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Evaluation of *In vivo* and *In vitro* Antioxidant properties of the Ethanol Leaf Extract of *Culcasia falcifolia*

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Abstract

Objective: To evaluate the *in vivo* antioxidant potential of the ethanol leaf extract of *Culcasia falcifolia* against Pentylenetetrazole induced oxidative stress as well as its *in vitro* antioxidant effect.

Method: The extract was prepared by maceration using ethanol. Four groups of six mice were used for the *in vivo* studies. Group I (control group) received 0.1% CMC. Group II (positive control) received diazepam on the experimental day (5mg/kg body wt.). Group III and IV received 200 and 400 mg/kg body wt. of ethanol extract of *C. falcifolia* orally for fourteen days. On the fifteenth, all mice were sacrificed to remove the brains after the injection of PTZ (60 mg/kg i.p). MDA, SOD, CAT, GPX and GR levels were estimated from the brain tissue. DPPH radical scavenging assay and ferrous ion chelating assay were used to evaluate the *in vitro* studies.

Result: The study suggests that the extract exhibit *in vivo* antioxidant property by increasing in the levels of SOD, CAT, GPX and GR and decreasing in the MDA level of the brain tissue significantly ($p<0.05$*). *The in vitro* studies also showed that the extract was able to scavenge free radical and chelate metal.

Conclusion: The ethanol extract of *Culcasia falcifolia* possesses *in vivo* and *in vitro* antioxidant activity against Pentylentetrazole induced seizure in mice.

Keywords: Antioxidant activity; *Culcasia falcifolia*; Epilepsy

Introduction

Epilepsy is one among the common neurological disorders that is characterized by repetitive seizure. Oxidative stress, free radical production and membrane lipid peroxidation occur as a result of epilepsy which causes damage to the brain tissue [1]. Seizure generation can be associated with the homeostatic imbalance between antioxidants and oxidants [2]. Oxidative stress is the most prominent mechanism in the development and progression of epilepsy [3]. Oxidative stress is a consequence of the increased oxidant burden which overwhelms the endogenous antioxidants and repair capacity or a consequence of diminished endogenous antioxidants and repair capacity which cannot encompass the normal oxidant burden [4]. Several experimental rodent models of epilepsy such as amygdala kindling model, the kainic acid model, the Pentylenetetrazole (PTZ) model and the electroshock model has been shown to have oxidative stress in the central nervous system [5]. In the present study Pentylenetetrazole induced seizure model was selected as it is one of the standardized simple, reliable and most validated techniques for the evaluation of antiepileptic drugs. *Culcasia falcifolia* is a perennial climber, epiphytic on trees and growing to several meters, stem with adventitious roots, penetrating bark with short clasping roots at the nodes. The leaves are lanceolate to narrowly ovate or elliptic to oblong, leathery, dark glossy green, acuminate to 30 cm long. Spathe is greenish-white, rigid and waxy, 6 cm long; spadix almost as long as spathe, cream-yellowish, foetid. Inflorescences are short separate branches, surrounded by a thick leathery, boat-shaped spade, greenish or yellowish white. Fruits are in a cluster of obovoid berries orange-red when ripe. The plant is native to damp evergreen forest in shade; riverine and swamp forests; marshy forest. It is found in Kenya, Ethiopia, Malawi, Tanzania, Uganda, Zambia, and Zimbabwe [6]. The leaves of *Culcasia falcifolia* are used as ash (internal) for dry cough, edema, and epilepsy. It is used as a tonic or ashes taken with porridge. *Culcasia falcifolia* is locally called as Chepnamobon/Kipnamobon [7]. The aim of this study was to evaluate the antioxidant property of ethanol extract of Culcasia falcifolia against PTZ induced seizure in laboratory mice.

Materials and Methods

Collection of material

The leaves of *Culcasia falcifolia* were collected along the river Kinģwal in Kaptildil, Nandi County, Rift Valley, Kenya. The leaves were identified and authenticated from the National Herbarium, Kenya (PS 22/05).

Preparation of the ethanol extract

The leaves were washed, dried and powdered and sieve to obtain finer particles. 100 grams of the sample was mixed and macerated with 1 liter of absolute ethanol for seven days with frequent stir. The extract was then filtrated through Whatman No 1 filter paper and the supernatant was evaporated using the rotary evaporator to obtain dried extract. The dried extract was then stored in sterile universal glass bottle until further use.

Chemicals used in the study

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Xantine and Xanthine oxidase; Potassium phosphate buffer; NBT; Hydrogen peroxide; dichromate-acetic acid; Sodium Azide; Thiobarbituric acid reactive, Butylated hydroxyl toluene, 2, 2-diphenyl-1-picrylhydrazyl, hydrogen peroxide, Ethylene Diammine Tetra Acetate, Ferrozine, ferrous sulphate.

Animals used in the study

Swiss albino mice were used for the study. The animals were placed in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24 ± 2°C and relative humidity of 30-70%. A 12:12 light: day cycle was followed. All animals were supplied water and food ad libidum. The animals were housed for one week in polypropylene cages prior to the experiments to accustom to laboratory conditions.

Drug treatment

The animals were randomly distributed into four different groups with six animals in each group. Group I served as control received 0.1% CMC. Group II served as positive control received diazepam (5 mg/kg body wt. only once, on the day of the experiment). Group III and Group IV received 200 and 400 mg/kg body wt. of the extract orally for fourteen days. On the day of the experiment, the animals were administered 60 mg/kg body wt. pentylenetetrazole intraperitoneally (45 min after the extract administration for group III and IV and 30 min after the administration of standard drug to group II).

Preparation of brain tissue sample

All the animals were sacrificed by decapitation. The brains were removed, washed in cold saline twice and placed on a sterile glass plate. The brains were cut into pieces using surgical scissors. The brain tissue pieces were homogenized with five ml of ice cold 0.1 M Tris-hydrochloric buffer 7.4 pH. Then the homogenate was centrifuged for 20 minutes at 5°c at the speed of 10000 rpm. After centrifugation, the overlaying organic phase was removed and the supernatant was used for the evaluation of antioxidant enzyme. The reading was obtained using spectroflorimeter.

In vivo Antioxidant Study

Lipid peroxidation assay

Two ml of thiobarbituric acid reagent was added to 100 µl of the tissue homogenate and mixed well. After incubating the mixture for 40 minutes in a boiling water bath, the mixture was centrifuged for 10 minutes at the room temperature at 3500 rpm. The development of pink colour was estimated against a reagent at 535 nm in a spectrophotometer. Lipid peroxidation was expressed as nmol of MDA [8].

Estimation of superoxide dismutase (SOD)

Estimation of superoxide dismutase was measured using the method described by Sun. After incubating the sample for 40 minute with xanthine and xanthine oxidase in potassium phosphate, nitro blue tetrazolium chloride (NBT) was added. The inhibition of the reduction of nitro blue tetrazolium chloride to 50% by SOD enzyme activity was measured at 550nm spectrophotometrically. SOD activity was defined as 1 nitrite unit (NU) [9].

Estimation of catalase

To 0.1ml of tissue homogenate, 0.9 ml of phosphate as buffer (0.01 M, pH 7.0), 0.4ml of hydrogen peroxide were added. After 60 seconds, 2.0 ml of dichromate-acetic acid mixture was added and incubated in boiling water bath for 10 minutes. The fluorescence developed was read at 620nm in a spectrophotometer. The activity of Catalase was expressed as µ mol /mg protein [10].

Evaluation of glutathione

To 2 ml of homogenate, 2.5 ml of 0.02 M Sodium acetate as buffer (pH 6. 9) is added and mixed well. Then, 4 ml of cold distilled water and 1 m of 50% trichloroacetic acid is added and shaken well for 10 minutes. Then, the mixture was centrifuged at 3000 rpm for 15minutes. After centrifugation, 2 ml of the supernatant tissue sample was mixed with 0.4 M tris- hydrochloric buffer (pH 8.9) and 0.1 ml of 0.01M Elman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) was added. The absorbance was read at 412 nm against reagent blank with no homogenate. For blank reading, 2 ml of distilled water was substituted for homogenate. The level of glutathione was expressed as µ mol/g of tissue [11].

Estimation of glutathione peroxidase

To 0.5 ml of tissue homogenate, 0.2 ml of Tris buffer (0.4 M, pH 7.0), 0.2 ml of Sodium acetate buffer (PH6. 9) and 0.1 ml of sodium azide were added. To this mixture, 0.2 ml glutathione and 0.1 ml hydrogen peroxide were added and incubated in water bath at 37°C for 10 minutes. 0.5 ml of 10% TCA (trichloro acetic acid) was added to stop the reaction after 10 minutes. The mixture was then centrifuged and the supernatant was assayed for glutathione. The level of glutathione peroxidase was expressed as µmol/mg of protein [12].

In vitro Antioxidant Activity

Free radical scavenging activity (DPPH•)-Method

The radical scavenging activity of ethanol extract of *Culcasia falcifolia* was determined by using DPPH radical (1, 1-diphenil-2-picrylhydrazyl) method described by Blois in 1958. 100 µg of 0.2 mM solution of DPPH was added to 50, 100, 150, 200 and 250 µg/ml concentrations of ethanol extract of *Culacasia falcifolia*. The mixture was incubated for 30 minutes at room temperature. The absorbance was measured at 517 nm after 30 minutes. BHT (butylated hydroxy toluene) was used as the standard control. All the tests were performed in triplicate and percentage of inhibition was calculated by comparing the absorbance values of the control and test samples [13].

$$\%\,\text{inhibition} = \left[\left(\text{Ab - As}\right) / \text{Ab}\right] * 100$$

Ferrous ion chelating activity-method

The method described by Singh and Rajini in 2004 was used to estimate the chelation of ferrous ions by the extract [14]. 100 µl of 2 mM ferrous sulphate solution was mixed with different concentrations of ethanol extract of *Culcasia falcifolia* (1000, 2000, 3000, 4000 and 5000 µg/ml) followed by the addition of 500 µl of 5 mM ferrozin. The mixture was incubated at room temperature for 10 minutes. After 10 minutes, the absorbance of the solution was measured at 562 nm. Ethylene Diammine Tetra Acetate was used as standard control. All the tests were performed in triplicate and percentage of inhibition was calculated by using this formula

$$\text{Percentage of inhibition} = \frac{\text{Abs}_{\text{cntrl}} - \text{Abs}_{\text{test}}\,\text{X}\,100}{\text{Abs}_{\text{cntrl}}}$$

Results

In vivo antioxidant studies

Effect of the extract on Malondialdehyde (MDA) levels in mice brain tissue : A difference was found in the brain MDA levels between the groups. The extract at 200 and 400 mg/kg body wt. reversed the increased MDA level (2.52 ± 0.28* and 1.97 ± 0.45*) in comparison to PTZ group (3.89 ± 0.47**) with statistical significance (p<0.05*, p<0.01**). Results shown in Table 1 Figure 1.

Effect of the ethanol extract of ***Culcasia falcifolia*** on levels of superoxide dismutase (SOD) in mice brain tissue : Low SOD activity was observed in PTZ treated group (9.13 ± 0.46**). The extract at 200 and 400 mg/kg body wt. significantly increased the SOD (12.47 ± 0.62* and 13.59 ± 0.62*) as compared to PTZ (9.13 ± 0.46**). Results are shown in Table 2 Figure 2.

Group	Drug treatment	Lipid peroxidation nmol MDA/mg protein
Group I	control 0.1% CMC	1.62 ± 0.32
Group II	PTZ 1 ml/100 gms	3.89 ± 0.47**
Group II	Diazepam (5 mg/kg)	4.24 ± 0.52
Group III	EECF (200 mg/kg)	2.52 ± 0.28**
Group IV	EECF (400 mg/kg)	1.97 ± 0.45*

Note: EECF: Ethanol leaf extract of *Culcasia falcifolia;* Values are expressed as mean ± SEM; p<0.05*, p<0.01**, Statistical significance test was done by ANOVA, followed by Dunnet's t-test.

Table 1: Effect of the extract on Malondialdehyde (MDA) levels in mice brain tissue.

Effect of the ethanol extract of ***Culcasia falcifolia*** on levels Catalase in mice brain tissue: There was a significant (p<0.01**) decrease in the levels of catalase in the PTZ treated group (15.45 ± 0.33**) as compared to control (20.47 ± 0.34). The extract at the doses of 200 and 400 mg/kg body weight showed significant (p<0.05*) increase in the levels of Catalase (18.63 ± 0.53* and 19.13 ± 0.26*) as compared to PTZ (15.45±0.33**) and diazepam treated group (16.05 ± 0.27**). Results are shown in Table 2.

Effect of the ethanol extract of ***Culcasia falcifolia*** on levels of glutathione reductase in mice brain tissue: There was a significant (p<0.01**) decrease in the levels of glutathione reductase in the PTZ treated group (22.86 ± 0.54**) as compared to the control group (29.12 ± 0.41). The extract at 200 and 400 mg/kg body weight significantly (p<0.05*) increased glutathione reductase levels (26.49 ± 0.41* and 27.63 ± 0.67*) as compared to the PTZ (22.86 ± 0.54**) and diazepam treated group (21.98 ± 0.36**) Figure 3. Results are shown in Table 2.

Effect of ethanol extract of Culcasia falcifolia on glutathione peroxidase level in mice brain tissue: The glutathione peroxidase level was significant (p<0.01**) decreased in the PTZ treated group (17.43 ± 0.29**) as compared to the control group (23.13 ± 0.28). The extract at the doses of 200 and 400 mg/kg body weight significantly (p<0.05*) increased the Glutathione peroxidase levels (21.43 ± 0.57* and 22.74 ± 0.49*) as compared to PTZ (17.43 ± 0.29**) and diazepam treated group (17.72 ± 0.49**). Results are shown in Table 2 Figure 4.

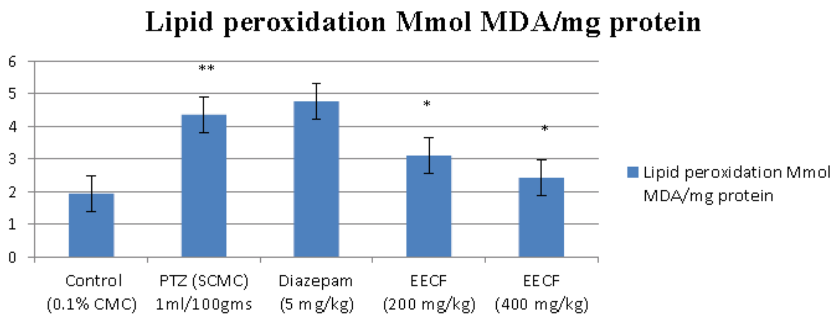

Figure 1: Effect of the extract on Malondialdehyde (MDA) levels in mice brain tissue.

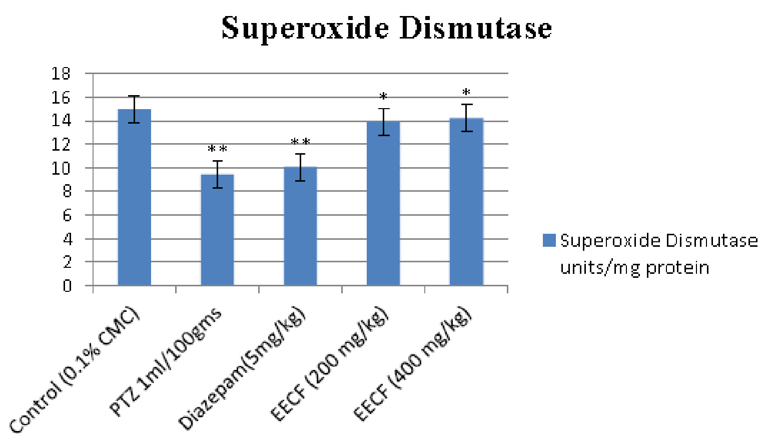

Figure 2: Effects of ethanol extract of *Culcasia falcifolia* on levels of superoxide dismutase seizure by Pentylenetetrazole in mice.

Group	Drug treatment	SOD U/mg protein	Catalase units/mg protein	Glutathione reductase µmol/mg protein	Glutathione peroxidase µmol/mg protein
Group I	control 0.1% CMC	14.42 ± 0.53	20.47 ± 0.34	29.12 ± 0.41	23.13 ± 0.28
Group II	PTZ (SCMC) 1 ml/100gms	9.13 ± 0.31**	15.45 ± 0.33**	22.86 ± 0.54**	17.43 ± 0.29**
Group III	Diazepam (5 mg/kg)	9.62 ± 0.46**	16.05 ± 0.27**	21.98 ± 0.36**	17.72 ± 0.49**
Group IV	EECF (200 mg/kg)	12.47 ± 0.62*	18.63 ± 0.53*	26.49 ± 0.41*	21.43 ± 0.57*
Group V	EECF (400 mg/kg)	13.59 ± 0.62*	19.13 ± 0.26*	27.63 ± 0.67*	1.74 ± 0.49*

Note: EECF: Ethanol extract of the leaf of *Culcasia falcifolia*; Values are expressed as mean ± SEM; *p<0.05;**p<0.01, Statistical significant test for comparison was done by ANOVA, followed by Dunnet's test.

Table 2: Effects of ethanol extract of *Culcasia falcifolia* on oxidative stress marker after the induction of seizure by Pentylenetetrazole in mice.

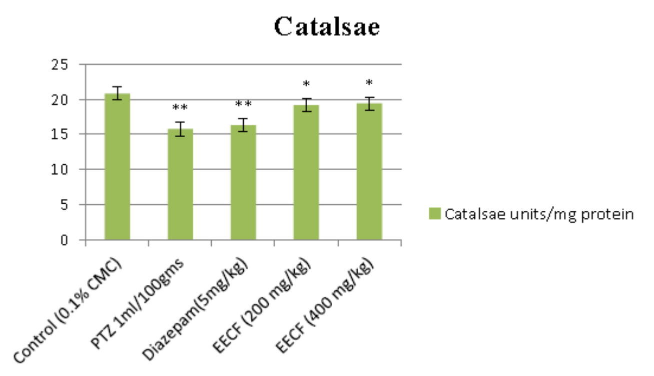

Figure 3: Effects of ethanol extract of *Culcasia falcifolia* on levels of catalase seizure by Pentylenetetrazole in mice.

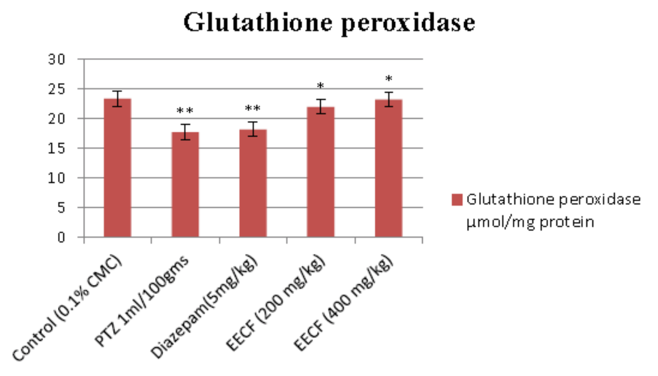

Figure 5: Effects of ethanol extract of *Culcasia falcifolia* on levels of glutathione peroxidase seizure by Pentylenetetrazole in mice.

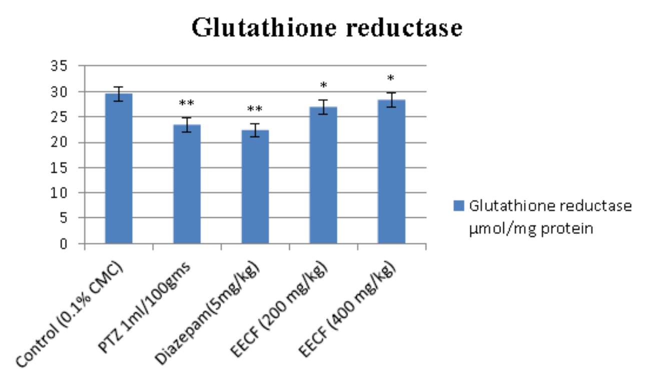

Figure 4: Effects of ethanol extract of *Culcasia falcifolia* on levels of glutathione reductase seizure by Pentylenetetrazole in mice.

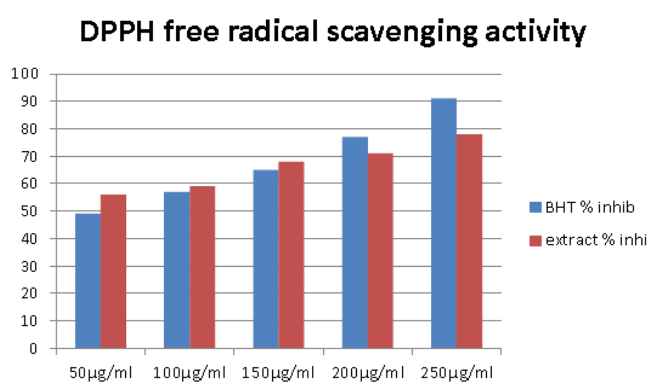

Figure 6: DPPH free radical activity of leaf extract of *Culcasia falcifolia*.

S.No	Sample concentration (µg/ml)	BHT % of inhibition	Leaf extract % of inhibition
1	50	49.39 ± 0.34	56.53 ± 0.33
2	100	57.15 ± 0.23	59.63 ± 0.31
3	150	65.56 ± 0.25	68.55 ± 0.25
4	200	77.79 ± 0.35	71.59 ± 0.28
5	250	91.74 ± 0.42	78.67 ± 0.26

Table 3: Free radical scavenging activity (DPPH).

S. No.	Sample concentration (µg/ml)	EDTA % of inhibition	Leaf extract % of inhibition
1	1000	52.28 ± 0.16	38.62 ± 0.23
2	2000	74.55 ± 0.37	43.47 ± 0.26
3	3000	84.48 ± 0.24	47.59 ± 0.32
4	4000	92.14 ± 0.28	52.84 ± 0.21
5	5000	95.53 ± 0.18	56.73 ± 0.31

Table 4: Ferrous ion chelating activity.

In vitro antioxidant studies

The finding of this study shows that with the increase in the concentration of the extract from 50 to 250 µg/ml there was increase in the percentage of scavenging effect on the DPPH radical. The percentage of inhibition was existed from 56.53 at 50 µg/ml to 78.67 at 250 µg/ml the extract. Result is shown in Table 3. Iron binding capacity of the ethanol extract of leaves of *Culcasia falcifolia* in terms of percent inhibition (56.73%) was shown at 5000 µg/ml. Result shown in Table 4 Figure 5.

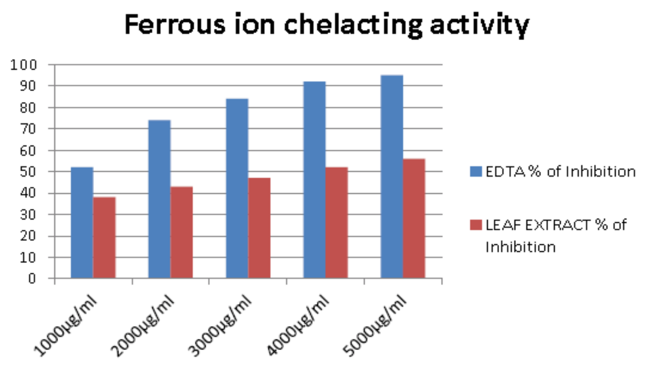

Figure 7: Ferrous ion chelating activity of the leaf extract of *Culcasia falcifolia.*

Discussion

In vivo enzymatic antioxidant activity of the ethanol extract of *Culcasia falcifolia* on PTZ induced seizure in mice

During seizure there is elevation of free radical, decrease in antioxidant defense mechanism which induces oxidative stress leading to brain damages [15,16]. The oxidative stress can be effectively neutralized by enhancing cellular defences in the form of antioxidants. Certain compounds act as *in vivo* antioxidants by raising the levels of endogenous antioxidant defences. The enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) increase the level of endogenous antioxidants [17]. Researches have shown that administration of a single dose of PTZ increase oxidative stress and lipid peroxidation [18]. The present study also showed that PTZ-induced seizure increased MDA level, and decreased SOD, CAT, GSR and GPX in the seizure control group (PTZ treated group) compared to normal control group. Attenuation of antioxidant defence leads to ROS-induced damage which decrease in SOD, CAT, GSR and GPX and elevated level of MDA. Free radical generation in the brains of PTZ treated group was measured through estimation of MDA which indicates lipid peroxidation [19]. Superoxide dismutase protects the cell against oxidative stress by catalyzing the conversion of superoxide to hydrogen peroxide [20]. Catalase, an endogenous antioxidant enzyme protects the cells against damage caused by H_2O_2 is generated inside the cells as a result of oxidative stress [21]. Glutathione peroxidase another antioxidant enzyme reacts with the free radicals and prevents the generation of hydroxyl radicals [22]. In the present study, the ethanol extract of *Culcasia falcifolia* at 200 and 400 mg/kg body weight increased the superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase levels relative to PTZ mice Figure 6.

In Vitro Antioxidant Activity of Culcasia Falcifolia

Free radical scavenging activity (DPPH$^{\bullet}$): DPPH is a preliminary radical scavenging activity to evaluate antioxidant activity in plant extract which is related to inhibition of lipid peroxidation [23]. The scavenging test is based on the decolorizing ability of DPPH (free radical) in the presence of antioxidant [24]. The principle of this assay is based on the reduction of purple colored methanol DPPH solution in the presence of hydrogen donating antioxidants by the formation of yellow colored diphenyl-picryl hydrazine. The antioxidant activity present in the extract is more efficient as the absorbance decreases and more DPPH reduction will occur [25]. The results of the DPPH scavenging of free radical showed maximum activity at 5000 µg/mL concentration. Presence of bioactive phytochemical in the extract attributed to the radical scavenging effect. The extract showed free radical scavenging effect similar to that of the standard BHT. Further, from the result it was noticed that extract possess hydrogen donating capabilities for scavenging free radicals. The reducing capacity of compounds could serve as indicator of potential antioxidant property [26]. The metal chelating capacity may significantly influence the course of oxidative reactions, thus metal binding compounds are included to the class of oxidation inhibitors. Iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components [27]. By stabilizing transition metals thereby reducing damage caused by free radical chelating agents inhibit the generation of free radicals. Some phenolic compounds have the ability to chelate metal ions to exhibit antioxidant activity [28]. Secondary antioxidants are effective chelating agents that form a bond with a metal to stabilize the oxidized form of the metal ion by reducing their redox potential [29]. The metal chelating ability of the extract of *Culcasia falcifolia* was measured by the formation of ferrous ion ferrozine complex. Ferrozine combines with ferrous ions forming a red colored complex which absorbs at 562 nm. The absorbance was a measure for metal chelating ability of the leaf extract of *Culcasia falcifolia* which was comparable to that of the reference standard, EDTA (Figure 7) [30].

Conclusion

The findings of this study show that the extract has the ability to attenuate the oxidative stress caused by PTZ-induced seizure. The extract has active phytochemicals which may be responsible for *in vivo* and *in vitro* antioxidant activity against PTZ-induced seizure in mice.

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