

## Study the neuroprotective effect of tyrosol in an experimental model of huntington's disease as a subsidy for the development of new medicines

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### ABSTRACT

The increase in the elderly population has led to an increasing incidence of neurodegenerative diseases worldwide. This has provided a keen interest in studies aimed at new strategies for the prevention and cure of diseases. Huntington's disease (hd) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiform movements, emotional disturbances and dementia .the 3-nitropropionic acid (3-np) is an irreversible inhibitor of succinate dehydrogenase enzyme complex ii of the respiratory chain provoked alterations similar to hd. The tyrosol is a phenolic compound that is present in olive oil and wine, it is known as a potent antioxidant and anti-inflammatory. “(a) objectives tyrosol can exert neuroprotection on behavioral and biochemical against oxidative stress induced by 3-np”, “(b) materials and methods adult rats were treated by gavage with tyrosol (3-15mg/kg) or vehicle 1h before 3-np for 7 consecutive days. Behavioral parameters were analyzed using open field, rotarod and elevated plus maze test. Lipoperoxidation in the nigroestriatal system and cerebral cortex were also evaluated.”, “(c) results the tyrosol at doses of 10 and 15 mg/kg, was able to improve the behavioral and biochemical parameters of hd induced by 3np.” And “(d) conclusion the tyrosol has an effective protection of neurons against huntington's disease.”

**Keywords:** rotarod; open field; plus maze; oxidative stress; acid 3-nitropropionic

## INTRODUÇÃO

In recent years the increase in the elderly population has led to an increasing incidence of neurodegenerative diseases worldwide. This has provided a keen interest in studies aimed at new strategies for the prevention and cure of diseases. Several natural products are recognized to possess antioxidant properties, therefore, they have been employed in the formulation of pharmaceuticals, targeting a potential neuroprotective effect. Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiform movements, emotional disturbances and dementia (Ryu et al, 2004). The HD is associated with an abnormal number of repetitions CAGs the short arm of chromosome 4 responsible for encoding the huntingtin protein, constitutively expressed in the central and peripheral nervous tissues, both in vesicles as in mitochondria (Li et al. 1996). Experimental models have been used to study neurodegenerative diseases, including some pharmacological and other transgenic models. The 3-nitropropionic acid (3NP) is an irreversible inhibitor of succinate dehydrogenase enzyme complex II of the respiratory chain and the treatment produces inhibition of this complex, with consequent release of mitochondrial calcium, oxidative stress and neuronal death, resulting in similar to changes HD (BEAL et al. 1993; ROSENSTOCK et al. 2004). The tyrosol (2-(4-hydroxyphenyl) ethanol) is a phenolic compound that is present in olive oil and wine. It is known as a potent antioxidant and anti-inflammatory (MUKHERJEE, 2009). Previous studies were designed to test the possibility for a neuroprotective effect, but results were not conclusive (BU, 2007). The present study aims to investigate the neuroprotective effect of tyrosol in an experimental model of HD.

## MATERIAL AND METHODS

### 2.1. Animal model

We used male wistar rats from the department of physiology and pharmacology, from the federal university of pernambuco (ufpe) with 3-5 months of age and weighing between 280-350g. Were also used swiss albino mice approximately 2 months of age weighing between 25-30g, from the research center aggeu magalhães (cpqam). The animals received water and food ad libitum and standard environmental conditions (12 h dark/light cycles) and were kept under controlled temperature ( $22\pm 2$  °C). Two days before the experiments, the animals were transferred to the experimental

laboratory. The experiments were performed in a room with temperature control and in the light phase of the cycle. All experimental protocols were approved by the ethics committee on animal use at the center for biological sciences ufpe under license number 23076.01977/2012-82, according to standard by brazilian college for animal experimentation.

### 2.2. Reagents

3NP was purchased from sigma (St. Louis, MO, USA), and dissolved before the beginning of the treatment in saline (pH 7.4). In subsequent days, solutions were stored in refrigerator (4 °C). The tyrosol was purchased from sigma, and dissolved in the distilled water. Butylated hydroxytoluene (bht), 2,2-diphenyl-1-picrylhydrazyl (DPPH+), thiobarbituric acid (TBA), and 1,1,3,3-tetrametoxipropane (TMP), used in biochemical analysis, were also purchased from sigma.

### 2.3. Oral acute toxicity

In this test, male and female mice ( $n = 5$ ) were housed in cages at  $22\pm 2$  °C temperature and deprived of food overnight with free access to water. One dose limits of 2000 mg/kg body weight of tyrosol, dissolved in distilled water, was administered orally to animals of both groups. In each case, the volume administered was 10 ml/kg body weight. After administration, the animals were carefully observed during the first three hours, and from there, daily, during 14 days, were observed signs and toxic symptoms and death. Signs and symptoms of toxicity included the following: (1) autonomic effects such as reflexions, salivation and piloerection; (2) central nervous system effects such as tremors and convulsions and (3) changes in the level of activity, posture, strength and bizarre behavior (Malone, 1977). The weight of the animals was measured daily.

### 2.4. Evaluation of antioxidant activity test *in vitro*

Radical scavenging activities by antioxidants in the tyrosol was determined using DPPH+ radical (2,2-diphenyl-1-picrylhydrazil) according to the method of Brand-Williams and collaborators (1995). Briefly, aliquots of 0.5 ml of solution containing different concentrations of tyrosol (5-250 µg/ml) were added to 1.5 ml of methanolic solution of DPPH+ (60 mM). The solutions were homogenized and incubated for 30 min at room temperature, and the absorbance of the resulting solution was read at 515 nm. The drop in reading the optical density of the samples and the

positive control bht was correlated to the control, establishing the percentage of DPPH+ discoloration, according to the formula below (Equation 1):

$$\frac{(\text{absorbance of control} - \text{absorbance of test}) \times 100}{\text{absorbance of control}}$$

(Equation 1)

Was calculated the value of ic 50 (concentration of sample required to inhibit 50% of radical).

## 2.5. Experimental groups

The animals were randomly divided into six experimental groups (n = 8). Group 1 were treated intraperitoneally (i.p.) for seven days with vehicle (nacl 0.9% i.p.) and water orally (10 ml/kg, p.o.); group 2 received 3-np (20 mg/kg, i.p.) for seven days and water (p.o.); groups 3-6 received 3-NP as the same in group 2, plus the animals received, one hours before treatment with 3-NP, tyrosol oral doses in levels of 3, 5, 10 and 15 mg/kg, respectively. Twenty four hours after the last day of treatment, the rats were evaluated in behavioral tests. After the behavioral assessments animals were anestesiaded, sacrificed and used for biochemical assays.

## 2.6. Evaluation of body weight

Animal body weight was recorded on the first day and last day of the experimentation. Percentage change in body weight was calculated in comparison to the initial body weight on the first day of experiment (Equation 2).

$$\frac{(\text{body weight 1day} - \text{body weight 9day}) \times 100}{\text{body weight 1day}}$$

(Equation 2)

## 2.7. Experimental procedures

The animals were submitted to behavioral tests twenty four hours after the seven day of treatment.

### 2.7.1 The locomotor activity on open field test

To quantify general activity, each rat was placed individually in the center of an open-field arena (a circular wooden box with 100 cm in diameter and 40 cm high, with floor divided into 19 regions). The frequency of locomotion (number of floor units entered by the animal with four paws),

immobility time (time that the animal stood still without making any movement), grooming (number of times that animal self-cleaning) rearing frequency (number of times the animal stood on its hind legs, with the trunk perpendicular to floor of arena) and start the movement time (start time for the animal to move), were assessed for five min. The cleaning of arena was made with alcohol at 5%, before each test.

### 2.7.2 The elevated plus maze test for spatial memory

The test was performed according to the method of Kumar et al. (2006). The elevated plus maze consists of two opposite open arms (50x10 cm), crossed with two closed arms of same dimensions with 40 cm high walls. The arms are connected with central square (10x10 cm). The rats were placed individually at one end of an open arm facing away from the central area. The time taken by animal to move from open arm until enter one of the closed arms was registered 24 hours after the last day of treatment with water or tyrosol and with vehicle or 3-np, and called for transfer of initial latency. Rats were allowed to explore the maze for 30 seconds after initial registration of acquisition latency, and then they were put back in their cages. The retention latency was evaluated again the next day.

The percentage of memory retention was calculated by the formula (Equation 3):

$$\frac{\text{transfer of initial latency} - \text{transfer latency repetition} \times 100}{\text{transfer of initial latency}}$$

(Equation 3)

### 2.7.3 Rotarod test

All animals were tested for motor skills and balance using the rotary axis or rotarod. For execution of this test, animal was placed with the four paws on a bar of diameter 7.0 cm and 25 cm above the floor, rotating at speed of 25 rpm. Before dividing each experimental group, the rats were trained in two sessions of 180 seconds for acclimatization. The animals were placed on rotating bars and length of stay was recorded. The cut off time used was of 180 seconds (kumar & kumar , 2009)

## 2.8. Biochemical studies

### 2.8.1 dissection and homogenization

After behavioral assessments animals were anesthetized and scarified for decapitation, the brains removed, placed on ice and the striatum,

cortex and substantia nigra dissected. These regions were weighed and homogenized in potter elvehjem type homogenizer with 1x pbs buffer (10% w/v), to which was added bht (0.004% w/v) to prevent autoxidation of the samples. The homogenate was centrifuged at 10.000 g for 15 minutes at 4° c, and an aliquot of supernatant was separated for biochemical analyzes.

### 2.8.2 Measurement of lipid peroxidation

Quantitative measurement of lipid peroxidation in regions dissected was assessed according to method of buege and aust (1978). The amount of malonaldehyde (mda) present in samples was quantified by reaction with tba. Aliquots of 500 µl of supernatant were added to 1 ml of reagent tba 0.38% (w.v.), 250 ml of 1n hydrochloride acid (hcl), trichloroacetic acid (tca) 15% and 20 ml of ethanolic bht (2%). The solution was heated at 100 °c for 15 minutes, followed by the cooling in an ice bath. 1.5 ml of n-butanol was added, shaken and centrifuged to 3000 g. After centrifugation, was collected the upper layer that was analyzed in spectrophotometer (cary 3e - uv - visible spectrophotometer varian) at 532 nm. All determinations were made in triplicate and contained only white n-butanol. For calculations, was made a standard curve with 1,1,3,3-tetramethoxypropane. Results were expressed as nmol mda/mg protein.

### 2.9. Statistical analysis

Results were expressed as MEAN ± SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by post hoc tukey's multiple comparison tests to determine the significance level. Statistical analyses were performed using Graph Pad Prism® 5.0. Values less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION:

### 3.1. Reduction of 2,2-diphenyl-1-picrylhydrazyl radical

Significant DPPH+ radical scavenging activity was evident at all concentrations tested. The preparation was able to reduce the stable free radical DPPH+ to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl with an IC50 8.51 µg/ml. The synthetic antioxidant bht at similar conditions showed an IC50 62.71 µg/ml.

### 3.2. Acute toxicity

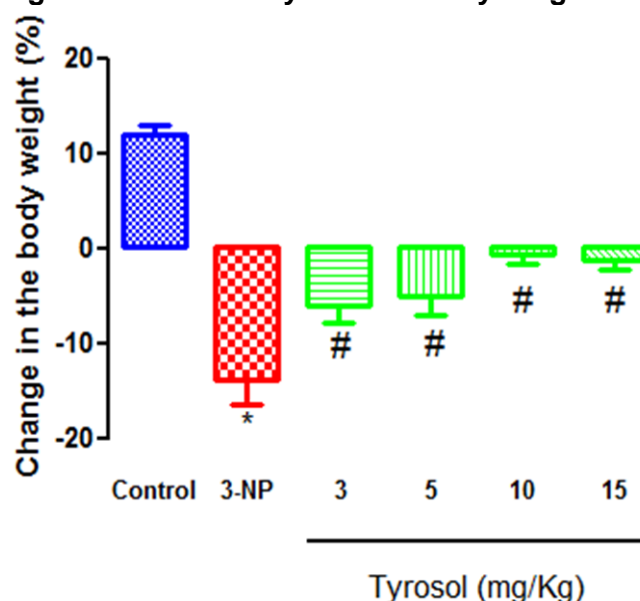
It was observed that, during the 14-day experiment, tirosol produced no death, no toxic

signs or negative symptoms in any animal. There were no changes in the corporal weight and all animals exhibited a gain in body weight. In the daily consumption of water and food, there were no changes in both sexes. Therefore, the acute minimum fatal dose of the tyrosol for swiss mice is higher than 2000 mg/kg b.w.

### 3.3. Effect of tyrosol on body weight in 3-NP treated rats

There was no change in initial and final body weight of vehicle treated animals. However, 3-np treatment caused a significant decrease in body weight on last day as compared to vehicle treated group. Daily treatment with tyrosol (3, 5, 10 and 15 mg/kg, p.o.), for seven days, significant decrease the body weight loss in 3-np treated rats (figure 1).

Figure 1 – Effect of tyrosol on body weight.

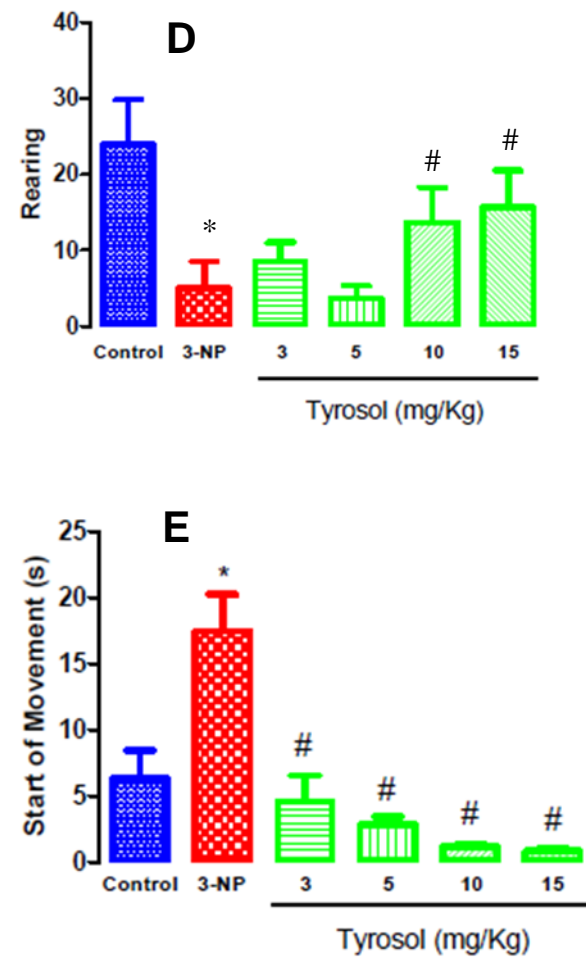
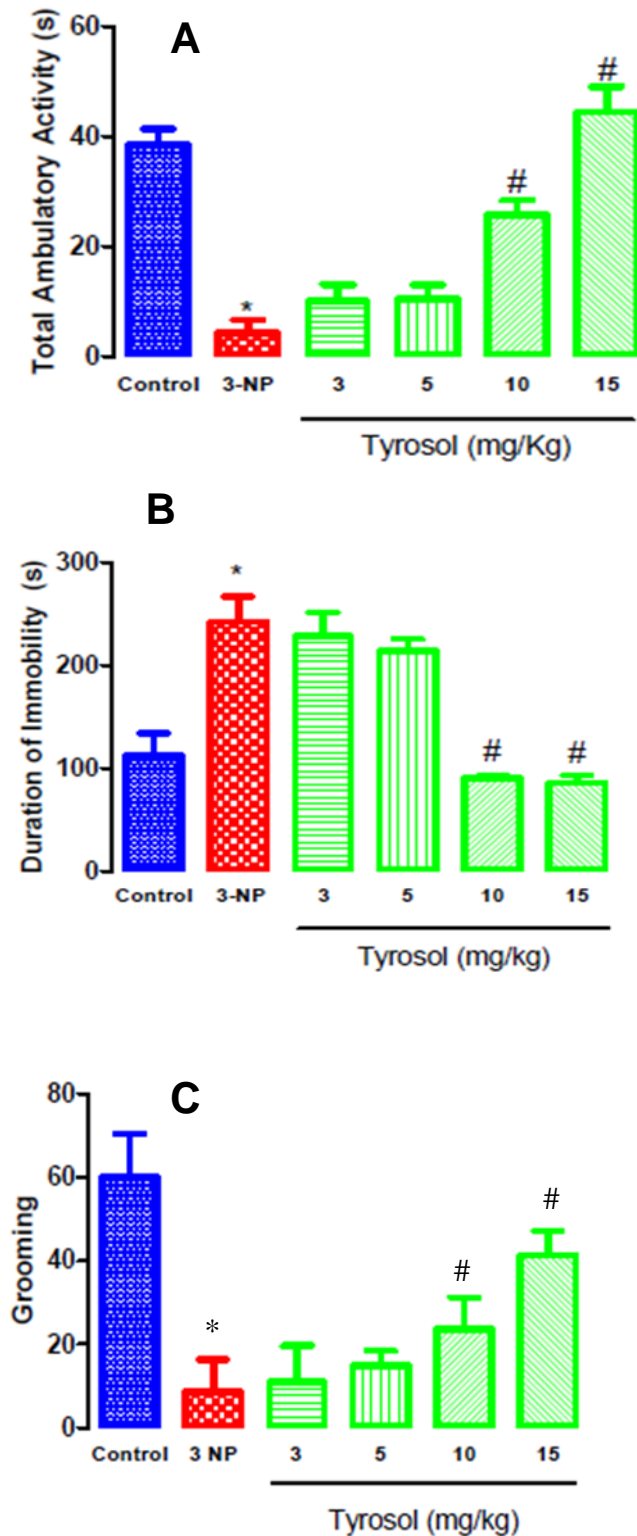


Legend: Each bar represents the mean ± standart error of the mean \*p<0,05 as compared to control group; #p<0,05 as compared to 3-NP group. One-Way analysis of variance followed by Tukey's comparsion test.

### 3.4. Effect of tyrosol on locomotor activity in 3-NP

Administration of 3-np for seven days in open field test, significantly reduced the total ambulatory activity, rearing and grooming and increased the time to start the movement and duration of immobility. Treatment with tyrosol (10 and 15 mg/kg, p.o.) significantly improved all parameters (figure 2). Daily treatment with tyrosol increased the ambulatory, grooming and rearing movements, and decreased the time to start to movement and duration of immobility.

**Figure 2 – Effect of treatment with tyrosol in open field test**

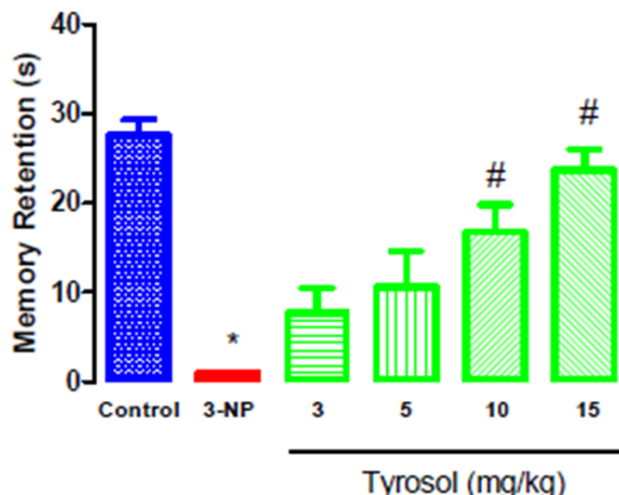


Legend: Total ambulatory activity (A), duration of immobility (B), grooming (C), rearing (D) and start of movement (E) in 3-nitropropionic acid (20 mg/kg i.p.) treated. Each bar represents the mean  $\pm$  standard error of the mean \* $p < 0,05$  as compared to control group; # $p < 0,05$  as compared to 3-NP group. One-Way analysis of variance followed by Tukey's comparison test

### 3.5. Effect of tyrosol on memory performance in elevated plus maze

Administration with the 3-np (20 mg/kg., i.p.) for seven days caused marked memory loss as shown by a significant decrease in the % retention of memory in rats as compared to vehicle control group (figure 3). Daily administration of tyrosol (3, 5, 10 and 15 mg/kg, p.o.) one hour before the treatment with 3-NP for seven days increased the % retention memory in rats as compared to 3-NP alone.

**Figure 3 – Effect of tyrosol in the test of elevated plus maze**

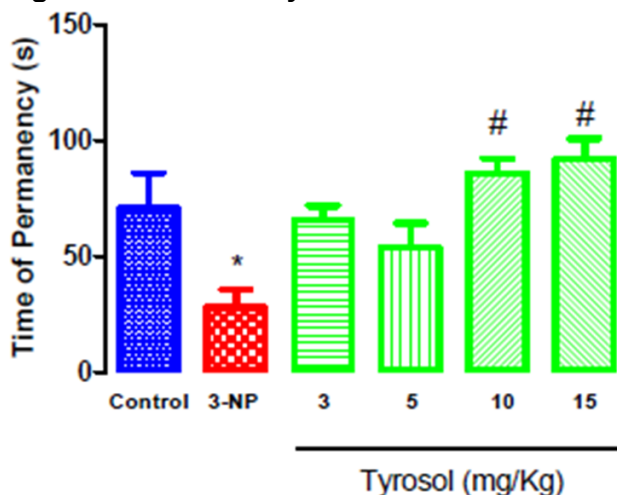


Legend: Each bar represents the mean  $\pm$  standard error of the mean \* $p < 0,05$  as compared to control group; # $p < 0,05$  as compared to 3-NP group. One-Way analysis of variance followed by Tukey's comparison test

**3.6. Effect of tyrosol on rotarod activity in 3-NP treated rats**

Treatment with 3-np (20 mg/kg, i.p.) significantly decreased muscle grip strength and significantly decreased the time of permanency (s) as compared to control animals (figure 4). Daily treatment with tyrosol (10 and 15 mg/kg, p.o.) significantly increased the time of permanency as compared to 3-nitropropionic acid alone treated group.

**Figure 4 – Effect of tyrosol in the rotarod test**

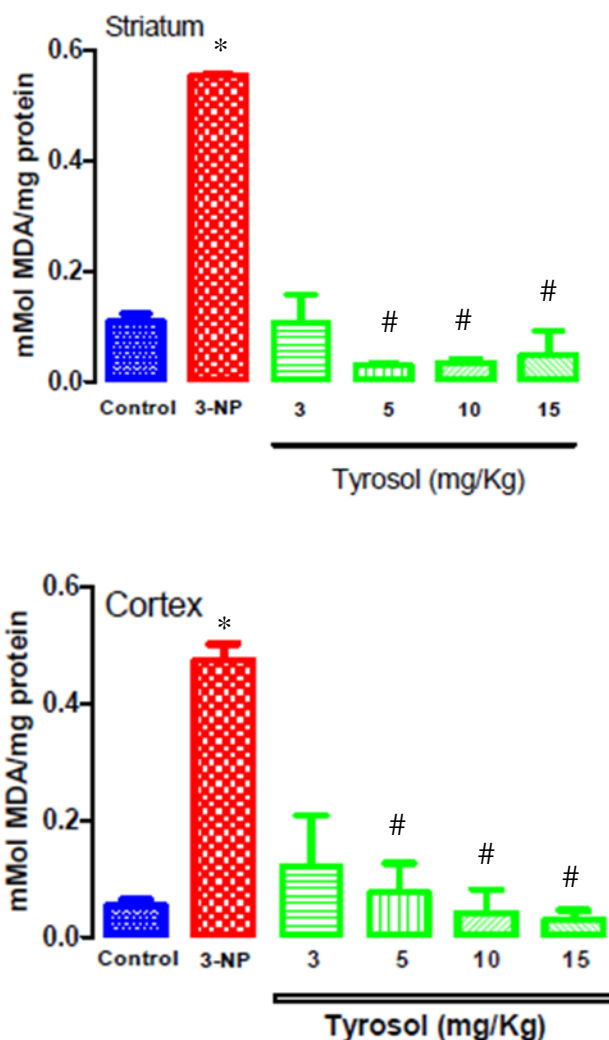


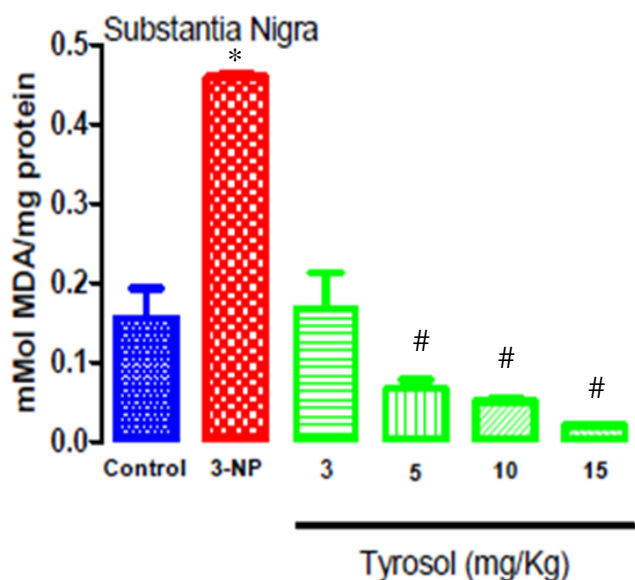
Legend: Each bar represents the mean  $\pm$  standard error of the mean \* $p < 0,05$  as compared to control group; # $p < 0,05$  as compared to 3-NP group. One-Way analysis of variance followed by Tukey's comparison test

**3.7. Effect of tyrosol on brain lipid peroxidation in 3-NP treated rats**

3-NP administration resulted in significant changes in biochemical parameters as compared to control animals. Administration of 3-NP induced oxidative stress as indicated by a significant increase in the striatum, cortex and substantia nigra mda levels as compared with vehicle control group. Tyrosol treatment (3, 5, 10 e 15 mg/kg, p.o.) one hour before the treatment with 3-NP attenuated the increase in lipid peroxidation in striatum, cortex and substantia nigra as shown by a significant decrease mda levels (figure 5).

**Figure 5 – Effect of treatment with tyrosol lipoperoxidation**





Legend: Each bar represents the mean  $\pm$  standard error of the mean \* $p < 0,05$  as compared to control group; # $p < 0,05$  as compared to 3-NP group. One-Way analysis of variance followed by Tukey's comparison test

In this study, treatment with tyrosol in levels of 3, 5, 10 and 15 mg/kg, p.o. During seven days in which was administered 3-np sharply reduced the toxicity caused by systemic administration of neurotoxin. Treatment with 3-np (20mg/kg, i.p.) Produced deficits in motor and cognitive functions, including bradykinesia, abnormal gait, hypoactivity, weakness and muscle stiffness, and significant reduction in body weight. Caused also neurochemical changes, in specific brain regions. These findings are in agreement with previous investigations who also noted a variety of neurobehavioural and motor abnormalities in rats after administration of 3-np (kumar and kumar, 2009). 3-np has as main mechanism of neurotoxicity the irreversible inhibition of mitochondrial enzyme succinate dehydrogenase, resulting in reducing the levels of atp and metabolic impairment that leads to cell death (beal et al. 1993; rosenstock et al, 2004).

Normally, the course of hd also begins with involuntary movements (chorea), followed by decrease on motor coordination and loss of control of voluntary movements that eventually evolves to rigidity and dystonia similar those of model induced by administration of 3-np. Effects on the gait abnormalities and movements of body are also supported in the evidence that interstitial injection of 3-np causes the reduction in markers for both striatal intrinsic neurons such as gaba, substance p, and somatostatin as well as a decrease in markers for striatal afferents such as dopamine and its metabolites (nam et al., 2005). Loss of body weight and hypoactivity can be simply because of energy

metabolism depressed after treatment with 3-np. The hypoactivity together with neuronal loss in striatum dorsolateral showed that disturbances of rigidity and movement are related to lesions of basal ganglia (beal et al., 1993).

Treatment with tyrosol significantly improved weight loss when compared to group treated only with 3-np. The weight reduction observed after injection of 3-np may be due to impaired energy metabolism, mobilization of energy reserves and lipid peroxidation that are peripheral effects of administration of 3-np (kumar and kumar, 2009). However, striatal lesions and bradykinesia can be partly responsible for reducing the food intake, appetite and motor incoordination in rats (fontaine et al, 2000).

In the evaluation of acute toxicity, our results show that the minimum lethal dose is more than 2000 mg/kg. Previous acute toxicity tests with tyrosol and showed minimum lethal dose is higher than 2000 mg/kg by the oral route can be considered practically nontoxic (konan et al, 2007; carvalho et al., 2011).

Administration of 3-np is associated with both hyperactivity and hypoactivity, depending on frequency and duration of treatment (borlongan et al., 1997). Animals that received only 3-np exhibited hypoactivity significant, incoordination motor and cognitive deficits that resemble clinical symptoms related to hd. Daily administration of tyrosol for seven days significantly attenuated hipolocomotion, the motor activity impairment and cognitive disorder, compared to animals treated with 3-np. The musculoskeletal injury may be related to reduced energy levels and consequently to changes in neural processing (seaman et al., 2000). Motor changes evidenced by decreasing the fall-off time in rotarod are attributed mainly to the degeneration of neurons of striatum, region functionally connected by afferences motor cortex (brown, 1992). The memory of patients with hd often decreases with degeneration of neurons in brain (barquero-jiménez e gómez-tortosa, 2001). Cognitive dysfunction can be due to disruption of circuit striatum-frontal in patients with hd (becker et al., 2003).

3-np binds irreversibly the enzyme succinate dehydrogenase inhibiting routes of oxidation of fatty acids. Inhibition of these pathways contributes to energy deficiency of metabolism. Therefore, the 3-np causes an energy deficit that leads to depolarization of membrane potential, followed by release of substrate for production of reactive oxygen species (ros) and, consequently, oxidative stress (tunez et al., 2010). Previous investigations

clearly demonstrate that the increased oxidative stress may be major deleterious events in hd (borlongan et al., 1997). Shinomol and muralidhara (2008) observed that various brain regions exposed to 3-np showed significantly elevated levels of ros, mda and hydroperoxide, and marked reduction in activity of antioxidant enzymes (sod, catalase and glutathione), suggesting induction of oxidative stress. Normally oxidative stress is counteracted by endogenous antioxidants and free radical scavengers (stack et al., 2010). Therefore, the administration of several antioxidants have demonstrated significant improvements in oxidative stress and lesions produced by 3-np (fontaine et al, 2000; kim et al, 2005; kumar, kumar, 2009; mutairy et al., 2010). In this study, after administration of 3-np, there was increase in level of mda (indicator of lipid peroxidation) in the striatum, cortex and substantia nigra, suggesting increased central oxidative stress. These effects were attenuated by treatment with tyrosol suggesting the antioxidant action of compound. The antioxidant activity of compound was also demonstrated in vitro, by dpph+ radical assay, which evaluates the antioxidant capacity of study compound to capture hydrogen radical, since the dpph+ is already clearly recognized by their ability to donate hydrogen (shinomol and muralidhara, 2008). It has scavenging potential towards oxygen and nitrogen reactive species, including hydroxyl radical, peroxy nitrite, superoxide radical, hydrogen peroxide and hypochlorous acid (rietjens et al, 2007).

## CONCLUSION

Taken together, these results confirm that administration of 3-NP in rats induce neurobehavioral and biochemical changes similar those found in hd and suggests that neurodegeneration occurred due to increased oxidative damage. Furthermore, treatment with tyrosol showed protective effect against 3-NP induced neurotoxicity, probably due to its antioxidant activity.

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