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Research Article

Protective Activity of Esculetin Against 3-Nitropropionic Acid Induced Neurotoxicity Via Scavenging Reactive Oxygen Species in Male Wistar Rats

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ABSTRACT

Huntington's disease (HD) is a devastating neurodegenerative disorder with no cure till date. Many genetic or chemically induced models have been developed in rodents to study the disease. 3-Nitropropionic (3-NP) acid is a well-known neurotoxin to induce Huntington's disease (HD) in rodents. It replicates the pathology of HD by causing oxidative stress. Esculetin is a natural compound, a coumarin, known to have neuroprotective effect in a mouse model of Parkinson's disease. In the present study, the neuroprotective effect of esculetin on 3-NP induced oxidative stress in rat striatum was determined by behavioral and biochemical parameters. Rats were induced with 3-NP (10mg/kg) intraperitoneally for 14 days and rats induced with 3-NP were treated with esculetin (25mg/kg and 50mg/kg) for 14 days. Esculetin attenuated the behaviour of rats in morris water maze, open field, forced swim, narrow beam walk and grip strength test. Biochemical effect of esculetin was also studied on oxidative stress markers, SDH and acetylcholinesterase. Esculetin treatment alleviated the increased values of acetylcholinesterase, protein carbonyls and lipid peroxidation. On treatment with esculetin, we observed that the levels of SOD, GSH, catalase, glutathione peroxidase, SDH were increased. The present study shows that the antioxidant activity of esculetin may be responsible for its neuroprotective activity against 3-nitropropionic acid induced neurotoxicity in rats.

Keywords: Esculetin, 3-nitropropionic acid, Huntington's disease, oxidative stress.

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder characterized by chorea, motor abnormalities, incoordination and bradykinesia. HD is prevalent in western population[1], but recent reports suggest that HD may also be prevalent in India[2]. HD is caused due to expansion of CAG repeats in Huntingtin (Htt) gene. The expansion of polyglutamine repeats brings about a conformational change in mutant huntingtin causing it to aggregate. Huntingtin protein shows ubiquitous expression, with highest levels in central nervous system (CNS)[3]. Htt is required for various cell processes like cell trafficking, proper orientation of mitotic spindle, cell adhesion and embryonic development[4].Alteration in levels of neurotransmitters such as dopamine, acetylcholine with loss of acetylcholinesterase levels is hallmark of HD[5]. The mechanism of the pathology of HD involves mitochondrial dysfunction and oxidative stress which leads to degeneration of medium spiny GABAergic striatal neurons[6].

3-nitropropionic acid (3-NP) is a neurotoxin produced by *Aspergillus flavus*, *Astragalaarthrinium and Indigoferaendecapylla*. Humans exposed to 3-NP can develop dystonia and acute encephalopathy.3-NP irreversibly inhibits succinate dehydrogenase, complex II of mitochondrial electron transport chain, and ultimately results in inhibition of oxidative phosphorylation, causing oxidative and nitrative stress due to overproduction of reactive oxygen species (ROS)/reactive nitrate species (RNS) or depletion of antioxidant system[7]. Systemic administration of 3-NP results in replicative neurodegenerative symptoms of HD [8]. Administration of 3-NP results in sparing of NADPH- diaphorase neurons and death of medium spiny neurons in striatum, ultimately,resulting in oxidative stress. Oxidative stress causes alterations in the levels of enzymatic antioxidants and behavioural deficits[9].

Esculetin is a natural coumarin which is a secondary metabolite of esculin. It is present in a variety of plants like *Foeniculum vulgare* (fennel),*Aesculushippocastanum, Salvia officinalis* (garden sage) etc.[10] .It is known to have anti-diabetic, neuroprotective, anti- cancer and anti-ischemic activity.

In the present study, an attempt has been made to evaluate the neuroprotective activity of esculetin against 3-nitropropionic acid induced Huntington's disease like symptoms.

MATERIALS AND METHODS

Chemicals

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3-nitropropionic acid and esculetin were purchased from Sigma- Aldrich Co. (St. Louis, USA). All other chemicals and reagents used in this study were of analytical grade.

Animals

Male Wistar rats (250-300 gm) were obtained from Central Animal House, University of Madras, Taramani, Chennai. All animals were maintained in 12h light/ dark cycle. The rats had free access to food and water. All the experiments were conducted according to the ethical rules approved by Institutional Animal Ethics Committee (IAEC no-01/12/2016).

Experimental Procedure

The rats were randomly divided into five groups as below:

Group I- Control- Rats administered with saline.

Group II- Rats administered with 3-nitropropionic acid (10 mg/kg b.w.) intraperitoneally (i.p.) for 14 days.

Group III- Rats simultaneously treated with 3-NP (10 mg/kg b.w. i.p) & Esculetin (25 mg/kg b.w. orally) for 14 days.

Group IV- Rats simultaneously treated with 3-NP (10 mg/kg b.w. i.p.) & Esculetin (50 mg/kg b.w. orally) for 14 days.

Group V- Rats treated with Esculetin (50 mg/kg b.w.) orally for 14 days.

Measurement of Body Weight

Body weight of the animals was recorded on day 1 and day 14. Percentage change in body weight was calculated.

Behavioural Analysis

Open field test

Open field test apparatus was made up of wood and had dimensions 100 cm x 100 x 40 cm, divided into 25 rectangular squares. The experimental room was illuminated by a 40-watt white bulb fixed 150 cm above the test apparatus. Each rat was placed into the centre and allowed to explore the apparatus for 12 mins. Each crossing was considered only when all the four paws moved to another square. The number of squares crossed by rats in the last 10 mins was recorded[11].

Grip strength test

Grip strength was measured by suspending rat with the forepaws to a thick steel wire of 2mm in circumference and 80 cm in length, placed 50 cm above a cushioned support. The length of the time for which rat remain suspended on wire was measured. The latency to loss is considered as an indirect measure of grip strength and the cut-off time was taken 90 sec[12].

Morris water maze test

This test was performed to evaluate cognitive performance. The maze was made of a circular water tank with diameter 160 cm and height 35 cm. The tank was divided into four points assigned as 4 quadrants. The tank was filled with water upto 20 cm and made opaque by adding milk. The tank comprised a square platform (escape platform) kept at 2 cm below the water surface and attached at one end along the circumference of tank. Animals were given prior training for 4 days for swimming to the platform by gently placing them in anyone of the quadrant not containing the escape area with their face towards wall of the tank. In case the rat failed to find the platform within 90sec it was guided to platform and made to stay there for 20sec. After the trials, the escape latency, that is, the time taken by the animals to reach the platform was recorded on day 5, 10 and 14[13].

Narrow beam walk test

This task was performed to measure the motor coordination ability of the animal. In this task, a beam consisting of two platforms (8cm in diameter) connected by a narrow wooden beam (0.5 cm in thickness, 2 cm in width and 120 cm in length) was used. The beam was elevated 50 cm above the ground. A box filled with sawdust was placed below the beam, for serving as cushioning protection for a falling rat. The rat was acclimatized by allowing it to explore the beam for 5 min before training. When a rat walked across the beam, the time taken to cross the beam was recorded[14].

Forced swim test

Forced swim test was carried out according to the method of Porsolt et al. The rats were placed in Plexiglas cylinders of height 20 cm and diameter 10 cm containing 15 cm water, maintained at 23-25^{0}C. Animals were released in water and the time of immobility was calculated. Animals were considered to be immobile when they swam[15].

Dissection and Homogenization

On day 15, after behavioural assessments, animals were sacrificed by decapitation. The brains were removed, put on ice and striatum was separated. A 10% (w/v) tissue homogenate was prepared in 0.1M phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 15 min and aliquots of supernatant were separated and used for biochemical estimation.

Biochemical Estimation

Estimation of enzymatic antioxidants

Glutathione peroxidase activity was determined by the method of Necheles et al. (1969)[16]. The absorbance of assay mixture was recorded at 412 nm within 5 min of addition of DTNB. The results were expressed as µg of GSH consumed/min/mg protein. SOD activity was assayed according to the method of Marklund and Marklund (1974)[17]. Catalase activity was assayed by the method of Luck (1971)[18], in which the breakdown of hydrogen peroxide (H_2O_2) was measured at 240 nm. The results were expressed as mM of H_2O_2 decomposed per milligram of protein/min.

Estimation of non-enzymatic antioxidant

Estimation of glutathione (GSH) in striatum was done according to the method of Moron et al[19]. Briefly, 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) is reduced by –SH groups and 1 mole of 2-nitro-5-mercaptobenzoic acid is produced by per mole of SH. The intensity of released nitro mercaptobenzoic acid anion is measured at 412 nm.

Estimation of Lipid peroxidation and Protein Oxidation

The protein carbonyl content was determined by the method of Levine et al[20]. The absorbance was measured at 366 nm and the results were expressed as nmoles of carbonyl groups/mg of protein. Lipid peroxidation was measured by the method of Wills (1966)[21]. Malondialdehyde, which is a marker of lipid peroxidation, was measured at 532nm. The values were calculated using the molar extinction coefficient of the chromophore (1.56

x 10^5 /M/cm) and expressed as a percentage of the vehicle treated group.

Estimation of Succinate dehydrogenase

SDH activity was measured according to the method of King et al (1976)[22]. The reaction mixture consisted of 1.5ml phosphate buffer (0.2 M, pH 7.8), 0.2 ml succinic acid (0.6M, pH 7.8), 0.3 ml BSA (1% w/v), 0.1 ml of 0.03 M potassium ferricyanide and homogenate. The decrease in absorbance was recorded at 420 nm for 3 min. Results were expressed as nmol succinate oxidized/min/mg protein.

Estimation of acetylcholinesterase (AchE) activity

Acetylcholinesterase (AchE) activity was assessed in striatum by the method of Ellman et al. (1961)[23]. The assay mixture contained 0.05ml of supernatant, 3ml of sodium phosphate buffer (ph 8), 0.1 ml of acetylthiocholine iodide and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was measured for 2 min at 30 sec interval at 412 nm using a UV-VIS spectrophotometer [UV-Pharmaspec 1700 Shimadzu (Japan)]. Results were expressed as micromoles of acetylthiocholine iodide hydrolysed per min per mg of protein (percentage of control group).

RESULTS

Effect of esculetin on 3-NP induced changes in body weight

Fig.1 shows the percent changes in body weight on day 14 as compared today 1. Systemic administration of 3-NP caused a significant ($p<0.01$) decrease in body weight on day 14 as compared to control group. Esculetin (25mg.kg and 50mg/kg) when co-administered with 3-NP significantly ($p<0.05$) reversed the decreased in body weight.

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Behavioral parameters

Effect of esculetin on 3-NP induced changes in open field test

Fig. 2 shows the No. of squares crossed by 3-NP induced rats were significantly ($p<0.01$) less than those by control rats. On treatment with esculetin (25mg/kg and 50mg/kg), rats showed a significant ($p<0.05$) increase in motility by crossing more number of squares than 3-NP induced rats.

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test).

Effect of esculetin on 3-NP induced changes in morris water maze test

The transfer latency of all the groups on 5th day was almost same. The transfer latency of 3-NP induced group was significantly ($p<0.01$) more than the control groups on 10th and the 14th day. However, treatment with esculetin (25mg/kg and 50mg/kg) significantly ($p<0.05$) decreased the transfer latency i.e. the time required to search the hidden platform (Fig.3).

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Effect of esculetin on 3-NP induced changes in forced swim test

In forced swim test, the immobility time is directly proportional to the locomotory impairment. In our study, in 3-NP induced group, there was a significant ($p<0.01$) increase in immobility time as compared to control group. Further, esculetin treatment (25mg/kg and 50mg/kg) significantly ($p<0.05$) attenuated the increase in immobility time (Fig.4).

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Effect of esculetin on 3-NP induced changes in grip strength test

Fig. 5 shows that the ability to hold the wire by forepaws was significantly ($p<0.01$) reduced in 3-NP induced rats. On treatment with esculetin (25mg/kg and 50mg/kg), the time spent holding the wire was significantly increased.

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Effect of esculetin on 3-NP induced changes in narrow beam walk test

In our study, 3-NP treated rats took significantly ($p<0.01$) longer time to cross the beam than the control group. The time taken to cross the beam was significantly ($p<0.05$) reduced in esculetin treated (25mg/kg and 50mg/kg) group (Fig.6).

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Biochemical Parameters

Effect of esculetin on 3-NP induced changes on lipid peroxidation and protein

We found a significant ($p<0.01$) increase in malondialdehyde (MDA) (Fig.7) and protein carbonyl (Fig.8) levels in 3-NP treated rats as compared to control group. Esculetin (25mg/kg and 50mg/kg) treatment significantly ($p<0.05$) reverted the increased levels of MDA and protein carbonyls, thus decreasing the lipid peroxidation and protein oxidation.

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Effect of esculetin on 3-NP induced changes on the levels of enzymatic antioxidants

There was a significant ($p<0.01$) decrease in levels of SOD (Fig.9), Cat (Fig.10) and GPx (Fig.11) on treatment with 3-nitropropionic acid. However, rats treated with esculetin (25mg/kg and 50mg/kg) showed a significant ($p<0.05$) increase in SOD, Cat and GPx levels.

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

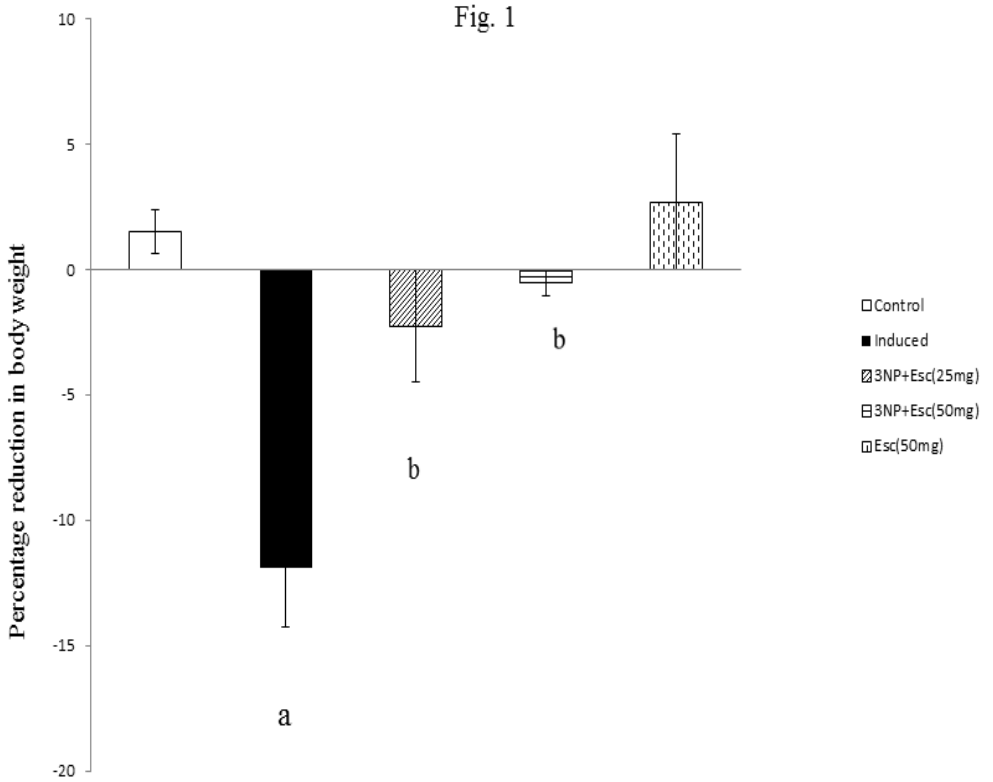

Figure 1: Effect of esculetin on 3-NP induced changes in body weight.

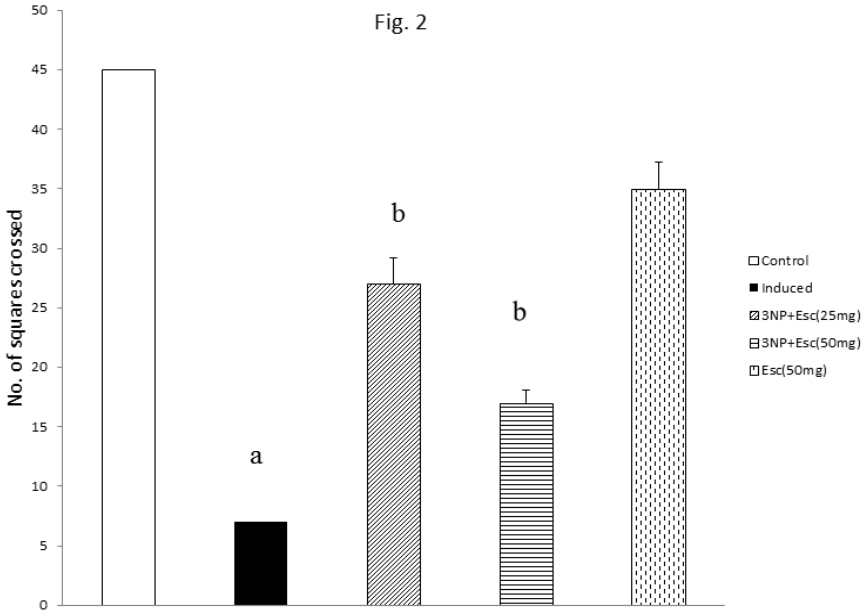

Figure 2: Effect of esculetin on 3-NP induced changes in open field test.

Effect of esculetin on 3-NP induced changes on the levels of succinate dehydrogenase

The activity of succinate dehydrogenase was significantly ($p<0.01$) reduced on treatment with 3-NP. There was asignificant ($p<0.05$) increase in SDH activity upon treatment with esculetin (25mg/kg and 50mg/kg) (Fig.12).

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Effect of esculetin on 3-NP induced changes on the levels of GSH

There was a significant ($p<0.01$) decrease in the levels of GSH in 3-NP treated group which was significantly

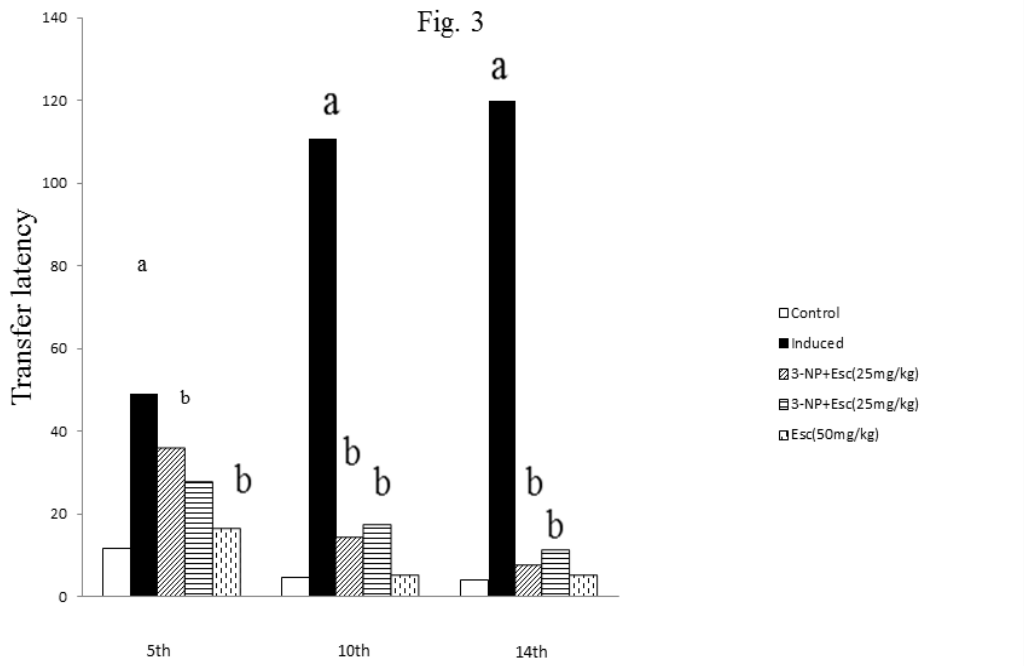

Figure 3: Effect of esculetin on 3-NP induced changes in morris water maze test.

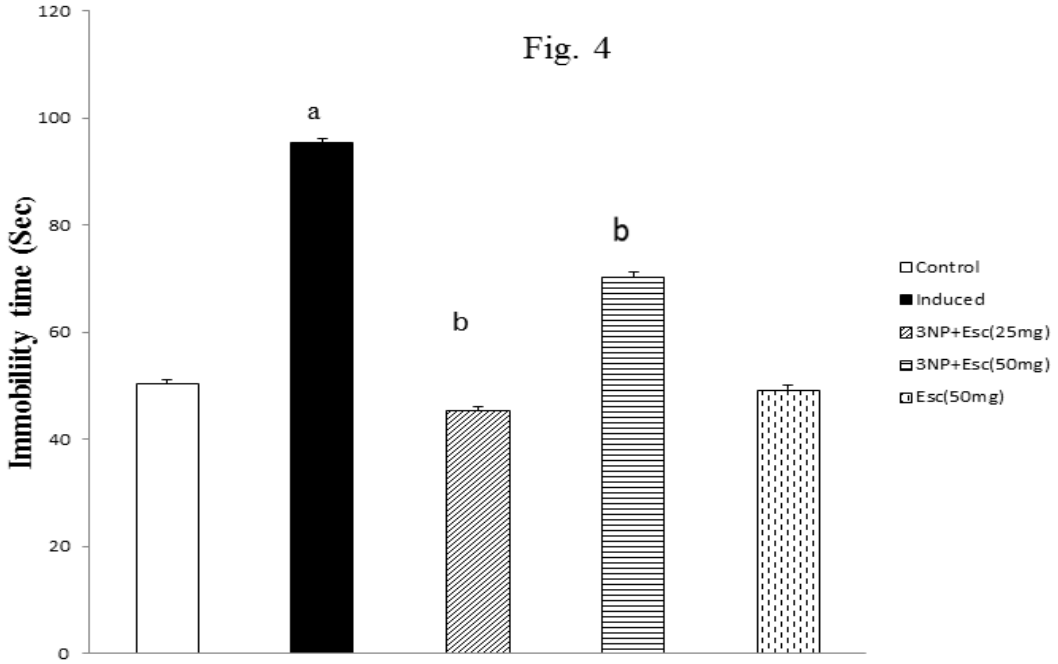

Figure 4: Effect of esculetin on 3-NP induced changes in forced swim test.

($p<0.05$) increased on treatment with esculetin (25mg/kg and 50mg/kg) (Fig.13).

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

Effect of esculetin on 3-NP induced changes on the levels of AchE

There was a significant ($p<0.01$) decrease in the levels of AchE in 3-NP treated group which was significantly ($p<.05$) increased on treatment with esculetin (25mg/kg and 50mg/kg) (Fig.14).

Data Represents mean ± SD (n=6). [a]$p<0.01$ compared to control group. [b]$p<0.05$ compared to 3-NP induced group (One way anova followed by Tukey's *Post hoc* test)

DISCUSSION

Oxidative stress has been linked to neurodegeneration. Oxidative stress results in production of free radicals which damage tissues and DNA, causes inflammation

Fig. 5

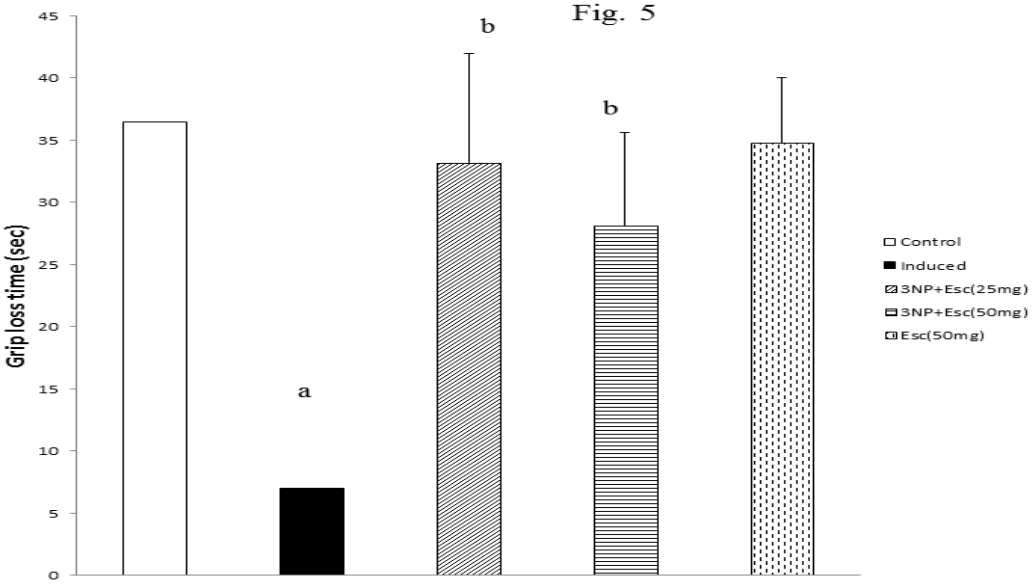

Figure 5: Effect of esculetin on 3-NP induced changes in grip strength test.

Fig. 6

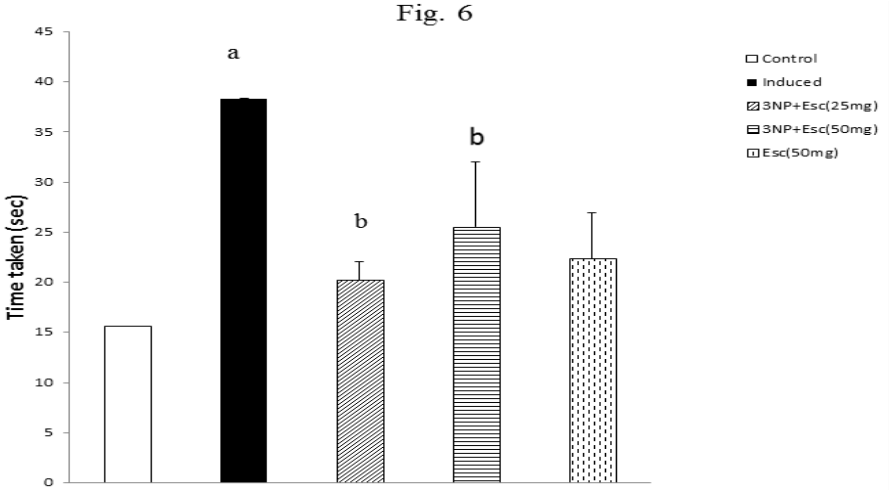

Figure 6: Effect of esculetin on 3-NP induced changes in narrow beam walk test.

and subsequently cellular apoptosis. Free radicals are molecules containing an unpaired electron. Free radicals are constantly produced in human body asby-products of aerobic metabolism. Brain is highly metabolically active with high oxygen demand and high levels of polyunsaturated fatty acids (PUFA), which makes brain highly vulnerable to oxidative stress[24].3-nitropropionic acid is a neurotoxininhibiting succinate dehydrogenase, targeting striatum, resulting in mitochondrial dysfunction[25]. SDH is Complex II of mitochondrial electron transport chain and also a membrane bound enzyme playing a role in Kreb's cycle by oxidising fumarate to succinate[26]. Inhibition of SDH leads to ETC inhibition and causes more electrons to be released from mitochondria, thus resulting in production of increased reactive oxygen species (ROS) including superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide leading to oxidative stress[27]. ROS production depletes the antioxidant levels causing oxidation of proteins and DNA[24]. 3- NP induction results in oxidative stress due to production of hydrogen peroxide, superoxide and hydroxide radicals[27]. These free radicals are normally scavenged by catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), the main antioxidant enzymes. Superoxide dismutase is known to convert two superoxide anions into one molecule each of hydrogen peroxide and oxygen[28]. This hydrogen peroxide produced is further converted to water either by CAT or by GPx[29].3-np induction causes a decrease in levels of these enzymes[30]. In our study, these enzyme levels were restored on treatment with esculetin (25mg/kg and 50mg/kg), which may be due to superoxide scavenging activity of esculetin[31].Our studies showed decreased levels of SDH in 3-np induced rats which were in accordance with earlier reports[26].

Oxidative stress results in lipid peroxidation and increased protein oxidation. As a result of increased protein oxidation, concentration of protein carbonyl produced increases[30]. Our study also reported an increase in thiol levels upon treatment with 3-NP which was significantly ($p<0.05$) attenuated on treatment with

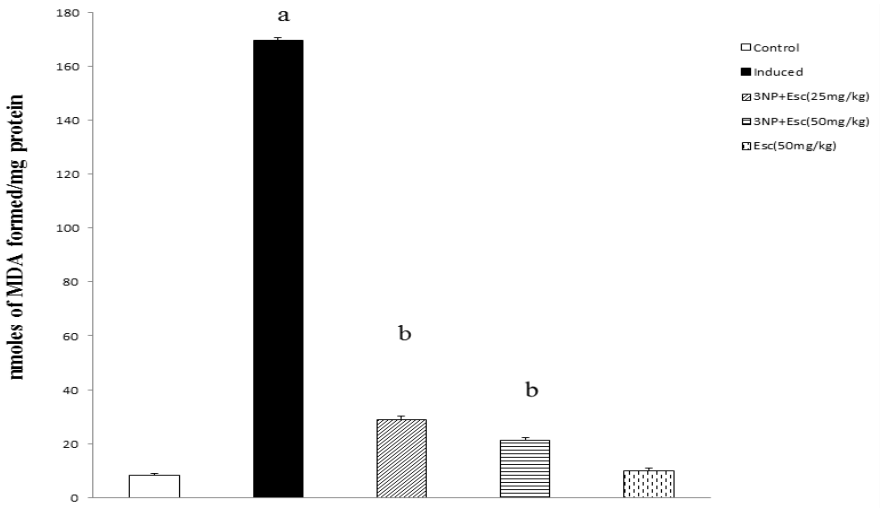

Figure 7: Effect of esculetin on 3-NP induced changes on lipid peroxidation.

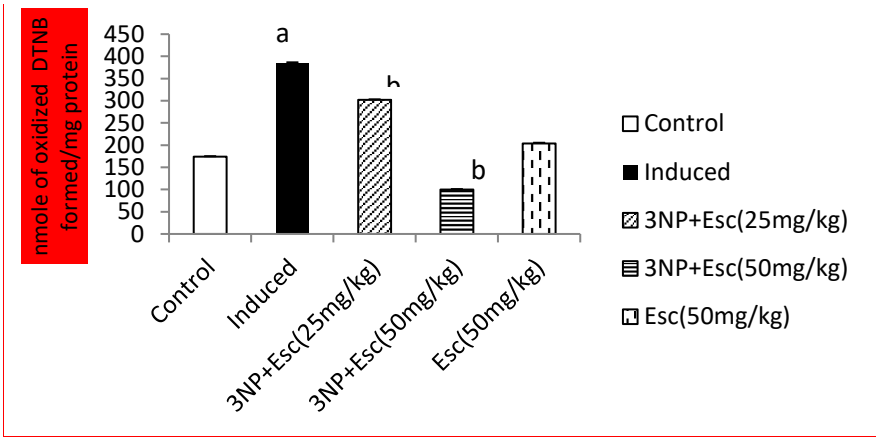

Figure 8: Effect of esculetin on 3-NP induced changes on the levels of protein carbonyl.

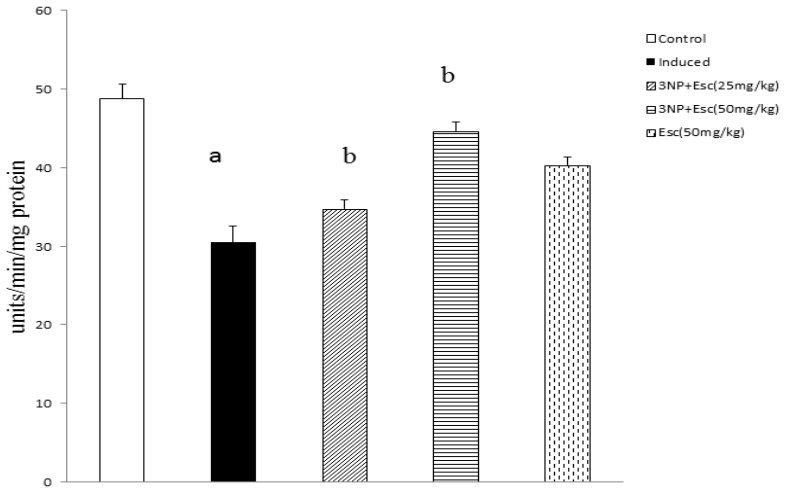

Figure 9: Effect of esculetin on 3-NP induced changes on the levels of SOD.

esculetin. In our study, the MDA levels which are a marker of lipid peroxidation were found to be elevated in 3-NP treated group which attenuated on treatment with esculetin (25mg/kg and 50mg/kg). As a result of oxidative stress, along with enzymatic antioxidants, levels of non-enzymatic antioxidants like GSH also decrease[32].

AchE is found in cholinergic synapses. There it hydrolyses the neurotransmitter acetylcholine to choline and acetate, thereby, playing an important role in cholinergic neurotransmission. Decreasing levels of acetylcholine has been implicated in many neurodegenerative disorders[33]. 3-NP being a potent neurotoxin causes a decrease in levels of AchE. We also

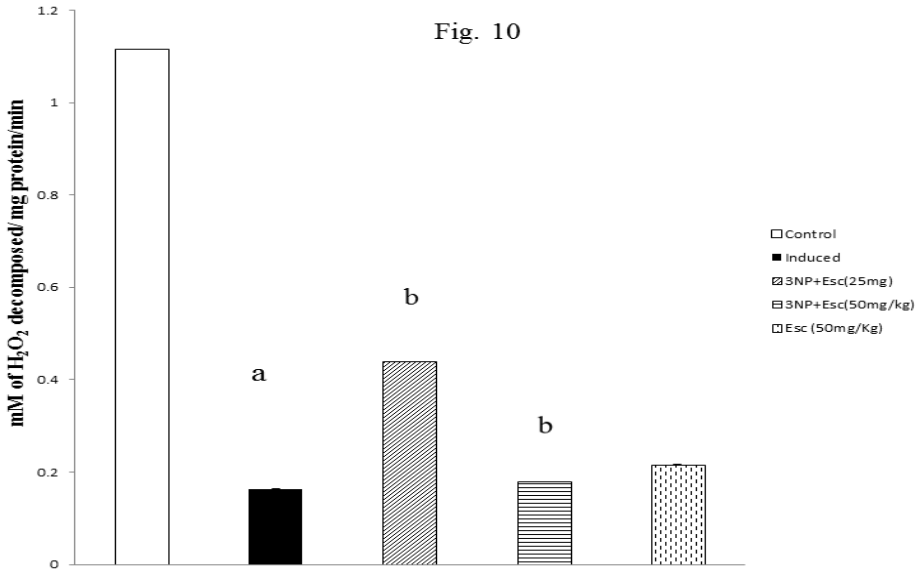

Figure 10: Effect of esculetin on 3-NP induced changes on the levels of Cat.

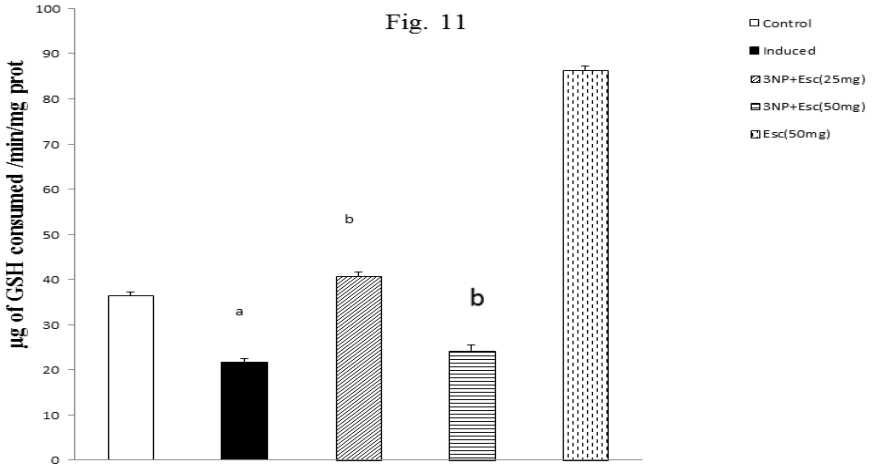

Figure 11: Effect of esculetin on 3-NP induced changes on the levels of GPx.

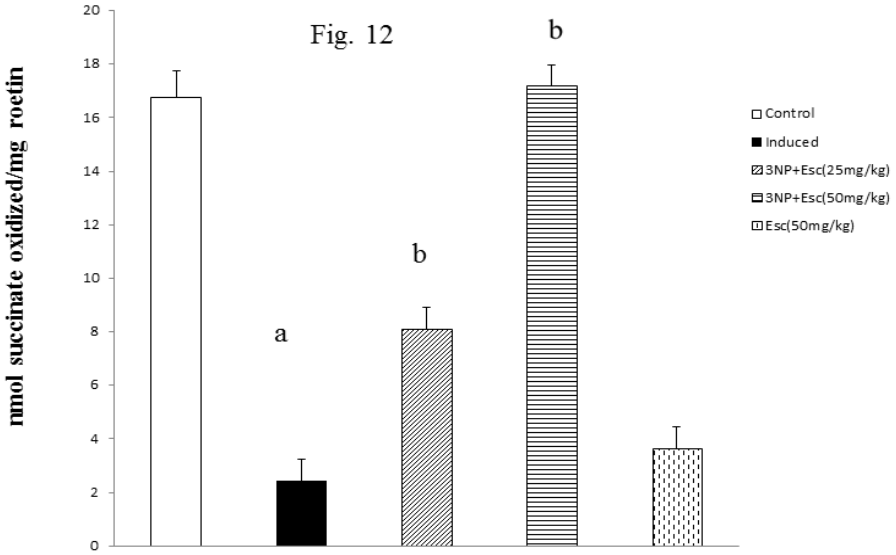

Figure 12: Effect of esculetin on 3-NP induced changes on the levels of SDH.

found aincrease in levels of AchE upon 3-NP treatment which was significantly ($p<0.05$) increased on treatment with esculetin[34].

As a result of biochemical alterations, behavioural changes occur upon 3-NP treatment. 3-NP treated group showed decrease in memory in morris water maze test as there transfer latency was more than control group whereas esculetin treated groups showed an improvement in transfer latency. Narrow beam walk test is performed to assess the motor coordination of animals. 3-NP treatment causes muscle weakness and rigidity. Subsequently animals take a longer time to cross the beam[35].

The animals were subjected to open field test for locomotory assessment. Our data shows that 3-NP treated rats had a decrease in locomotory behaviour which was significantly ($p<0.05$) attenuated on treatment with esculetin which is in accordance with previous reports[25]. Forced swim test is performed to assess the depressive behaviour. The time of immobility indicates the onset of behavioural despair.3-NP treatment causes an increase in immobility time. Our data shows a significant decrease in immobility time on treatment with esculetin (25mg/kg and 50mg/kg) which is in accordance with previous report[36].

CONCLUSION

The data combined together suggests that esculetin treatment protects against biochemical and behavioural deficits caused as a result of 3-np induced oxidative stress in striatum. Both the doses of esculetin(25mg/kg and 50mg/kg) significantly ameliorated the altered biochemical and behavioural parameters, but esculetin dose of 25mg/kg showed better results in most of the behavioural and biochemical parameters. This shows that esculetin scavenges free radicals and possess antioxidant properties.

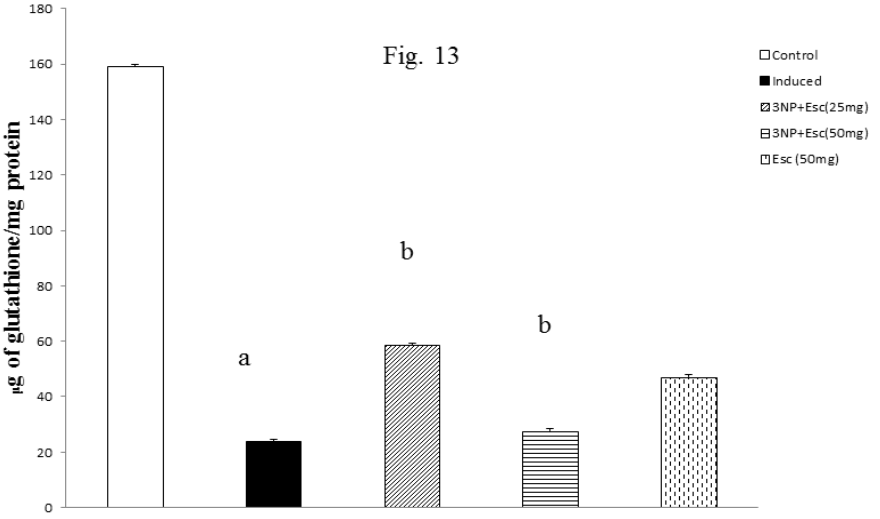

Figure 13: Effect of esculetin on 3-NP induced changes on levels of GSH.

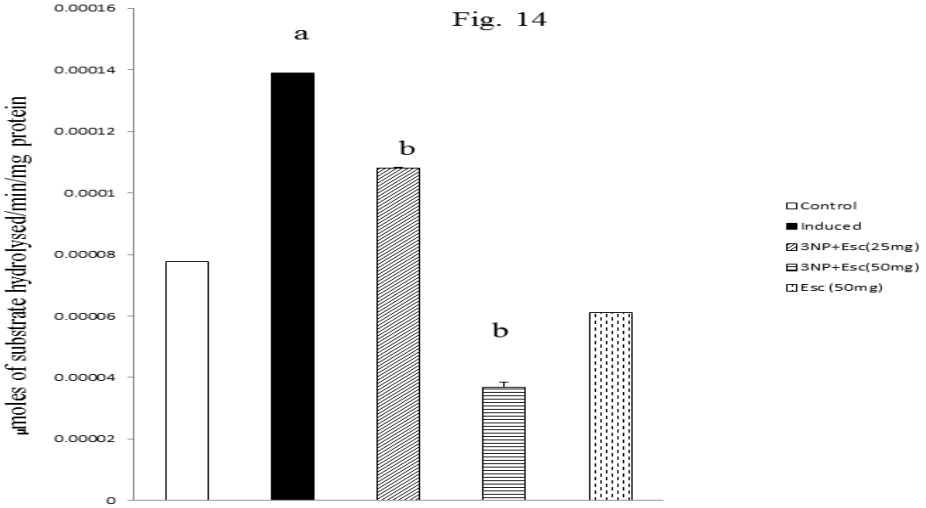

Figure 14: Effect of esculetin on 3-NP induced changes on the levels of AchE.

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