4: generalized clonic convulsions turn over into side position.
VI. 5: generalized convulsions with tonic extension episode and status epilepticus.
VII. 6: mortality.

Preparation of plant hydro-alcoholic extract

C. rotundus rhizome was provided from the local market and was scientifically identified by the department of Botany of Shahed University. To prepare the hydro-alcoholic extract, using percolation method; 50 g of cleaned rhizome was crushed and mixed at a ratio of 1 to 5 with ethanol 80% and kept for 24 h at room temperature. Then the solution was transferred to a percolator and then the deposit was separated using paper filter. The tab of percolator has been opened somehow that velocity of solution be hold at 2 to 3 drops per min. After filtration it was maintained in a water bath at 40°C for 16 h to let the alcohol be evaporated from filtered solution to reach a final concentration of 25%.

Sample preparation and biochemical assays

After the injection of challenge dose of PTZ and behavioral analysis, mice were decapitated. The brains were removed quickly and were washed in cold saline two times. They were placed in freezer (-30°C) in a glass bottle (less than 10 h). Then the brain pieces (cutting the brain tissue using the scissors) were homogenized using four times ice-cold Tris-Hcl buffer (50 mM, PH 7.4) for two minutes at 5000 rpm. MDA and NO levels were measured at this phase. The homogenized solution was then centrifuged for 60 min at 5000×g to remove debris. The supernatant solution was then extracted with a mixture of ethanol/chloroform (a volume with ratio of 5:3). After centrifugation at 5000×g for 30 min, the clear upper layer (the ethanol phase) was taken and used for evaluation of the SOD activity. All experiments were carried out at +4°C.

MDA evaluation

The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured according to the following protocol. Briefly, trichloroacetic acid and TBARS reagent were added to supernatant, then mixed and incubated at 100°C for 80 min. After cooling on ice, samples were centrifuged at 1000×g for 20 min and the absorbance of the supernatant was read at 532 nm (Fernandez et al., 1997).

NO evaluation

Supernatant NO content was assayed by the Griess method. Because NO is a compound with a short half life and is rapidly converted to the stable end products nitrate (NO^{3-}) and nitrite (NO^{2-}), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium (Cortas and Wakid, 1990). The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer (Ilhan et al., 2005).

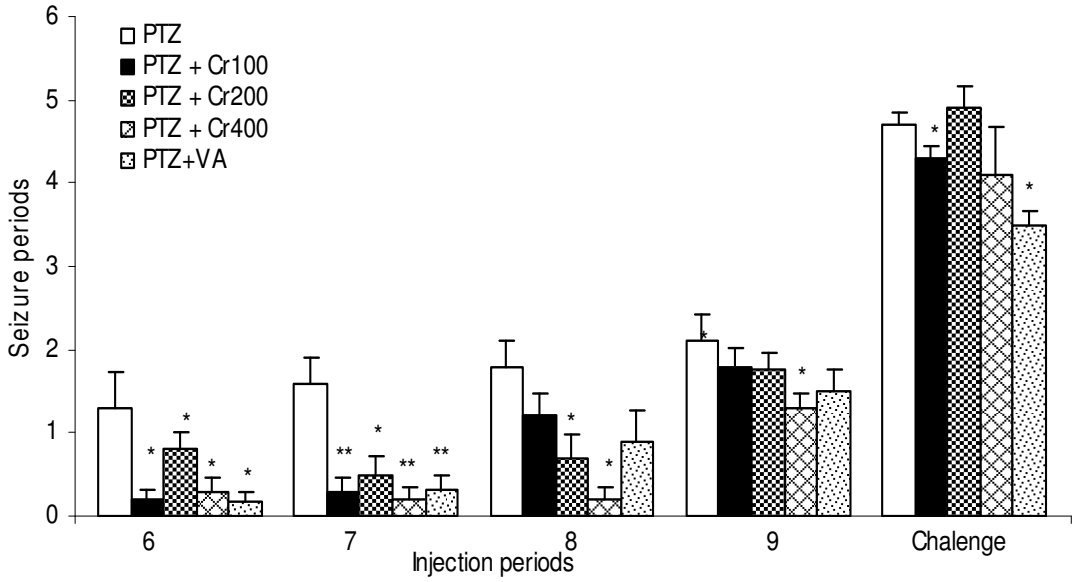

Figure 1. Effect of *C. rotundus* pretreatment on the PTZ-induced kindling intensity. Cr shows *C. rotundus*. **P* < 0.05 and ***P* < 0.01 indicate significant differences as compared to PTZ-kindled group.

SOD activity evaluation

SOD activity measurement was according to the following protocol. Briefly, supernatant was incubated with xantine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37°C) for 40 min and nitro blue tetrazolium (NBT) was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity (Burnham et al., 2010).

Statistical analysis

Data were expressed as means ± S.E.M. Statistical analyses was carried out using repeated measure ANOVA followed by post-hoc Tukey test and p values less than 0.05 were considered as significant differences.

RESULTS

Effect of *C. rotundus* on the PTZ-induced kindling intensity

Statistical analysis of results (Figure 1) indicates that there are no significant differences among experimental groups in the seizure intensity till 6th injection. As it is shown in Figure 1, hydro-alcoholic *C. rotundus* extract at doses of 100, 200 and 400 mg/kg and at 6 to 8th injection and 400 mg/kg dose at 9th injection were able to reduce significantly PTZ-induced seizure ($p<0.05$, 0.01 respectively).

However, valproate (100 mg/kg) significantly reduced seizure intensity in all periods ($p<0.05$). At 12th injection (challenge dose), valproate at a dose of 100 mg/kg had more significant reducing effect on seizure intensity than other treatments ($p<0.05$).

Effect of *C. rotundus* on PTZ-induced kindling factors

As could be seen in Figure 2, pretreatment of animals with *C. rotundus* (200 mg/kg) has a significant effect on the duration which the mice reach to phase 5 seizures. In addition, Figure 3 indicates that only pretreatment of mice with *C. rotundus* 400 mg/kg and valproate 100 mg/kg are able to reduce significantly the period that mice remain in phase 5 of seizure ($p<0.05$).

Effect of *C. rotundus* on biochemical indexes of oxidative stress and antioxidant defense system

Table 1 indicates the brain levels of biochemical factors that are usually indexes of oxidative stress in brain tissues of kindled and non-kindled groups with or without pretreatment with valproate and *C. rotundus* extract. PTZ-induced kindling significantly increased MDA level in the brain tissue of kindled mice relative to control group ($p<0.05$). However, the significant reductive effect of PTZ on SOD level in the brain as compared to control mice was also observed ($p<0.05$). Nonetheless, the NO level in the brain of kindled mice relative to control group was unchanged.

Valproate administration was only able to decrease the brain NO activity in the PTZ-kindled mice relative to control and non treated PTZ-kindled mice significantly ($p<0.05$) and did not change MDA and SOD level in brain

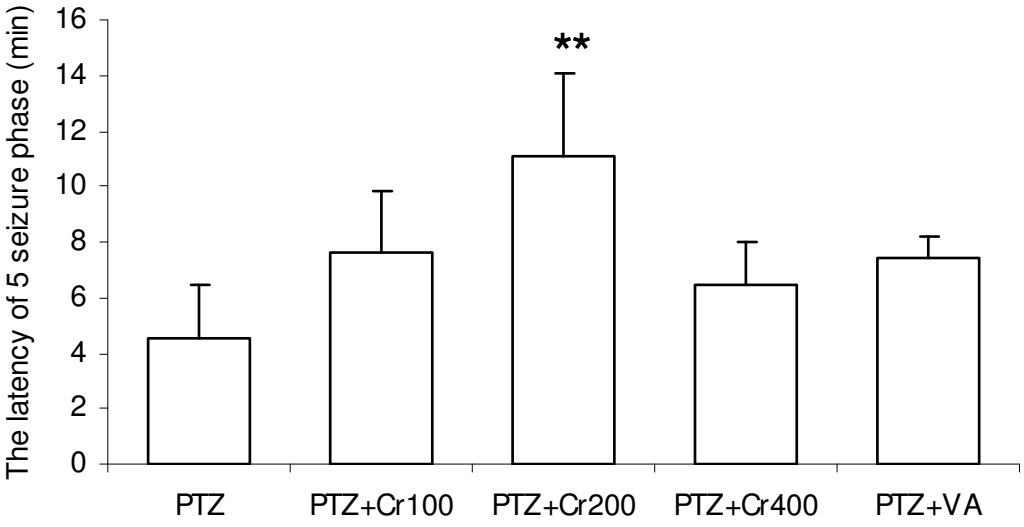

Figure 2. Effect of valproate (100 mg/kg) and three doses of *C. rotundus* (100, 200 and 400 mg/kg) on the latency of arriving to phase 5 of seizure. n=10 in each group. VA and Cr indicate *valproate and C. rotundus* respectively.

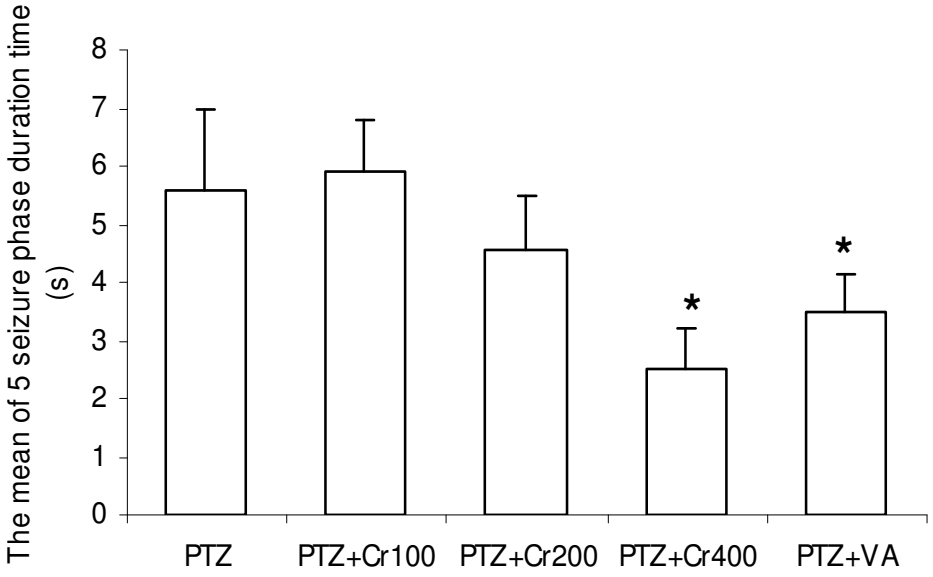

Figure 3. Effect of valproate (100 mg/kg) and three doses of *C. rotundus* (100, 200 and 400 mg/kg) on the remaining time in the phase 5. n=10 in each group. VA and Cr indicate *valproate and C. rotundus* respectively. *$P < 0.05$ shows significant difference as compared to PTZ-kindled group.

tissue. Interestingly, in the pretreated group with 100, 200 and 400 mg/kg doses of *C. rotundus* NO brain content had a significant increase relative to other groups ($p<0.05$). MDA level of brain tissue only in *C. rotundus* treated group (400 mg/kg) relative to PTZ-kindled mice significantly decreased ($p<0.05$). Finally, it was shown that *C. rotundus* at doses of 100, 200 and 400 could reduce the brain level of SOD relative to control and PTZ-kindled mice ($p<0.05$).

DISCUSSION

In the present study, it was found out that *C. rotundus*

Table 1. The effect of valproate and three doses of *C. rotundus* on the NO, MDA and SOD levels of brain tissue on the PTZ-kindled mice.

Enzymes/g protein/Groups test	NO (µmol)	MDA (nmol)	SOD (U)
control	0.027±0.564	1.89 ±17.69	0.127±004
PTZ	0.398 ±0.032 *	25.63±2.11 *	0.098±0.008 *
PTZ + Valproate	0.409±0.042 *	20.46±1.98 #	0.121±0.018 #
PTZ + Cr 100	0.376±0.051 *	24.46±1.23	0.133±005 #
PTZ + Cr 200	0.386±0.017 *	23.21±1.64	0.134±0.011 #
PTZ + Cr 400	0.527±0.052 *#	21.29±1.18 #	0.136±0.018 #

Brain levels of NO, MDA and SOD are compared in six groups. In each group n=10 and Cr indicates *C. rotundus*. * and # show significant differences as compared to control and PTZ-kindled groups respectively ($p<0.05$).

rhizome could reduce the intensity and duration of PTZ-induced seizure. Our data also showed that the hydro-alcoholic extract of this plant at higher dose could significantly reduce the duration of phase 5 seizure. Previous reports have shown that PTZ via blocking $GABA_A$ receptor channels have a key role in epileptic seizure development (Rebrov et al., 2004). However, attachment of benzodiazepine-like components of *C. rotundus to* $GABA_A$ receptors could explain its anti-epileptic effect. Also, other constituents of *C. rotundus including* sugeonol and cyperone, could yield a modulatory effect on glutaminergic system, especially lowering the opening of NMDA receptor channels (Ngo Bum et al., 2003), which could lead to anticonvulsant effects. Free radicals are involved in pathogenesis of many diseases such as epilepsy. The important effect of free radicals is membrane lipid peroxidation and tissue injury by which leads to cell membrane destruction and its dysfunction. Normally, biological effects of free radicals in the body are controlled by a lot of antioxidants and via anti-oxidant enzymes like SOD (Sudha et al., 2003; Ilhan et al., 2006). Free radical production act on seizure via inactivating glutamine synthetas that result in the increment of L-glutamate brain level (Alabadi et al., 1999; Halliwell and Gutteridge, 1991). However, some researchers suggest that only NMDA receptor activation and NO production, without glutamine synthetase inhibition are involved in the seizure (Lapouble et al., 2002). Generalized epilepsy is accompanied by reversible convulsing and can induce production of reactive oxygen species in the brain (Halliwell and Gutteridge 1991). Since it is supposed that free radicals mediate the convulsion improvement, nowadays searching for antiepileptic drugs with antioxidant and neuroprotective effects are of interest.

It has also been observed that antioxidants significantly inhibit PTZ-induced seizure and reduce seizure-induced oxidative stress (Ilhan et al., 2005). Furthermore, in epileptic patients, the serum level of antioxidants reduces and lipid peroxidation increases that are correctable with antiepileptic drugs (Sudha et al., 2001). In the chemical kindling model which is identified by an increase in the seizure induction potential due to blockade of GABA receptor and ionofor chloride complex, PTZ is capable to excite glutamate receptors in the brain area. Furthermore, possibly PTZ is a starter of various processes such as membrane phosphorylation, proteolysis, and nuclease and consequently release of free lipid peroxides and free radicals (Obay et al., 2008). In the present study, significant increase of MDA as an index of lipid peroxidation and significant reduction of antioxidant enzyme SOD in the PTZ-induced kindled group lead to excess production of free radicals and existence of oxidative stress in the brain. Therefore, this research is in accordance with the theory that in the PTZ-induced animals, the oxidative stress is possibly one of parameters that participate in the pathophysiology of epilepsy. In the present study, *C. rotundus* at a dose of 400 mg /kg decreased MDA level relative to PTZ mice that one can probably conclude the antioxidant effect of this plant could lead to reduction of oxidative injury, lipid peroxidation and MDA level. The enzyme SOD itself is an antioxidant enzyme which catalyses the conversion of superoxide to hydrogen peroxide and in this way protects the cell against the oxidative stress. In the present study, it was observed that *C. rotundus* at all doses could increase SOD level relative to PTZ mice. This can lead to the conclusion that probably *C. rotundus* with antioxidant effect is able to preserve the antioxidant enzyme SOD and consequently affect seizure intensity and duration.

Nowadays, NO is known as an important neurotransmitter that in addition to various physiological duties, is also related to synaptic plasticity, neuronal excitability regulation, and epileptic activity (Buisson et al., 1993). Involvement of NO in epilepsy is approved via different experiments and systemic injection of NOS (NO synthase) inhibitors (Starr and Starr, 1993). Controversial effects of NO on the PTZ-induced convulsant have been obtained. Oliveria and colleagues have shown that NOS inhibition in kindling model amplifies the 60 mg/kg PTZ-induced seizure intensity, but has protective effect against 80 mg/kg PTZ-induced tonic seizure (Tsuda et al., 1997). So, they have concluded that preconvulsant or anticonvulsant activity of NOS and NO inhibitors is

dependent on the PTZ dose and the seizure model. They have attributed the protective and inhibitory effect of NOS on the high dose of PTZ to the proconvulsant effect of NO in the limbic system (Del-Bel et al., 1997). It has also been reported that PTZ-induced seizure are modulated by endogenous NO production and glutamate ionotropic receptors (Itoh and Watanabe, 2009). Using nNOS $^{-/-}$ mice (lacking nNOS gene) and nNOS (neuronal NO synthase) inhibitors, they have concluded that basic and enhanced levels implies negative and positive modulatory effects respectively (Itoh and Watanabe, 2009). PTZ via NMDA glutamate receptors activates calcium release via NMDA receptor that consequently activates calcium-calmodoline pathway to increase nNOS protein expression and NO increment in brain different area. The higher NO level is able to increase the induction of generalized epilepsy. NO has complex effect on the neurotransmitters, by which it could mediate the cGMP activation resulted from NMDA receptor activation but simultaneously inhibit the intracellular $Ca^{2+,}$ NOS activation and NMDA receptor (Swamy et al., 2010). It has also been shown that NO is able to inhibit synaptic membrane connected L-glutamate in mouse brain (Fujimori and Pan 1991). It is therefore suggested that anticonvulsant role of NO is related to an implied feedback of NO on NMDA receptor activation via different mechanisms (Buisson et al., 1993). However, NO is known as a molecule that can easily react with O_2. - radicals in the brain and reduce the oxidative stress-induced damage via deleting free radicals (Sudha et al., 2001). Controversial results make it difficult to predict pro- or anti-convulsant effect of NO molecule. Anyway, in the present research, the NO level decreased in PTZ group relative to control and significantly increased in *C. rotundus* treated group (400 mg/kg) relative to PTZ mice. This indicates that probably this extract could have suppressed the seizure via activation of NO synthesis pathway. Probably, the reduced level of NO in PTZ mice is resulted from free radicals production at seizure time and its consumption due to its cleaning effect. Also, the enhanced level of NO in treated group with *C. rotundus* extract is due to its antioxidant effect by which eliminates O_2.- radicals, and consequently prevents lipid per-oxidation and oxidative stress-induced injury that leads to increased level of NO.

In conclusion, the present research indicates that hydro-alcoholic *C. rotundus* extract has anticonvulsant effect on PTZ-induced kindling in mice which is mediated via attenuation of oxidative stress and augmentation of antioxidant defense system.

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