

## Original Article

# Protective Effects of Water Extract of *Morus Nigra* L. on 6-Hydroxydopamine Induced Parkinson's Disease in Male Rats

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## Abstract

**Background:** Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Although the etiology of PD is unknown, but major biochemical processes such as oxidative stress is largely described. Angiotensin II activates NADPH depending oxidases and produce superoxides formation. *Morus nigra* L. extract is an Angiotensin Converting Enzyme (ACE) inhibitor and tested for anti-Parkinsonism effects by biochemical and behavioral evaluations.

**Materials and Methods:** In total 48 Male Wistar rats weighting 200-250 g were divided into 4 groups: (1) Sham (normal saline was injected in the left SNC), (2) Neurotoxin (injection of 6-hydroxydopamine into left SNC), (3) *Morus nigra* L. aqueous extract and (4) captopril. *Morus nigra* (10 mg/kg) and captopril (5 mg/kg) were daily-injected i.p. from 6 days before neurotoxin injection, until one day after 6-hydroxydopamine injection. Muscle stiffness and apomorphine test were assessed in 6 rats of any groups after two weeks. Protein oxidation, lipid peroxidation and ACE activity were assessed in brains of 6 rats of each group after 24 hours.

**Results:** Rotation test with apomorphine, Rigidity with Murprogo's test, and lipid peroxidation in sham, captopril and *Morus nigra* groups were significantly lower than neurotoxin group. Protein oxidation in *Morus nigra* group was significantly lower than neurotoxin group. Brain ACE activity in neurotoxin, captopril and *Morus nigra* groups were inhibited.

**Conclusion:** *Morus nigra* L. extract had protective effects on neuronal oxidation and death and improved signs of PD possibly by ACE inhibition.

**Keywords:** Angiotensin, Medicinal plants, Parkinson's disease, Oxidative stress, *Morus nigra*

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## Introduction

Parkinson's disease (PD) is the most prevalent neurodegenerative disease after Alzheimer's disease (AD) that affecting approximately 1% of people over 65 years of age<sup>1</sup>, through which the basal ganglia cells and substantia nigra cells are destroyed and then the level of dopamine is decreased<sup>2</sup>. Oxidative stress

is mentioned as an important casual factor for PD<sup>3-5</sup>, and it is defined as an imbalance between production of free radicals and reactive metabolites, so-called reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidative systems<sup>6</sup>.

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II activates

oxidases related to NADPH and creates superoxides. Therefore angiotensin II destroys the dopaminergic neurons by creation of ROS through AT1 receptors activation, and inhibition of Renin Angiotensin System (RAS) is important in the treatment of PD<sup>4,7</sup>. Dopamine levels increase in different areas of brain has been shown after administration of captopril, so ACE inhibitors like captopril may be useful in the treatment of PD<sup>5,8</sup>. It has been shown that subcutaneous injection of captopril to PD rats decrease oxidative stress in midbrain<sup>3</sup>. We have previously shown *in vitro* ACE inhibition by *Morus nigra* L. in 135 medicinal plants, which screened for ACE inhibitory effect<sup>9</sup>. Some of these plants tested and exhibited PD alleviation in 6-hydroxy dopamine (6-OHDA) model of PD in rats<sup>10,11</sup>. In this study we assessed the *Morus nigra* aqueous extract on the rat model of Parkinson's disease to evaluate its possible anti-Parkinsonism effects.

## Methods

**Chemicals:** 1,1,3,3-Tetraethoxy propane; 2,4-Dinitrophenyl Hydrazine 97%; Apomorphine hydrochloride; Cresyle violet acetate; Guanidine hydrochloride minimum 98%; Hippuryl-His-Leu; Streptomycin sulfate; Igepal; Desferrioxamine; 6-Hydroxy dopamine (6-OHDA) were purchased from Sigma Co.; Ketamine; Magnesium acetate tetrahydrate; Sucrose; Thiobarbituric acid; Trichloroacetic acid, Xylazine were purchased from the Merck Co.

**Experimental design:** In total, 48 adult male Wistar rats (weighing about 200–250 g) were used. The rats were divided randomly into four groups ( $n=12$  each):

1. Rats in sham group ( $n = 12$ ) were used as controls and received i.p. and left SNC (substantia nigra pars compacta) injections of saline.
2. Rats in neurotoxin group ( $n = 12$ ) were received 5  $\mu$ L of 6-OHDA (12.5  $\mu$ g in 0.2% ascorbic acid in normal saline) into the left SNC at a rate of 1  $\mu$ L/min.
3. Rats in Captopril group ( $n = 12$ ) were received captopril (5 mg/kg) i.p. at 144, 120, 96, 72, 48, 24, and 2 hr. before and 4 and 24 hr. after the injection of 6-OHDA.
4. Rats in *Morus nigra* L. group ( $n = 12$ ) were received aqueous extract of *Morus nigra* L. (10 mg/kg)<sup>12</sup> at 144, 120, 96, 72, 48, 24, and 2 hr. before

and 4 and 24 hr. after the injection of 6-OHDA.

This project was approved by Shahid Beheshti University of Medical Sciences. Stereotaxic coordinates for injection in the left SNC was measured accurately as antero-posterior -4.8 mm, lateral -2 mm and dorso-ventral -8.3 mm relative to bregma according to the atlas of Paxinos and Watson. All surgery was performed under ketamine/xylazine anesthesia.

Since oxidative stress indices, estimated by determination of lipid peroxidation and protein oxidation, reach to their highest levels during 24 hr. after neurotoxin injection<sup>13</sup>. In each group 6 rats were decapitated and analyzed for oxidative stress and ACE activity 24 hr. after neurotoxin injection and 6 rats kept and examined for movement and thereafter histology study 14 days after neurotoxin injection.

100 g of *Morus nigra* leaves were powdered and soaked into 1 liter of boiling water in a beaker and kept in room temperature for 2 hr., after that the solution was filtered and freeze-dried, 10 g extract powder were yield.

### Behavioral testing

**Apomorphine test:** The animals were tested for rotational behavior by apomorphine hydrochloride (2.5 mg/kg, i.p.) 2 weeks after the surgery. The animals were allowed to habituate for 10 min and then 1 min after the injection of drugs, full rotations were counted in a cylindrical container (33 cm diameter & 35 cm height) at 10-min intervals for the first 60 min in a quiet isolated room. The number of contralateral rotations was counted as positive scores for apomorphine. Net number of rotations was defined as the positive scores minus the negative scores.

**Murprogo's test:** First rat was placed on the bench surface to study the rigidity. The animal received the score of 0.5 when it didn't move by touch. Then two wood platforms with the heights of 3 and 9 cm were used to evaluate the rigidity. Then the right hand of the animal was put on the wood-platform with the height of 3 cm, if it didn't take its hand off the platform after 10 seconds, the score of 0.5 was included. This test was repeated for the left hand of the animal as well. In the next stage, the right hand of the animal was placed on the wood-platform with the height of 9 cm, so that any other parts of the animal did not touch the platform. The animal was given score of 1, if it did not

take its hand off the platform after 10 seconds. Finally, the test was repeated in the same way for the left hand. Full rigidity in each animal was given a total score of 3.5.

### Biochemical analysis

**Lipid peroxidation:** Animals were decapitated and their brains removed 24 hours after neurotoxin injection, and kept in  $-80^{\circ}\text{C}$  freezers. Lipid peroxidation was assessed by determination of the concentration of thiobarbituric acid reactive substances (TBARS) by spectrophotometer<sup>13</sup>. The brain tissue sample was homogenized with three volumes of homogenization fluid ( $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  buffer (pH 7.4) isotonized with KCl and containing butylated hydroxytoluene (200 mM) and desferrioxamine (200 mM), for 40 sec and 4000 rpm (Tomy micro smash MS-100). 200  $\mu\text{l}$  of the resulting sample was treated with 200  $\mu\text{l}$  sodium dodecyl sulfate (SDS; 8%, w/v), followed by 750  $\mu\text{l}$  acetic acid (20%), and the mixture was vortexed for 1 min. 750  $\mu\text{l}$  of thiobarbituric acid (0.8%) was then added and the resulting mixture incubated at  $95^{\circ}\text{C}$  for 60 min. After cooling to room temperature, 3 ml of n-butanol were added, and the mixture was shaken vigorously. After centrifugation at 4,000 rpm for 5 min, the absorbance of the supernatant (organic layer) was measured at 532 nm with spectrophotometer (Cecil Instruments 2000 series). Standard curve of malondialdehyde (MDA) (5–150 nM) was generated by acid hydrolysis ( $\text{SO}_4\text{H}_2$ ; 1.5%, v/v) of 1,1,3,3-tetraethoxypropane and the TBARS results expressed as nanomoles MDA per milligram protein. The protein concentration of the sample obtained was determined according to Bradford method, with BSA as the standard<sup>14</sup>.

**Protein oxidation:** Protein oxidation was estimated by determination of protein carbonyl content by spectrophotometer<sup>15</sup>. Preparation of homogeny was done as same as lipid peroxidation. Nucleic acids were percipitated by streptomycin sulphate 1% (1:9 v/v), and centrifuged at 13000 rpm for 5 min. 200  $\mu\text{l}$  trichloroacetic acid 1M was added to supernatant and sonicated. Solution was centrifuged at 13000 rpm again. Pellet was dissolved in NaOH (0.5M) with gently vortex for 3 min. After that 800  $\mu\text{l}$  of 2,4-dinitrophenyl hydrazine 10mM in chloridric acid 2M was added and mixed in darkness for 1 hour at room

temperature. After addition of trichloroacetic acid 1M, it was centrifuged and pellet was washed with ethanol /ethylacetate (1:1 v/v). Pellet was dissolved again in guanidine 6M with  $\text{KH}_2\text{PO}_4$  buffer (pH=2.3, 20mM). Solution was assayed at 370 nm. Carbonyl groups were defined as standard with use of  $\epsilon=22000 \text{ M}^{-1}\text{cm}^{-1}$ . Protein level was assayed based on Bradford method<sup>14</sup>.

### ACE enzyme activity in brain tissue homogenate:

Brain tissue was homogenized and enzyme activity was started by incubation of 10  $\mu\text{l}$  of homogenate with 40  $\mu\text{l}$  substrate (hippuryl L- histidyl L- leucine) in a thermo mixer (Eppendorf- MTP model) for 30 min at  $37^{\circ}\text{C}$  and 300 rpm and then stopped by adding 150  $\mu\text{l}$  phosphoric acid 5M. We measured enzyme product - hippuric acid- by HPLC (Shimadzu, pump: LC-10ADVP, detector: SPD-10AV, controller: SCL-10AVP, and class-VP software). 20  $\mu\text{l}$  of each sample was injected into C18 column, washed by mobile phase (1:1 methanol and  $\text{KH}_2\text{PO}_4$  0.1M, pH=3) with 1ml/min flow rate and detected at 228nm. One unit of enzyme activity was defined as nanomol hippuric acid liberated per min at  $37^{\circ}\text{C}$  per mg total protein.

**Brain histology:** At the end of behavioral experiments, each rat was deeply anesthetized with a high dose of ketamine (150 mg/kg) and perfused through the ascending aorta with 50-100 ml of 0.9% saline followed by 150-200 ml of fixative solution containing 4% paraformaldehyde and 0.2 M phosphate buffer (pH 7.4) followed by 100 ml of 0.2 M phosphate buffer containing 10% sucrose. Following perfusion, the brain was removed from the skull, the blocks of midbrain and substantia nigra were prepared, embedded in paraffin, and then sections were cut at a thickness of 40  $\mu\text{m}$  on a freezing microtome at  $-20^{\circ}\text{C}$  and collected in phosphate buffer 0.2 M. Every other section was Nissl-stained with 0.1% cresyle violet.

**Neuronal counting:** Nissl-stained neurons of the SNC were counted manually (Light microscopy;  $\times 200$ ) using a superimposed grid to facilitate the procedure. At least two sections representative of each of four Paxinos–Watson planes (4.2, 3.8, 3.2, 2.96; Interaural) were examined by scanning the entire extent on each side. Counting was done blind to the treatments received. Number of SNC neurons was expressed as the average of counts obtained from the representative sections.

**Statistical analysis:** All data were expressed as mean  $\pm$  SEM. All data were analyzed by non-parametric Kruskal-Wallis test and each two groups were compared using Mann-Whitney test. For each group, the values of Nissl-stained cells for the injected and non-injected sides were compared. The inter-group differences were analyzed using one-way ANOVA. In all analyses, the null hypothesis was rejected at the 0.05 level.

## Results

**Apomorphine-induced circling behavior:** Stereotaxic injection of 6-OHDA had significant effect in apomorphine-induced rotational behavior at 1 hour period and it was higher than captopril, *Morus nigra* and sham group (Figure 1).

**Murprogo's test:** Rigidity in the *Morus nigra*, captopril and sham groups were significantly lower than 6-OHDA toxin group (Figure 2).

**Lipid peroxidation:** Treatment with Captopril or *Morus nigra* significantly reduced the levels of lipid peroxidation induced by the injection of 6-OHDA (Figure 3).

**Protein oxidation:** The effect of 6-OHDA injection on the carbonyl content of proteins was more

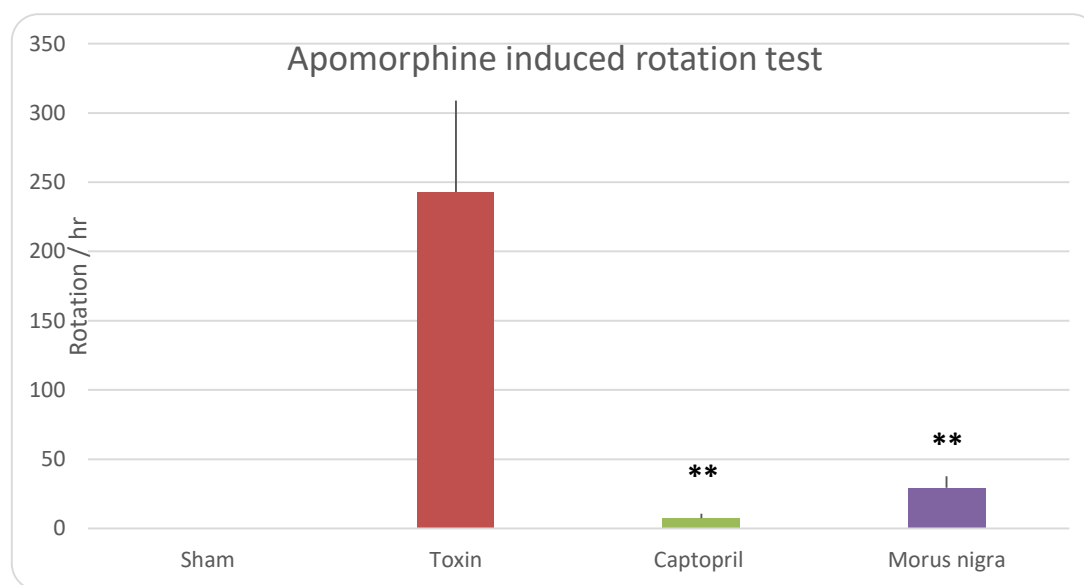
pronounced. Treatment with *Morus nigra* significantly reduced the 6-OHDA induced increase in protein carbonyl content as same as in sham (Figure 4).

**Brain ACE activity:** ACE activity of brain homogenates in neurotoxin, captopril and *Morus nigra* groups were lower than sham group non-significantly (Figure 5).

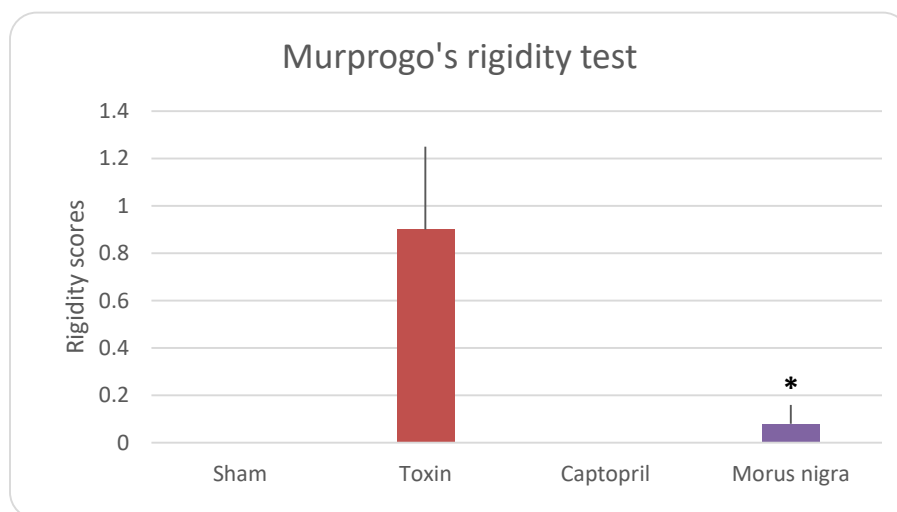
**Histological test:** The results of histological studies (Table 1 and Figure 6) showed that although there was no significant difference for the number of Nissl-stained dopaminergic neurons on the right and left sides of SNC in sham group, a significant reduction in the left side of SNC was observed for toxin group. Meanwhile, this reduction for treatment group was lower than toxin group. Injected 6-OHDA alone resulted in almost complete loss of dopaminergic neurons in toxin group compared to the other groups.

## Discussion

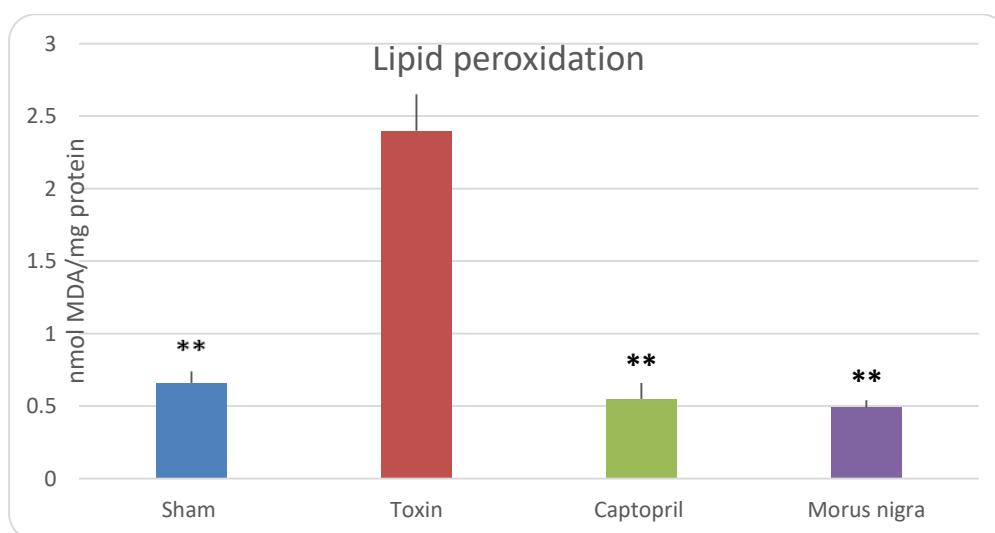
In this study we investigated the therapeutic effect of *Morus nigra* L. extract on male rat Parkinsonism model induced by 6-OHDA. Data have shown that *Morus nigra* L. aqueous extract (10 mg/kg) improved movement criteria in the diseased rats. Lipid peroxidation and protein oxidation in *Morus nigra* L



**Figure 1. Rotational test after apomorphine injection:** Rotations per hour significantly increased in toxin group but there were not any differences between captopril, *Morus nigra*, and sham group (\*\*P<0.01 related to toxin group).



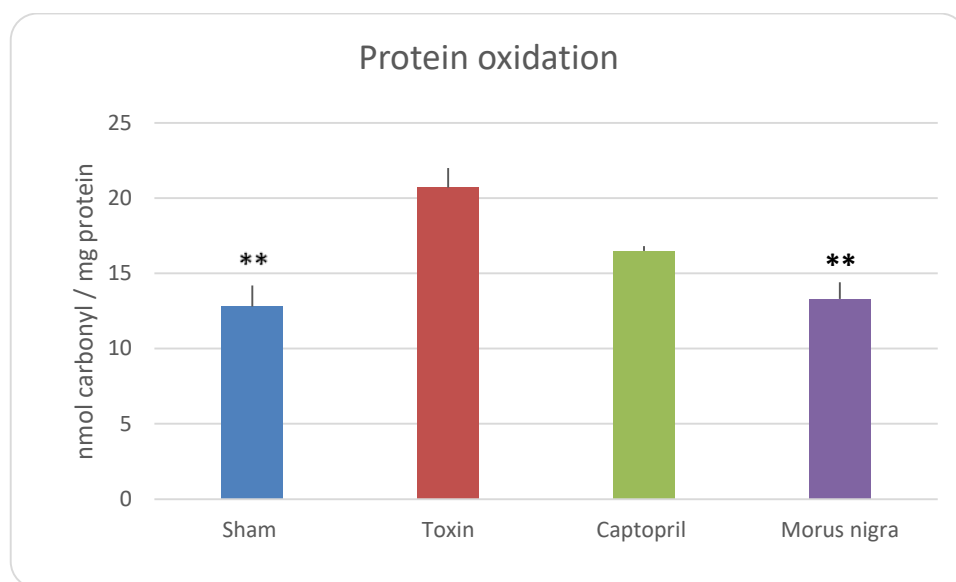
**Figure 2.** Muprogo's test: rigidity was seen in toxin and *Morus nigra* group (\* $P < 0.05$  related to toxin group).



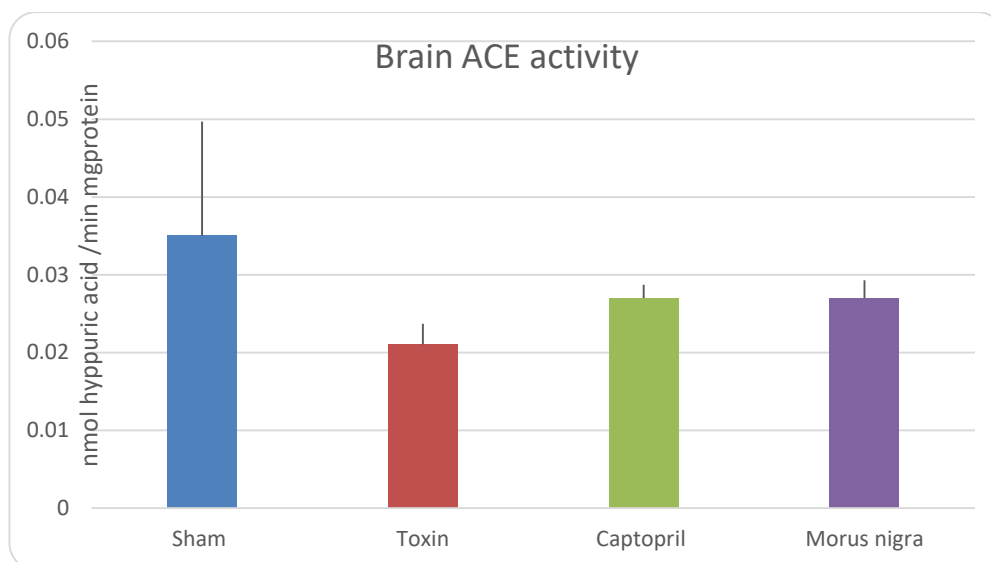
**Figure 3.** Lipid peroxidation: MDA production was decreased in captopril and *Morus nigra* group versus toxin group (\*\* $P < 0.01$  related to toxin group).

group was lower than toxin group. This may be related to ACE inhibition or direct antioxidant property of the plant. Histological study confirmed the clinical and biochemical benefit of the *Morus nigra*. Our results are supported by previous works on plants with ACE inhibitory effects<sup>10,11</sup>. We previously studied 135 medicinal plants for their ACE inhibitory effect<sup>9</sup>. Water extract of the leaves of *Morus nigra* L. was one of the most potent ACE inhibitors<sup>9</sup>.

6-OHDA is a catecholaminergic neurotoxin that widely used as a laboratory chemical in PD studies. Many data showed that 6-OHDA has a close relationship with free radicals, because MDA is increased in the presence of 6-OHDA<sup>5,16</sup>. NADPH oxidase has distributed in brain<sup>17</sup>. Non-toxic doses of some neurotoxins can help to destroy dopaminergic neurons related to NADPH, and ROS production<sup>18</sup>. Although the etiology of PD is unknown, but major biochemical processes such as oxidative stress is



**Figure 4. Protein oxidation:** Concentration of carbonyl groups in *Morus nigra* treated and sham group rats were significantly lower than toxin group (\*\*P<0.01 related to toxin group).



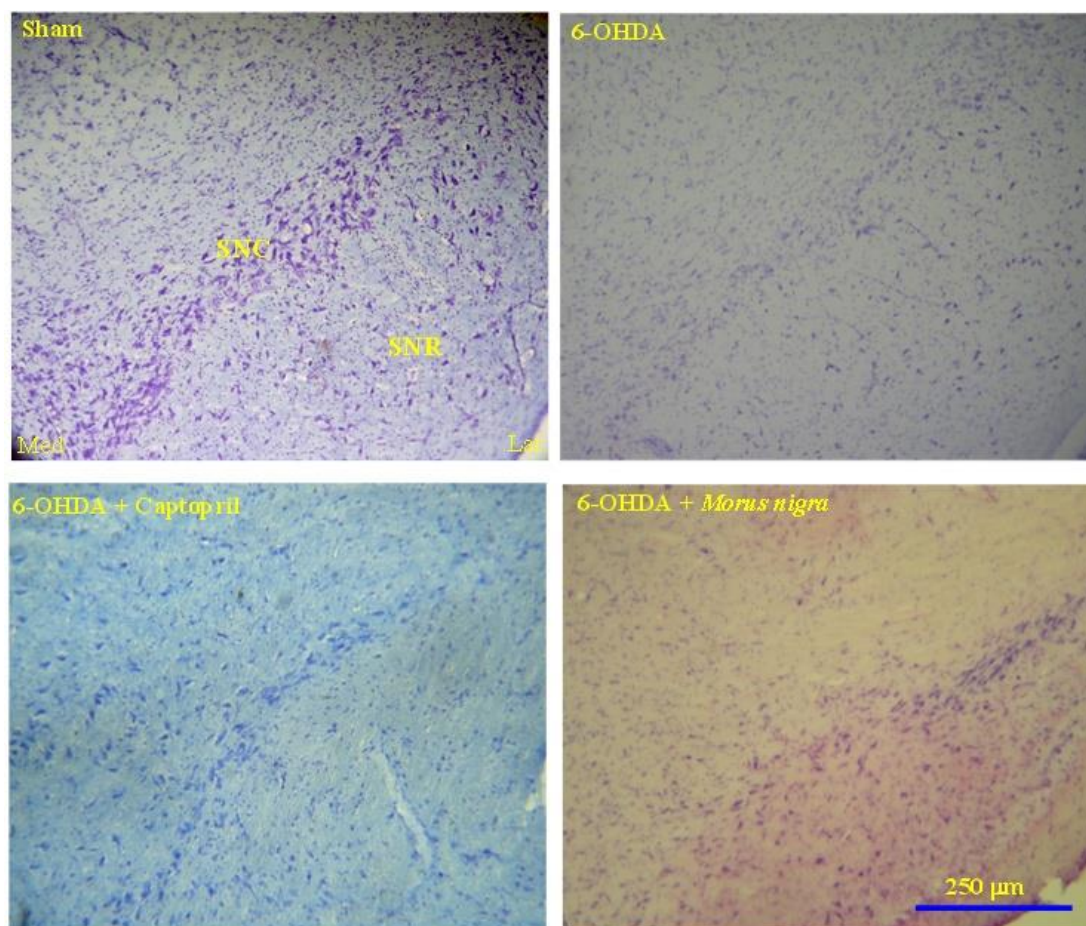
**Figure 5. Brain ACE activity in toxin, *Morus nigra* and captopril non-significantly decreased related to sham group.**

largely described<sup>1</sup>. Many studies showed that free radicals are destructive chemical substrates in PD<sup>19</sup>. Recent studies suggest that, PD is a dopaminergic neurodegenerative disease that is created by oxygen free radicals and lipid peroxidation<sup>18</sup>. Free radicals especially ROS, can produce defects by damaging DNA, proteins, enzymes and lipid membranes<sup>20</sup>. Because oxidative stress and free radicals destroy neurons and induce PD and AD, so antioxidants such vitamin E and C, can improve memory, learning and

rigidity in PD and prevent neurodegeneration in animal and human models<sup>19</sup>.

The possible explanation for the mechanism involved in ACE inhibitors' beneficial effects might be that they prevent chronic and/or toxic angiotensin II signaling via AT<sub>1</sub> receptors. AT<sub>1</sub> receptors stimulation through the activation of NADPH complex can result in ROS release (mainly the superoxide anion)<sup>21,22</sup>. ROS converted into H<sub>2</sub>O<sub>2</sub> by superoxide dismutase or it make a combination with nitric oxide to generate





**Figure 6.** Dopaminergic neurons on SNC in sham, toxin plus *Morus nigra*, and toxin plus captopril groups. Scale bar = 250 µm. SNC: substantia nigra pars compacta; SNR: substantia nigra pars reticulata.

**Table 1:** Total number of Nissl-stained neurons on the left (injected) and right sides of SNC in sham, toxin and *Morus nigra* groups were shown as mean of stained cells  $\pm$  SEM. (\*\*  $p < 0.01$  compared to the left side of the sham SNC).

SNC	Sham	6-OHDA	6-OHDA + <i>Morus nigra</i> L.	6-OHDA + captopril
Left	124.5 $\pm$ 9.7	49.2 $\pm$ 10.3**	83.4 $\pm$ 9.2	69.4 $\pm$ 7.6
Right	131.2 $\pm$ 8.9	127.8 $\pm$ 9.5	121.8 $\pm$ 7.8	135.5 $\pm$ 8.2

peroxynitrite and promotes lipid peroxidation and protein oxidation<sup>7,23</sup>. Increase in ROS production or decrease in inactivation, bring about oxidative stress and finally apoptosis. Captopril, can reduce oxidative stress by 6-OHDA significantly and is suggested that this ACE inhibitor can reduce dopaminergic neurodegeneration and progression of disease<sup>5</sup>. Treatment with captopril can prevent destruction of dopaminergic neurons significantly. This effect was

seen 7 days after treatment with captopril<sup>5</sup>. Many studies showed that ACE inhibitors are efficient by scavenging ROS<sup>20</sup>. This effect of ACE inhibitors may be related to prevention of angiotensin II synthesis and NADPH dependent oxidase activation<sup>24</sup>.

## Conclusion

In this study we showed that *Morus nigra* L. extract

reduced oxidative stress and improved signs and symptoms of PD in rats possibly through ACE inhibition.

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