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Synergistic Neuroprotective Effect of Garlic Acid Extract and Sulforaphane in 6-Hydroxydopamine-lesioned Rat Model of Parkinson's Disease

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- ERK1/ERK2
- Oxidative stress
- Apoptosis

Abstract

Parkinson's disease (PD) is characterized by selective neuronal death. One of the first toxins used to degenerate the dopaminergic neurons in the substantia nigra is possibly 6-Hydroxydopamine (6-OHDA). Our study aimed to investigate the neuroprotective effect of garlic acid extract and sulforaphane in a 6-hydroxydopamine-lesioned rat model of Parkinson's disease. the rats were assigned to five groups in the following manner: The Sham Group received saline only. The 6-OHDA group received 6-OHDA injection. The 6-OHDA /SFN group received 6-OHDA and SFN. The 6-OHDA /GE Group received both 6-OHDA and GE. The 6-OHDA /SFN /GE Group received 6-OHDA, SFN and GE. Apomorphine-induced rotational test and Hanging test in rats were performed. Oxidative stress biomarkers were measured in SN samples using a colorimetric assay. Additionally, the levels of ERK1/ERK2 were quantitatively measured. In addition, histological examinations were conducted. Both SFN and GE resulted in a significant improvement in motor tests and a decrease in oxidative stress markers. Furthermore, it led to the inactivation of ERK1/ERK2 expressions and improved structural damage in the substantia nigra. Furthermore, there was a decrease in caspase-3 expression in SN. The neuroprotective effect of both SFN and GE when given together was more than each separately indicating synergistic effect of them. Therefore, SFN and GE can be considered to be used as a combination treatment for parkinsonism.

Abbreviations: PD, Parkinson's disease; **6-OHDA,**6-Hydroxydopamine; **SFN,**sulforaphane;**GE,** Garlic Acid Extract;**SN,** substantia nigra;**ERK1/2,**extracellular signal-regulated kinase 1 and 2;**APO,** apomorphine-induced-rotational testing; **GST,**glutathione-S-transferase; **GR**, glutathione reductase; **GSH,** reduced glutathione; **MAPK**, mitogen-activated protein kinase.

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Introduction

Parkinson's disease (PD) is an age-related disorder during which there is degeneration of dopaminergic neurons in the substantia nigra **[1]**.PD is characterized by motor dysfunction such as resting hand tremors **[2]** bradykinesia, muscle rigidity**[3]**, and postural instability**[4],** non-motor dysfunctions such as rapid eye movement, anxiety, drowsiness, depression, and cognitive deficiencies **[5].**

PD is currently being treated with two different kinds of medications that use both dopaminergic and non-dopaminergic pathways. Levodopa injection is the usual therapy approach to improve PD via the dopaminergic system. Despite levodopa being the most successful treatment for PD, there is no way to completely rule out the serious long-term side effects**[6].**

Nowdays herbal medicine and natural products have shown significant role in alleviating neurodegenerative disorders **[7].**A food plant belonging to the onion family, garlic (Allium sativum) is widely used as a spice or seasoning ingredient in cookery all over the world. According to studies, garlic extract (GE) and chemicals obtained from garlic may be used to treat a variety of pathological illnesses, such as cancer **[8]**, cardiovascular problems**[9],** and neurological disorders **[10].**

Interestingly, oxidative stress and inflammation are two clear components involved in the occurrence and progression of PD **[11]**, and GE has anti-oxidation and anti-inflammatory properties **[12].** Additionally, it has been demonstrated that garlic can reverse disease-induced alterations in the levels of monoamine neurotransmitters in the brain, such as dopamine**[13].**According to a prior study, giving mice garlic may raise their dopamine levels in the brain **[13].**

A powerful dietary bioactive substance, sulforaphane (SFN) is found in cruciferous vegetables, including cauliflower, broccoli, watercress, kale, cabbage, collard greens, mustard greens, and Brussels sprouts **[14].**

SFN is a phytocompound that has anti-inflammatory, anti-apoptotic, and antioxidant properties. The scientific community has become more interested in it since neurodegenerative illnesses are linked to oxidative stress, inflammation, and mitochondrial dysfunction**[15].**Thus, SFN was studied about the major neurodegenerative illnesses, such as multiple sclerosis (MS), PD, and Alzheimer's disease (AD), based on its characteristics **[15]**.

Therefore, this study evaluated the efficacy of SFN and GE synergistic effects on this neurodegenerative disease and possible mechanisms to have naturaland appropriate treatment for PD.

1. Materials and methods

2.1 Chemicals and drugs

6-Hydroxydopamine (6-OHDA) was obtained from (Sigma–Aldrich, St. Louis, MO, USA, powder), Sulforaphane (SFN) from (Lkt Laboratories, St. Paul, MN, USA, powder), and saline solution was used as a vehicle. Before use, One kilogram of peeled garlic was combined with 70 % alcohol (300 mL of alcohol for every 100 grams of garlic). After that, the resultant solution was incubated for 72 hours at 40 °C. Using Whatman filter paper, the solution was filtered, and then Speed Vac was used to dry it (Thermofisher) **[16]**. Lastly, animals were given

garlic extract by gavage while it was stored at +4 °C.

2.2 Experimental animals and study design

This study was conducted on 50 adult male Wistar rats weighing 250–300 g, following the guidelines of our institution. The study was approved by the Research Ethics Committee of the Faculty of Medicine at Tanta University (approval code number 36264PR867/9/24). All of the rats were purchased from Animal house of Faculty of Medicine, Tanta University and kept in mesh cages from wire with free access to food and water for a week. The cages were set up on a 12-hour light-dark cycle. Rats were then randomly divided into five equal groups (10 rats each):

(i) Sham group (Group I): Rats underwent the same injection procedures as 6-OHDA group and received the same volume of saline solution as a vehicle. Also received normal saline by gavage for one week with intraperitoneal (i.p.) administration of saline twice a week for 4 weeks.

(ii) 6-OHDA group (Group II): the rats underwent unilateral lesion surgery and injected 6-OHDA (16 µg/4 µl 0.2 % ascorbate saline) into medial forebrain bundle (mfb) **[17].**

(iii) 6-OHDA /SFN group (Group III): the rats underwent unilateral lesion surgery and injected 6-OHDA into medial forebrain bundle (mfb) and after one hour, we started intraperitoneal (i.p.) administration of 5 mg/kg SFN**[18]**Neuroprotective effect of sulforaphane in 6-hydroxydopamine-lesioned mouse model of Parkinson's disease twice a week for 4 weeks**.**

(iv) 6-OHDA /GE (Group IV): 500 mg/kg of GE administered by gavage for one week, along with a 16 µg/ 4 µl injection of 6-OHDA into the left MFB at a 0.2% ascorbate-saline concentration and another week of GE administration following the injection **[17].**

(v) 6-OHDA /SFN /GE (Group V): 500 mg/kg of GE administered by gavage for one week, along with a 16 µg/ 4 µl injection of 6-OHDA into the left MFB at a 0.2% ascorbate-saline concentration and another week of GE administration following the injection concomitant with intraperitoneal (i.p.) administration of 5 mg/kg SFN twice a week for 4 weeks.

1.3 Behavioral assessments

2.3.1 Apomorphine-induced rotational test

Apomorphine-induced-rotational testing (APO) is frequently used in rat models of Parkinson's disease (PD) to validate the effects of specific neurotoxins (6-OHDA). The damage to the nigrostriatal pathway is closely correlated with the number of rotations brought on by an apomorphine injection. The APO test was conducted in this study at the end of the experimental period. Once the rats were given APO (2 mg/kg, i.p.), their contralateral rotations were observed. For thirty minutes, every rat was tested. The Parkinsonian model animals were those that rotated seven or more times per minute**[5, 6].**

2.3.2 Hanging test

Every rat was placed on a wire that measured 100 cm in length and 1 cm in diameter, and the wire was held in place by supports. The animal was then suspended from the inverted wire. Every rat's hanging time was noted, and eventually, the data were compared between the groups**[3, 7]**.

2.4 Tissue sampling

At the end of the experiment (once behavioral analysis was completed) the rats were weighted and anaesthetized with 60 mg/kg sodium pentobarbital intraperitoneally, and their heads were decapitated. Then, the skull vaults were removed, and the brains were extracted from the skull carefully, dipped in ice cold saline and left to dry. The right and left SN were dissected on an ice-cold plastic dish. After being snap frozen in liquid nitrogen, the right SN was stored at -80 °C for analysis. The Bradford method was used to assess the protein content after the tissues were homogenized in lysis buffer (50 mM Tris, pH 7.5, 0.4% NP-40, 150 mM NaCl, 10% glycerol, 10 mg/mL aprotinin, 20 mg/mL leupeptin, 10 mM EDTA, 1 mM sodium orthovanadate, and 100 mM sodium fluoride).The homogenate was centrifuged at 10000 x g for 10 minutes at 4°C and oxidative stress biomarker were measured in the supernatant aliquot. The left SN was used for histopathological and immunohistochemical analysis.

2.5 Biochemical Assays

Oxidative stress biomarkers

GST activity (Cat# No BA0028), GR activity (Cat# No TFAB00118) and GSH content (Cat# No E-BC-K030-M) were measured in SN samples using a colorimetric assay.

2.6 Quantitative assessment of brain tissue relative ERK1/2 mRNA expression by quantitative real-time reverse transcription PCR (rt-PCR):

Total RNA was extracted using the Qiagen RNeasy Total RNA isolation kit (Qiagen, Hiden, Germany) in accordance with the manufacturer's procedure from frozen brain tissue that had been treated as directed by the manufacturer. According to the OD260 and OD260/280 ratios, respectively, the total RNA concentration, purity, and integrity were calculated using a Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, USA). RNA has an A260/A280 that is preserved at -80 ° C. The first strand was then synthesized using the Super-Script III First-Strand Synthesis System for real-time PCR kit (Life Technologies, Carlsbad, California, USA) under the manufacturer's instructions. Following the manufacturer's instructions, PCR reactions were carried out using Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, California, USA). **ERK1/2** was quantified relative to the housekeeping gene **GAPDH** gene, which was used as an internal control. Primers with specified sequences were designed as the following ERK1/2 (accession number NM_138957.3) Forward: 5′-TCAAGCCTTCCAACCTC-3′ Reverse: 5′-GCAGCCCACAGACCAAA-3′ with product length 200 and GAPDH (accession number NM_002046.7) Forward: 5′-AGCCACATCGCTCAGACA-3′ Reverse: 5′-TGGACTCCACGACGTACT-3′ with product length 315. The setting for thermal cycling was as follows: After the first denaturation at 95 °C for 10 minutes, 40 cycles of initial denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds were carried out. After the final cycle, the temperature was increased from 60 to 95 °C for melting curve examination. The comparative threshold (Ct) using the $2^{-\Delta\Delta CT}$ formula was utilized to automatically calculate the relative gene expression based on the values of the target and reference genes.

2.7 Histological and Immunohistochemical studies

The tissue specimens were fixed in 10% formal saline. Dehydration in ascending alcohol grades, then treatment with xylol and immersion in paraffin were done. 5μm- thickened sections were cut and stained with:

2.7.1 Haematoxylin and eosin staining (H&E) for evaluation of the morphological characters of pars compacta substantia nigra.

2.7.2 Immunohistochemical analysis of activated caspase 3 in substantia nigra

After being deparaffinized, the specimens were rehydrated. Antigen retrieval was then completed. The samples were cleaned with phosphate-buffered saline (PBS) after being treated in 3% hydrogen peroxidase in methanol. Following that, anti-active caspase-3 antibody (Cat #: ab13847, Abcam, Cambridge, MA, USA) was incubated with the specimens. Subsequently, PBS was used to rinse the sections both before to and subsequent to incubation with biotinylated secondary antibody (LSAB kit, Dako Carpinteria, CA, USA). Subsequently, the specimens were incubated with streptavidin HRP (LSAB kit, Dako, Carpinteria, CA, USA) and then rinsed with PBS.

In order to maintain the stain, the slides were lastly cleaned in distilled water, dried, and immersed in xylene for five minutes before being mounted with a mixture of xylene, plasticizer, and distyrene. Apoptosis is indicated by the brownish coloration in neurons that test positive for active caspase-3**[20]**.

2.8 Statistical analysis

Statistical significance was determined by ANOVA with a Tukey post-hoc test for multiple comparisons. Results were considered statistically significant with P value <0.05. (GraphPad Prism, San Diego, CA).

3. Results

3.1 Effect on apomorphine-induced rotational test (fig 1A):

The number of rotations was significantly higher in the 6-OHDA group ($p<0.05$) compared to the control group. Though the 6-OHDA/SFN and 6-OHDA/GE groups caused a significant ($p<0.05$) reduction of the number of rotations compared to 6-OHDA group, they still showed a significant elevation of the number of rotations compared to the control. 6-OHDA/SFN/GE group caused a significant ($p<0.05$) reduction in the number of rotations compared to all groups including the control group.

3.2 Effect on Hanging time(fig1B):

Our results showed a significant ($p<0.05$) reduction in the time of hanging in the 6-OHDA group, 6-OHDA/SFN, and 6-OHDA/GE groups compared to control groups. The time of hanging was significantly higher ($p<0.05$) in OHDA/SFN, and 6-OHDA/GE groups compared to the 6-OHDA group. The 6-OHDA/SFN/GE group showed higher hanging time compared to the 6-OHDA group, 6-OHDA/SFN, and 6-OHDA/GE but it wasn't restored to the basic level compared to the sham group.

3.3 Effect on oxidative stress markers(fig.2):

We have demonstrated that 6-OHDA group caused significant ($p<0.05$) reduction in the level of GSH, GST and GR compared to control group. OHDA/SFN, and 6-OHDA/GE groups showed significant elevation ($p<0.05$) of the

aforementioned parameters compared to 6-OHDA group.

6-OHDA/SFN/GE group has shown remarkable results as it restored the level of oxidative stress markers as it caused significant ($p<0.05$) elevation of GSH and GST compared to 6-OHDA group, 6-OHDA/SFN and 6-OHDA/GE. And it showed significant ($p<0.05$) elevation of GR content as compared to control group showing the antioxidant potential for the combined treatment with SFN and GE.

3.4 Effect on ERK1/2 gene expression(fig.3):

We have demonstrated that the 6-OHDA group caused a significant ($p<0.05$) increase in the level of ERK1/2 gene expression compared to the control group. OHDA/SFN and 6-OHDA/GE groups showed a significant ($p<0.05$) reduction of ERK1/2 gene expression compared to 6-OHDA group. On the other hand, the OHDA/SFN/GE group showed a remarkably significant reduction ($p<0.05$) of ERK1/2 gene expression compared to the 6-OHDA group.

3.5 Histopathological Observation of substantia nigra:

***3.5.1 Hematoxylin and Eosin-staining* (Figure 4)**

Histopathological examination of H&E-stained midbrain sections revealed normal morphology of substantia nigra in sham group **(Figure 4A)**. Substantia nigra pars compacta were characterized by cell rich regions formed of multiple large and medium sized elongated or fusiform neuron with prominent vesicular nuclei.

While microscopic examination of Substantia nigra pars compacta of**6-OHDA group** showed multiple signs of neuronal degenerations **(Figure 4. B&C).** Some areas showed loss of neuronal tissue, and some sections showed marked reduction of cellular mass of pars compacta, Multiple edematous neurons were seen. While other neurons showed karyolysis of their nuclei and others lost their nuclei. On the other hand, Small pyknotic cells were also visible. Lewy bodies and macrophage infiltration were abundant.

On the other hand**,** microscopic examination of Substantia nigra pars compacta of **6-OHDA/SFN (Figure 4D) and 6-OHDA/GE (Figure 4E)** groups showed improvement of signs of neuronal degeneration. Elongated, fusiform normal neurons with prominent vesicular nuclei were seen. Some edematous neurons and small pyknotic cells were seen. Few neurons lost their nuclei. Few Lewy body and macrophage infiltration were also detected.

Pars compacta of **6-OHDA/SFN/GE group (Figure 4F)** showed marked improvement of signs of neuronal degeneration. A marked increase of neuronal cells mass were observed. Normal neuronal cells with Few Lewy bodies and loss of neuronal nuclei were detected.

***3.5.2 Immunohistochemical analysis of caspase 3* (Figure 5)**

Immunohistochemical analysis of activated caspase 3 in substantia nigra showed minimal reaction in **sham group** (Figure 5A) and **6-OHDA/SFN/GE group** (Figure 5E). Marked reaction was obvious in **6-OHDA group** (Figure 5B). **6-OHDA/SFN** (Figure 5C) and **6-OHDA/GE** (Figure 5D) groups show moderate reaction of caspase 3 positive cells when compared with other groups. These results confirmed the curative effects of **SFN** and **GE** and combination of both is the best choice in case of parkinsonism.

3.5.3Statistical analysis of %Caspase 3 area

Statistical analysis of %Caspase area showed highly significant difference between Sham group & 6-OHDA group and between 6-OHDA &6-OHDA/SFN/GE(P-value=0.000). Also, significant difference was noticed between 6-OHDA &6-OHDA/SFN and 6-OHDA &6-OHDA/ GE groups (P-value=0.000).

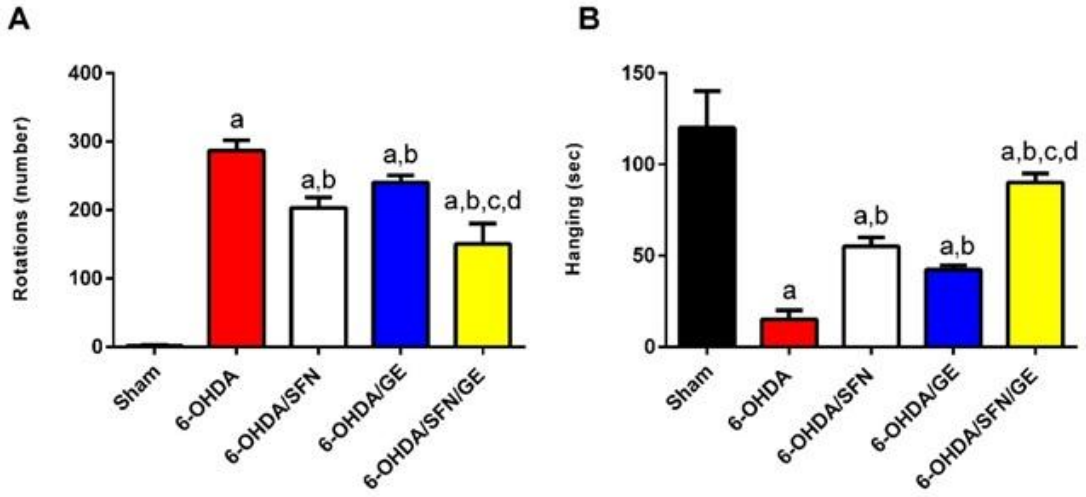

Fig. 1: Graphical representation of effects of SFN and GE on

A) Apomorphine-induced rotation test
B) hanging time
Data are represented as mean ± SD
a denotes statical significance (p<0.05) compared to control group
b denotes statical significance (p<0.05) compared to 6-OHDA group
c denotes statical significance (p<0.05) compared to 6-OHDA/SFN group
d denotes statical significance (p<0.05) compared to 6-OHDA/GE group

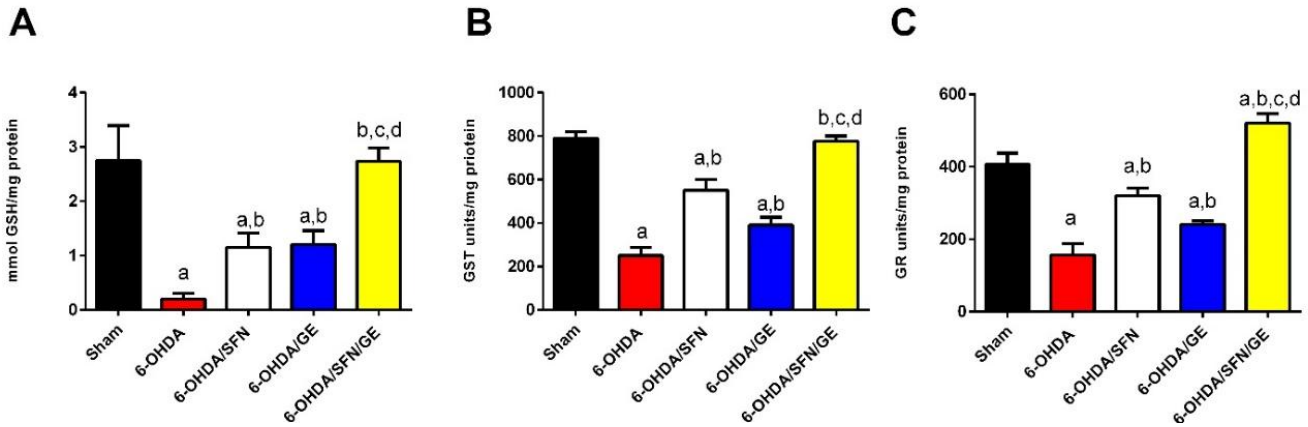

Fig.2: Graphical representation of effects of SFN and GE on
A) GSH content B) GST C) GR
Data are represented as mean ± SD
a denotes statical significance (p<0.05) compared to control group
b denotes statical significance (p<0.05) compared to 6-OHDA group
c denotes statical significance (p<0.05) compared to 6-OHDA/SFN group
d denotes statical significance (p<0.05) compared to 6-OHDA/GE group

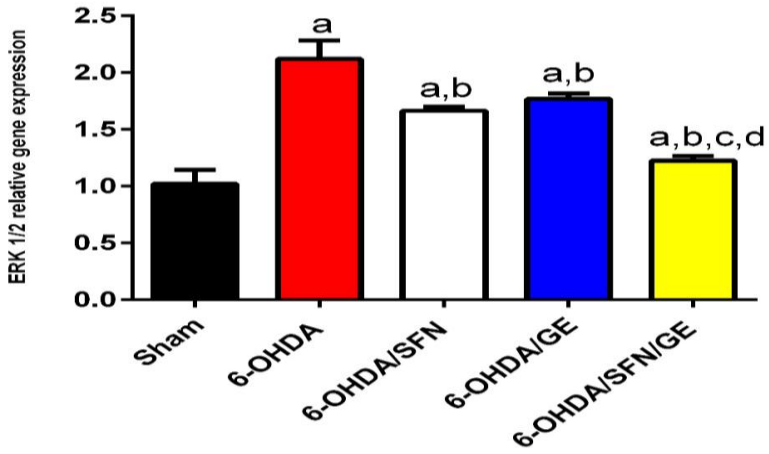

Fig.3: Graphical representation of effects of SFN and GE ERK1/2 relative gene expression
Data are represented as mean ± SD
a denotes statical significance ($p<0.05$) compared to control group
b denotes statical significance ($p<0.05$) compared to 6-OHDA group
c denotes statical significance ($p<0.05$) compared to 6-OHDA/SFN group
d denotes statical significance ($p<0.05$) compared to 6-OHDA/GE group

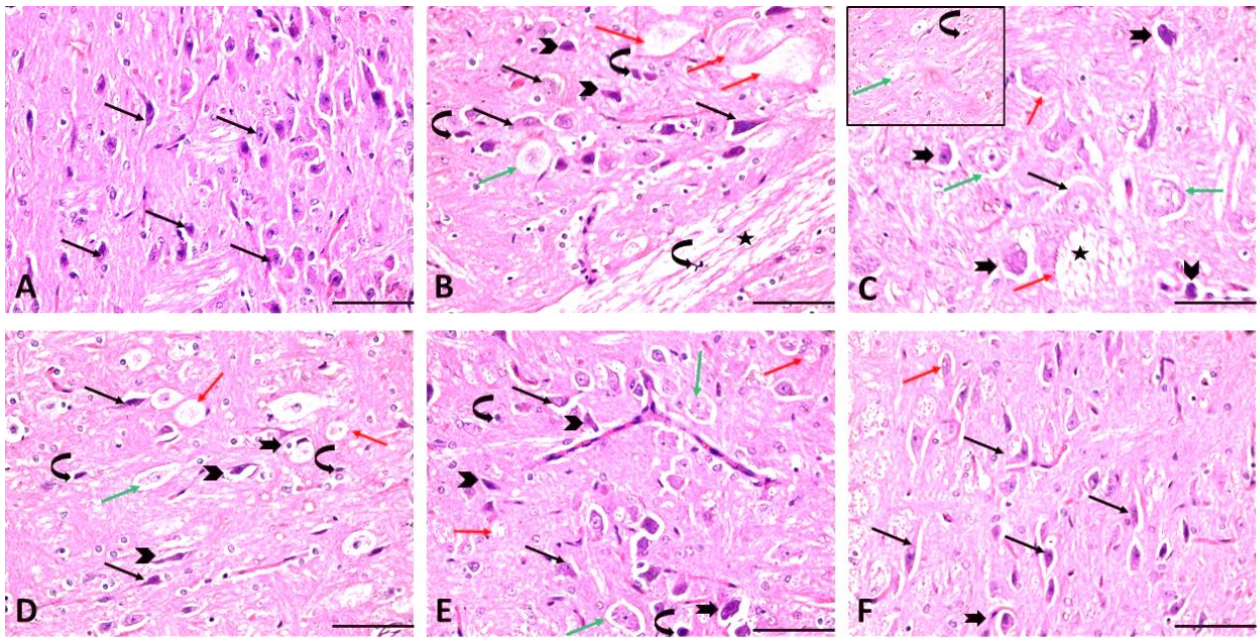

Figure **4:** Photomicrographs of substantia nigra of rats of all group (H. & E. × 400). Pars compacta of substantia nigra of sham group (Fig. A) shows cell rich region characterized by multiple large and medium sized elongated or fusiform neuron with prominent vesicular nuclei (arrows). Substantia nigra pars compacta of 6-OHDA group shows multiple signs of neuronal degenerations (Figs. B&C); some areas show loss of neuronal tissue (star), some sections show marked reduction of cellular mass (Fig. C inset), Multiple edematous neurons are obvious (black arrows), Other neurons show karyolysis of their nuclei (green arrows) and others lose their nuclei (red arrows). Small pyknotic cells are also visible (arrow heads). Lewy bodies (notched arrows) and macrophage infiltration (curved arrows) are abundant. Pars compacta of 6-OHDA/SFN (Fig. D) and 6-OHDA/GE (Fig. E) groups show elongated or fusiform neuron with prominent vesicular nuclei (black arrows), edematous neuron (green arrows). Other neurons show loss of their nuclei (red arrows). Small pyknotic cells (arrow heads), Lewy body (notched arrows) and macrophage infiltration (curved arrows) are also visible. Pars compacta of 6-OHDA/SFN/GE group (Fig. F) shows marked increase of neuronal cell mass as in sham group. Normal neuronal cells (black arrows), Few Lewy bodies (notched arrows) and loss of neuronal nuclei (red arrows) are visible.

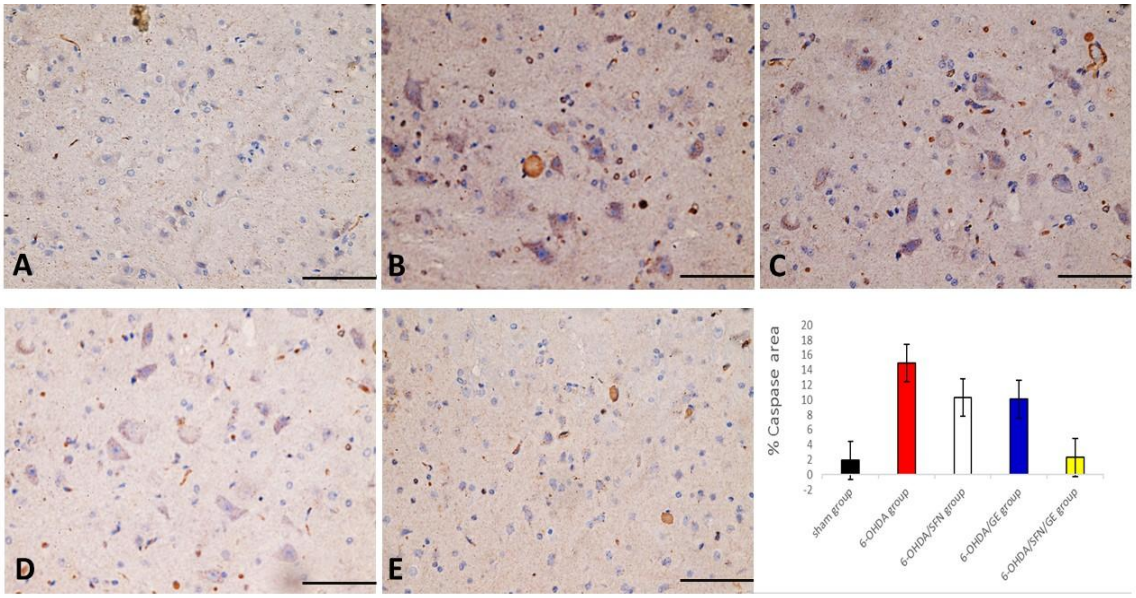

Figure 5: Photomicrographs of immunohistochemical analysis of activated caspase 3 in substantia nigra of rats of all group (Caspase 3 ×400). Minimal reaction (brown coloration) is noticed in sham group (Fig. A) and 6-OHDA/SFN/GE group (Fig. E). Marked reaction is obvious in 6-OHDA group (Fig. B). 6-OHDA/SFN (Fig. C) and 6-OHDA/GE (Fig. D) groups show moderate reaction of caspase 3 positive cells when compared with other groups. Statistical analysis of %Caspase area shows highly significant difference between Sham group & 6-OHDA group and between 6-OHDA &6-OHDA/SFN/GE(P-value=0.000). Also, significant difference is noticed between 6-OHDA &6-OHDA/SFN and 6-OHDA &6-OHDA/ GE groups (P-value=0.000).

4. Discussion

This study explores the possible molecular pathways by which SFN and garlic acid extract shield neurons from 6-OHDA-induced neurotoxicity and shows that they reverse SN cell loss in rats after injection of 6-OHDA. Also, we investigated the effect of the combination of both of them on a rat model of Parkinson's disease. To create an animal model of Parkinson's disease in rodents, 6-OHDA injection is a commonly used technique **[21-23]**.It was demonstrated that GE protects the dopaminergic neurons in PD**[17].** Additionally, SFN has been shown to have anti-apoptotic qualities, protect dopaminergic neurons, and postpone the beginning of PD or at least reduce its progression **[19].** In our study, we demonstrated the effect of the combination of SFN and garlic acid extract on PD.

We observed that the 6-OHDA group rats had a weak performance in apomorphine-induced rotational test and hanging wire, which is in line with the previous studies that have reported movement disabilities following the injection of 6-OHDA **[6]**. Our data also revealed that hanging time in the hanging test in the 6-OHDA/SFN group, 6-OHDA /GE group, and 6-OHDA /SFN /GE group animals were significantly higher than the 6-OHDA group. Also, the apomorphine-induced rotational test showed a decrease in the number of rotations for the animals in the 6-OHDA/SFN group, 6-OHDA /GE group, and 6-OHDA /SFN /GE group animals compared to 6-OHDA group rats. These beneficial effects of the GE and SFN on the motor functions in rat models of PD were proved before**[17, 19]**.

It has been confirmed that inflammation and oxidative stress have a major role in PD **[3, 11, 24]**. On the other hand, GE has been well-documented that it has a high potential to inhibit neuroinflammation and oxidative stress**[25-27]**.Additionally, it was previously believed that

Nrf2 activation was necessary for the neuroprotective action of SFN against toxin-induced Parkinson's damage, with autophagy and anti-oxidation both contributing to damage reduction **[28].**Concomitant with these findings in our study, 6-OHDA injection caused an overproduction of free radicals which, in turn, decreased GSH levels and GST and GR activities in the SN. However, following the oxidative stress induced by 6-OHDA, SFN, and GE restored the level of nigral GSH and the activities of GST and GR in the 6-OHDA/SFN group, 6-OHDA /GE group and 6-OHDA /SFN /GE group, suggesting that SFN and GE increase antioxidant potential in the brain and help it to fight against 6-OHDA-induced oxidative damage.

It is well known that the activation of the transcription factor Nrf2 is necessary for molecular mechanisms involving the "indirect antioxidant" effect of SFN. Nrf2 is linked to multiple kinase pathways in different tissues, including MAPK **[29].**

We focused in our study on the extracellular signal-regulated protein kinases (ERK1/2). ROS-induced oxidative stress is frequently associated with ERK1/2 pathway activation. In both acute neuronal traumas and neurodegeneration models, ERK1/2 activation seems to be mediated by redox processes **[30-32]**. In our model, we observed an increase in SN ERK1/2 levels after 6-OHDA injection and more importantly we found that SFN could inhibit ERK activation, suggesting the involvement of MAPK pathway in its mechanisms of neuroprotection. Also Elkhawas et al.,**[33]** reported that ERK1 and ERK2 levels were significantly decreased in rats receiving GE on multiple sclerosis rat model. The same effect was reported in our study that ERK1/2 levels significantly decreased in 6-OHDA/SFN group, 6-OHDA /GE group and 6-OHDA /SFN /GE group compared to 6-OHDA group.

Microscopic examination of pars compacta of substantia nigra of the 6-OHDA group showed multiple signs of neuronal degenerations in the form of loss of neuronal tissue in some areas and marked reduction of cellular mass of pars compacta in other areas, Multiple edematous neurons, karyolysis nuclei, and small pyknotic nuclei were seen and others lost their nuclei. Lewy bodies and macrophage infiltration were abundant. Marked reaction to caspase 3 was detected indicating an increased level of apoptotic cells. Loss of neurons was also detected by Becker et al.,**[34]**in mice injected with 6-OHDA which causes massive profound degeneration of cell bodies and also nerve terminals leading to progressive cell loss of substantia nigra pars compacta neurons, which are secondarily affected through dying back mechanism. In addition, **Yeni et al., [30]**also detected severe levels in pyknotic neurons and glial cells in the rat model of 6-OHDA-ınduced Parkinson's disease. They attributed neuronal degeneration in the 6-OHDA group to increased oxidative stress levels in the substantia nigra.

6-OHDA's pro-oxidant action is what makes it toxic. In the extracellular area, the toxin rapidly auto-oxidizes, which accelerates the production of reactive oxygen species**[35],** which is associated with the activation of the apoptotic process**[36].** Numerous lines of evidence suggest that apoptotic cell death following 6-OHDA injection into the striatum plays a major role **[37, 38]**. Degenerating neurons appear to undergo an apoptotic process

and trigger the caspase cascade, according to evidence from human PD brain tissue **[39]**. Consistent with these previous findings, our results also showed an increase in caspase-3 activity in the 6-OHDA group compared to the sham group. Our study demonstrated improvement of signs of neurodegeneration caused by 6-OHDA in in 6-OHDA/SFN group, 6-OHDA /GE group, and 6-OHDA /SFN /GE group in the form of increased neuronal cell mass with normal neurons and weaker reaction to caspase 3 when compared to 6-OHDA group. Bigham et al,**[17]** demonstrated that garlic acid extract improved neurotoxicity caused by 6-OHDA on rat substantia nigra through its anti-apoptotic effect. Schepici et al.,**[15]**demonstrated the neuroprotective effects of sulforaphane in neurodegenerative diseases such as Parkinsonism and illustrated its anti-apoptotic roles.

According to our results, the neuroprotective effect of using both SNF and GE together is more than each of them alone. This is evidenced by better motor improvement, less oxidative stress and apoptosis, and more inhibition of the ERK1/2 pathway in the 6-OHDA /SFN /GE group compared to the 6-OHDA/SFN group and the 6-OHDA /GE group. This proves the synergetic effect of using both of them together.

5. Conclusions

SFN and GE are neuroprotective in PD due to their anti-oxidative, anti-apoptotic, and anti-inflammatory effects. This neuroprotection is attributed to their ability to counteract ERK1/2 activation induced by 6-OHDA. Also, we demonstrated that both SFN and GE have a synergetic effect when used together which can be considered in using as a combination therapy in the treatment of Parkinsonism.

6. Declarations and statements

Ethics approval and consent to participate:

We conducted the study protocol according to The Local Committee of Research and Medical Ethics of the Faculty of Medicine, Tanta University.

Availability of data and material:

The corresponding author can provide the datasets used and/or analyzed during the current work upon request.

Competing interests:

The authors declare to have no conflicts of interest.

Funding:

The authors received no specific funding for this work.

Authors' contributions:

All authors contributed to the data analysis and interpretation of the data, drafted, and revised the manuscript, and approved the final version of the manuscript.

7. References

1. **Gonzalez-Rodriguez P, Zampese E, Surmeier DJ.** Selective neuronal vulnerability in Parkinson's disease. Progress in brain research.252:61-89,2020.
2. **Mu J, Chaudhuri KR, Bielza C, de Pedro-Cuesta J, Larrañaga P, Martinez-Martin P.** Parkinson's disease subtypes identified from cluster analysis of motor and non-motor symptoms. Frontiers in aging neuroscience.9:301,2017.
3. **Heidari Z, Mohammadipour A, Haeri P, Ebrahimzadeh-Bideskan A.** The effect of titanium dioxide nanoparticles on mice midbrain substantia nigra. Iranian journal of basic medical sciences.22(7):745-51,2019.

4. **Palakurthi B, Burugupally SP.** Postural instability in Parkinson's disease: a review. Brain sciences.9(9):239,2019.
5. **Haddadi H, Rajaei Z, Alaei H, Shahidani S.** Chronic treatment with carvacrol improves passive avoidance memory in a rat model of Parkinson's disease. Arquivos de neuro-psiquiatria.76:71-7,2018.
6. **Kumari N, Agrawal S, Kumari R, Sharma D, Luthra PM.** Neuroprotective effect of IDPU (1-(7-imino-3-propyl-2,3-dihydrothiazolo [4,5-d]pyrimidin-6(7H)-yl)urea) in 6-OHDA induced rodent model of hemiparkinson's disease. Neuroscience letters.675:74-82,2018.
7. **Haeri P, Mohammadipour A, Heidari Z, Ebrahimzadeh-Bideskan A.** Neuroprotective effect of crocin on substantia nigra in MPTP-induced Parkinson's disease model of mice. Anatomical science international.94(1):119-27,2019.
8. **Agbana YL, Ni Y, Zhou M, Zhang Q, Kassegne K, Karou SD, et al.** Garlic-derived bioactive compound S-allylcysteine inhibits cancer progression through diverse molecular mechanisms. Nutrition research.73:1-14,2020.
9. **Zhu Y, Anand R, Geng X, Ding Y. A mini review:** garlic extract and vascular diseases. Neurological research. 40(6):421-5,2018.
10. **Colin-Gonzalez AL, Ortiz-Plata A, Villeda-Hernandez J, Barrera D, Molina-Jijon E, Pedraza-Chaverri J, et al.** Aged garlic extract attenuates cerebral damage and cyclooxygenase-2 induction after ischemia and reperfusion in rats. Plant foods for human nutrition. 66(4):348-54,2011.
11. **Mohammadipour A, Haghir H, Ebrahimzadeh Bideskan A.** A link between nanoparticles and Parkinson's disease. Which nanoparticles are most harmful? Reviews on environmental health.35(4):545-56,2020.
12. **Mumtaz S, Ali S, Khan R, Shakir HA, Tahir HM, Mumtaz S, et al.** Therapeutic role of garlic and vitamins C and E against toxicity induced by lead on various organs. Environmental science and pollution research international.27(9):8953-64,2020.
13. **Hwang KA, Hwang YJ, Hwang IG, Song J, Jun Kim Y.** Low temperature-aged garlic extract suppresses psychological stress by modulation of stress hormones and oxidative stress response in brain. Journal of the Chinese Medical Association : JCMA.82(3):191-5,2019.
14. **Panjwani AA, Liu H, Fahey JW.** Crucifers and related vegetables and supplements for neurologic disorders: what is the evidence? Current opinion in clinical nutrition and metabolic care.21(6):451-7,2018.
15. **Schepici G, Bramanti P, Mazzon E.** Efficacy of Sulforaphane in Neurodegenerative Diseases. Int J Mol Sci.21(22),2020.
16. **FARSHBAF KA, MOHAMMADI GB, Shahnazi M, Asghari S, Javadzadeh Y, Azghani P.** Comparing the effect of garlic, Zataria multiflora and Clotrimazole vaginal cream 2% on improvement of fungal vaginitis: A randomized controlled trial. Iran Red Crescent Med J.18(12):e29262, 2016

17. **Bigham M, Mohammadipour A, Hosseini M, Malvandi AM, Ebrahimzadeh-Bideskan A.** Neuroprotective effects of garlic extract on dopaminergic neurons of substantia nigra in a rat model of Parkinson's disease: motor and non-motor outcomes. Metabolic brain disease.36(5):927-37,2021.
18. **Sandouka S, Shekh-Ahmad T.** Induction of the Nrf2 Pathway by Sulforaphane Is Neuroprotective in a Rat Temporal Lobe Epilepsy Model. Antioxidants.10(11), 2021.
19. **Morroni F, Tarozzi A, Sita G, Bolondi C, Zolezzi Moraga JM, Cantelli-Forti G, et al.** Neuroprotective effect of sulforaphane in 6-hydroxydopamine-lesioned mouse model of Parkinson's disease. Neurotoxicology.36:63-71, 2013.
20. **Erekat NS, Al-Jarrah MD.** Association of Parkinson Disease Induction with Cardiac Upregulation of Apoptotic Mediators P53 and Active Caspase-3: An Immunohistochemistry Study. Medical science monitor basic research.24:120-6, 2018.
21. **Fine JM, Stroebel BM, Faltesek KA, Terai K, Haase L, Knutzen KE, et al.** Intranasal delivery of low-dose insulin ameliorates motor dysfunction and dopaminergic cell death in a 6-OHDA rat model of Parkinson's Disease. Neuroscience letters.714:134567, 2020.
22. **Marin C, Bonastre M, Fuentes M, Mullol J.** Globus pallidus, but not entopeduncular nucleus, 6-OHDA-induced lesion attenuates L-Dopa-induced dyskinesia in the rat model of Parkinson's disease. Pharmacology, biochemistry, and behavior.197:173013, 2020.
23. **Zhang S, Tao K, Wang J, Duan Y, Wang B, Liu X.** Substantia Nigra Hyperechogenicity Reflects the Progression of Dopaminergic Neurodegeneration in 6-OHDA Rat Model of Parkinson's Disease. Frontiers in cellular neuroscience.14:216, 2020.
24. **Cenini G, Lloret A, Cascella R.** Oxidative Stress in Neurodegenerative Diseases: From a Mitochondrial Point of View. Oxidative medicine and cellular longevity.2019:2105607, 2019.
25. **Ghasemi S, Hosseini M, Feizpour A, Alipour F, Sadeghi A, Vafaee F, et al.** Beneficial effects of garlic on learning and memory deficits and brain tissue damages induced by lead exposure during juvenile rat growth is comparable to the effect of ascorbic acid. Drug and chemical toxicology.40(2):206-14, 2017.
26. **Kong X, Gong S, Su L, Li C, Kong Y.** Neuroprotective effects of allicin on ischemia-reperfusion brain injury. Oncotarget. 8(61):104492-507, 2017.
27. **Farzanegi P, Abbaszadeh H, Farokhi F, Rahmati-Ahmadabad S, Hosseini SA, Ahmad A, et al.** Attenuated Renal and Hepatic Cells Apoptosis Following Swimming Exercise Supplemented with Garlic Extract in Old Rats. Clinical interventions in aging. 15:1409-18, 2020.
28. **Zhou Q, Chen B, Wang X, Wu L, Yang Y, Cheng X, et al.** Sulforaphane protects against rotenone-induced neurotoxicity in vivo: Involvement of the mTOR, Nrf2, and autophagy pathways. Scientific reports. 6:32206, 2016.

29. **Owuor ED, Kong AN.** Antioxidants and oxidants regulated signal transduction pathways. Biochemical pharmacology. 64(5-6):765-70, 2002.
30. **Alessandrini A, Namura S, Moskowitz MA, Bonventre JV.** MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proceedings of the National Academy of Sciences of the United States of America. 96(22):12866-9, 1999.
31. **Yeni Y, Genc S, Ertugrul MS, Nadaroglu H, Gezer A, Mendil AS, et al.** Neuroprotective effects of L-Dopa-modified zinc oxide nanoparticles on the rat model of 6-OHDA-induced Parkinson's disease. Scientific reports.;14(1):19077, 202.
32. **Kuperstein F, Yavin E.** ERK activation and nuclear translocation in amyloid-beta peptide- and iron-stressed neuronal cell cultures. The European journal of neuroscience.16(1):44-54, 2002.
33. **Elkhawas YA, Seleem M, Shabayek MI, Majrashi TA, Al-Warhi T, Eldehna WM, et al.** Phytochemical profiling and mechanistic evaluation of black garlic extract on multiple sclerosis rat model. Journal of Functional Foods. 111:105900, 2023;.
34. **Becker B, Demirbas M, Johann S, Zendedel A, Beyer C, Clusmann H, et al.** Effect of Intrastriatal 6-OHDA Lesions on Extrastriatal Brain Structures in the Mouse. Molecular neurobiology. 55(5):4240-52, 2018.
35. **Hanrott K, Gudmunsen L, O'Neill MJ, Wonnacott S.** 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cδ. Journal of biological chemistry.281(9):5373-82, 2006.
36. **Ouyang M, Shen X.** Critical role of ASK1 in the 6-hydroxydopamine-induced apoptosis in human neuroblastoma SH-SY5Y cells. Journal of neurochemistry. 2006;97(1):234-44.
37. **Marti MJ, Saura J, Burke RE, Jackson-Lewis V, Jimenez A, Bonastre M, et al.** Striatal 6-hydroxydopamine induces apoptosis of nigral neurons in the adult rat. Brain research.958(1):185-91,2002.
38. **Mladenovic A, Perovic M, Raicevic N, Kanazir S, Rakic L, Ruzdijic S. 6-**Hydroxydopamine increases the level of TNFalpha and bax mRNA in the striatum and induces apoptosis of dopaminergic neurons in hemiparkinsonian rats. Brain research.996(2):237-45, 2004.
39. **Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, et al.** Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America.97(6):2875-80, 2000.