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Antioxidant, anti-apoptotic, and anti-inflammatory potential of Nigella sativa extract orchestrates its role against fipronil-induced toxicity in rats' brain

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ORIGINAL ARTICLE

Antioxidant, Antiapoptotic, and Anti-inflammatory Potential of *Nigella sativa* Extract Orchestrates its Role Against Fipronil-induced Toxicity in Rats' Brain

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Abstract

Introduction: Fipronil (FPN) a phenyl pyrazole insecticide, is widely applied in agriculture and veterinary products. However, its use is accompanied by neurotoxic effects.
OBJECTIVE: The current study was designed to assess the possible neuroprotective action of *Nigella sativa* extract (NS) against FPN-induced neurobehavioral toxicity in Wister male albino rats.
Methods: Thirty male albino rat weighing one hundred fifty to one hundred eighty grams. Animals were given FPN (12.6 milligrams per Bodyweight) orally or pre-treated using NS (100 and 200 mg/kg BW, respectively). The research study had been carried out within a four-week period.
Results: The findings demonstrated considerable promotions in neurobehavior in FPN-intoxicated rats and pre-treated with FPN in the open field, dark light test, and elevated plusmaze test. In addition, pre-treatment with NS lead to valuable rise in gamma-aminobutyric acid (GABA) together with substantial declines in malondialdehyde (MDA). Conversely, NS induced significant increases in catalase (CAT), super oxide dismutase (SOD), glutathione peroxidase (GSH-Px), besides Nrf2 upregulation. Marked decreases in tumor necrosis factor-alpha (TNF- α) and nuclear factor Kappa-B (NF-κB) were observed following NS treatment. These findings were confirmed by histopathologic imaging of brain cortex. Also, caspase-3 immunostaining was noticeable negative in the NS-treated groups.
Conclusions: Our findings suggest that, NS, in a dose-dependent manner, can be considered as a potent neuroprotective agent via its anti-inflammatory, antioxidant and anti-apoptotic activities.

Keywords: Fipronil, Neuroinflammation, Oxidative stress, Apoptosis, Nigella sativa, Nrf-2/Nf-Kb

1. Introduction

Frequent exposure to environmental xenobiotics, such as pesticides, is a proven cause of brain function impairment [1–4]. Fipronil (FPN), a broad-spectrum insecticide from the phenyl pyrazole chemical family, is categorized by WHO as a class II moderately dangerous pesticide. Its chemical name is 5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethyl-phenyl)-4-fluoromethylsulfinyl pyrazole. It is toxic by oral, inhalation, and dermal acute exposure. It is particularly toxic to rats and mice with oral LD50 ranging from 40 to 100 mg/kg [5,6].

FPN causes neurotoxicity by tightly binding to gamma-aminobutyric acid (GABA) chloride channels with subsequent hyperexcitability. Furthermore, the main FPN metabolite (FPN sulfone) has a stronger affinity for mammalian GABA receptors than it does for insect GABA receptors. The reduction in GABA release interferes with intracellular signaling and blocks presynaptic Ca2+ channels, which prevents the release of neurotransmitters [7].

FPN is reported to induce oxidative stress, inflammation, apoptosis, and finally leads to death in high doses; in addition, possible behavioral alterations were observed in animals following

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prolonged exposure to FPN [8,9]. It evokes its neurotoxic activity by increasing reactive oxygen species (ROS) production with subsequent disruption to the natural antioxidant defense mechanism leading to a decrease in the levels of antioxidant enzymes such as glutathione peroxidase (GPX), glutathione reduced (GSH), superoxide dismutase (SOD), and catalase (CAT) and an increase in the synthesis of oxidative ones such as malondialdehyde (MDA) and NO. In addition, vigorous inflammatory reaction is involved in FPN toxicity in the brain. Tumor necrosis factor-alpha (TNF- α) gene inhibition may minimize an inflammatory reaction by blocking nuclear factor Kappa-B (NF-κB) and mitogen-protein kinase activation in lipopolysaccharide-stimulated neurons [10].

Plant-based medicine is now the focus of study due to its efficacy, lack of side effects, and low cost; several phytochemicals have been found to provide significant neurotoxicity protection in animal models by restoring the antioxidant state [3,11,12]. *Nigella sativa* (NS) is extensively grown and has received substantial phytochemical research due to its vast variety of therapeutic benefits. The seeds of NS include 28–36% fixed oil, proteins, alkaloid, saponin, and 0.4–2.5% essential oil. The fixed oil consists primarily of unsaturated fatty acids such as arachidonic, eicosadienoic, linoleic, and linolenic acid. The oil contains palmitic, stearic, and other saturated fatty acids [13]. NS seeds may considerably protect spatial cognitive function in rats with chronic cerebral hypoperfusion. Furthermore, NS can protect spatial memory following scopolamine exposure by lowering acetylcholinesterase (AChE) activity and reducing oxidative stress in brain tissue in rats. Also, NS is well-known for its substantial antioxidative actions. The capacity of NS flavonoids to protect neurons from neurotoxic insult by enhancing memory, cognitive function, and learning, as well as the ability to diminish neurological inflammation, was demonstrated in the brain [14,15].

Based on the abovementioned considerations, the existing study focused on the investigation of the ameliorative impact of NS extract versus FPN-induced neural damage in the brain cortex of rats. We evaluated the behavioral disturbances as well as the oxidative, inflammatory, and apoptotic biomarkers in brain tissues.

2. Material and methods

2.1. Kits

Lipid peroxide MDA (Biodiagnostic, Egypt).

Glutathione reduced (GSH) (Biodiagnostic, Egypt).

Superoxide dismutase (SOD) (Biodiagnostic, Egypt).

Catalase (CAT) (Biodiagnostic, Egypt).

Glutathione peroxidase (GPx) (Biodiagnostic, Egypt).

2.2. Determination of median lethal dose LD_{50}

In all, 25 male albino rats weighing 150 ± 5 gm were randomly distributed into five groups for the determination of the median lethal dose (LD_{50}) of FPN.

These groups were orally intubated with FPN (Rado-X 80%) using a stomach tube dissolved in water at doses of 0, 100, 200, 400, 800 mg/kg b. w. The first served as the control group and received only water. The experimented animals were observed for 24 h. Clinical signs, mortalities, and gross lesions were recorded throughout the experimental period.

The LD_{50} value was determined using the approach outlined by Weil (1952) using the following formula:

$$\text{Log m} \pm 2.179\ \delta_{\log m}$$

$$\text{Log LD}_{50} = \text{Log D} + \text{d.}\ (\text{f} + 1) \text{ for k} = 3 \text{ or more}$$

where

Log D: the log of the lowest dosage levels utilized.

d: the logarithm of the constant ratio between dosage levels.

f: constant value obtained from special tables.

k: the number of dosage levels.

The confidence interval of 95% was determined according to the same method using the following formula.

$$\text{Log m} \pm 2.179\ \delta_{\log m}$$

where log m = log LD_{50}

δ log m = d. δ f

d: the logarithm of the constant ratio between dosage levels.

δ f: a constant value obtained from the special table (Weil, 1952).

Table 2. Summary of the experimental work of determination of Fipronil LD_{50}.

Group	Number of rats/group	Dose mg/kg b. w	Observation period
1	5	(Control) 0 ml of distilled water	
2	5	100 mg/kg b. w.	
3	5	200 mg/kg b. w.	24 h
4	5	400 mg/kg b. w.	
5	5	800 mg/kg b. w.	

2.3. Animals and experimental ethics protocol

In all, 30 male albino rats weighing 150–180 gm were bought from the animal house at the Faculty of Medicine, Mansoura University. Animals were maintained in metallic boxes with free access to water and diet, at a temperature of 22–25 °C, humidity level of 45–55%, and a 12 h light/dark cycle. Animal management and experimental design used in this study were authorized by the Ethical Committee of the Faculty of Veterinary Medicine, Mansoura University (Number of approvals: M/20).

2.4. Chemicals and reagents

FPN was obtained from the central laboratory of pesticides in Doki. All kits used for biochemical analysis were obtained from Bio Diagnostic Company (Giza, Egypt). Other chemicals were of analytical grade. The plant material (NS) was prepared in dried form, and it was crushed into powder. To obtain a 70% ethanolic extract (10% mass), 10 g of dried plant was mixed with 100 ml of 70% (v/v) EtOH, soaked for 1 h at room temperature, and then filtered.

2.5. Preparation of the plant extract

2.5.1. HPLC analysis method

HPLC (Agilent 1100) with two LC umps and a UV/Vis detector was used to analyze chromatographic data. A C18 column (5 m, 125 mm × 4.60 mm particle size) was used. The Agilent ChemStation was used to capture and evaluate chromatograms.

Ultrasounds were used to remove dissolved air from the mobile phase, which was subsequently filtered using a PTFE 0.2 m membrane. To improve ionization and resolution, the mobile phase for polyphenol identification contained formic acid in water (pH 3.0) as solvent A and formic acid in acetonitrile (pH 3.0) as solvent B. To separate polyphenolic chemicals, binary elution using gradients was used:

From 10 to 20 min, solvent B was at 5–30%; from 20 to 40 min, solvent B was maintained at 30%; from 40 to 50 min, solvent B was at 30–50%; and from 50 to 52 min, solvent B was at 50%. Flow rates varied as follows: between 0 and 5 min, 5–15 min, 0.1 ml/min, 15–35 min at 0.2 ml/min, 35–50 min, 0.1 ml/min 0.2 ml/min, and between 50 and 52 min, 0.1 ml/min [16].

2.5.2. Determination of polyphenol content

To separate the content polyphenolic, the mobile phase containing 0.1% methanol: phosphoric acid mixture (50: 50 v/v, isocratic mode) was used. Its flow rate had been fixed at 1.0 ml/min, and the recognition wavelength was fixed at 280 nm [17].

2.5.3. Flavonoid concentration determination

At an isocratic flow rate of 1.0 ml/min, the mobile phase was a binary mixture of methanol and water (50 : 50 v/v) with pH 2.8 phosphoric acid [18].

2.6. Experimental design and sampling

After a 2-week acclimatization period, rats were randomly assigned into five equal groups (n = 6 rats per group) and treated for 28 days as follows:

(a) Group (1): Animals in the control group were given carboxymethyl cellulose) Cmc(1% orally.
(b) Group (2): Received FPN at selected dosage based on a previous research) 1/10 LD50, which is equivalent to 12.6 mg/kg b.wt, as I applied a pilot LD50 experiment for the calculation of this dose.
(c) Group (3): received 200 mg/kg b.wt of NS ethanolic extract dissolved in Cmc 1% every day [19].
(d) Group (4): 1 h before FPN delivery, received NS at a dose of 100 mg/kg b.wt and FPN at a dose of 12.6 mg/kg b.wt
(e) Group (5): Administered NS (200 mg/kg b.wt) and FPN (12.6 mg/kg b.wt). Rats were assessed for behavioral abnormalities within 24 h after the last treatment and then all animals were killed by cervical dislocation after sodium pentobarbital (Sigma–Aldrich) at a dose of 300 mg/kg b.wt [20]. They were then decapitated, Furthermore, the brain is taken quickly and separated into three sections. The initial part was homogenized in ice-cold 10 mM phosphate buffer (pH 7.4) to provide a 10% (w/v) homogenate, which was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was subsequently subjected to biochemical tests. Histopathology and immunohistochemistry were performed on the second part of the brain which was preserved in formaline. For molecular studies (RNA extraction), the remaining component was frozen in the presence of liquid nitrogen and maintained at −80 °C.

2.7. Assessment of neurobehavioral alterations

Rats' neurobehavioral activities were investigated by applying the open-field test (OFT), dark/

light (DL), and elevated plus-maze tests, with no previous contact; the rats were simply examined once for each test. The tests were selected for their use in analyzing and assessing the hazards of CNS drugs. Behavioral styles were discovered between 9:00 a.m. and 11:00 a.m. as previously described [4,21].

2.7.1. Open-field test (OFT)

The evaluation assessed the attitude within a metallic box (40*40 cm) with opaque walls 40 cm high, in which the creatures had never previously been seen. This test was created to examine behavioral reactions in an unexpected context, including exploratory behavior and locomotor activity. The framework was divided into 16 comparable parts. Each rat spent 5 min in the cage and was assessed based on freezing length, rearing frequency, and grooming time. Grooming routines included foreleg shaking, genital grooming, paw scratching, body licking, nose, face, and head cleaning, head shaking, and scratching, which are all common behaviors. Notice the duration for each rat maintained in the box's periphery or center. Following the inspection of each animal, the OFT enclosure was cleaned by ethanol 5% to remove any feces or any kind of contaminants found as previously stated by Terçariol and Godinho [21].

2.7.2. Dark–light test

Following the previous OFT, DL) test was done. The rats were housed in a dark enclosure, an opening in a step-through cage design connected it to a bright enclosure. The amount of time which is required for setting the front legs on the bright box was documented for the utilization as a statistical measure. Each rat was assessed for more than 5 min in the lighted arena (width of 18 cm, depth of 20 cm, and height of 22 cm) and the black enclosure (20 cm wide, 20 cm deep, and 17 cm high). The test was performed aligned with Rock *et al*. [22].

2.7.3. Elevated plus maze test

This test is used to assess the anxiety effects caused by different toxins. 4 arms (10*50 cm in width and in length) (2 open and 2 closed). Closed arms had partially sealed walls that were 40 cm elevated whereas open ones had a 1-cm-high edge. It was around 50 cm above the floor. Each animal had been allocated to one of the open arms in the raised plus maze's center. Throughout the test period (5 min), the total number of ra entries into both their closed or open arms as well as the time spent (s) were measured to determine anxiety or fear-induced inhibition of normal investigative behaviors. With 5% ethanol, the device was fully sterilized between each pair of rats [23].

2.8. Evaluation of oxidant and antioxidant biomarkers within the brain homogenate

By using kits, the action of the GPx within the brain homogenate was evaluated (nmol/g tissue). The process of converting NADPH to NADP+ is then a decrease in absorption at 340 nm (A340), used for assessing GPx enzymatic activity spectrophotometrically. NADPH has a mass attenuation value of 6220 M−1 cm1 at 340 nm. The homogenate of tissue or cell was combined in a solution containing NADPH, glutathione reductase, and glutathione to test for c-GPx. The enzyme process begins with the addition of hydrogen peroxide and the substrate, recording the A340 value. The A340's rate of decrease is proportional to the sample's GPx action. This was established by following the processes of Paglia and Valentine [24] (Cat. GP 2524, Bio diagnostic). GSH was determined based on the reduction of 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB) to produce a yellow compound. The process of absorption of the decreased chromogen might be determined within 405 nm and is correlated directly with GSH concentration. As mentioned before, in Ref. [25]. MDA reacts with thiobarbituric acid (TBA) for 30 min at 95 °C in an acidic solution to create thiobarbituric acid reactive response products. The absorption coefficient of the colored pink compound is determined to be 534 nm [26]. CAT levels in U/g tissue were determined with an industrially accessible kit that involved interacting with H_2O_2 after stopping the interaction with an antagonist of CAT within 510 nm wavelength as reported previously [27]. SOD was measured based on the reduction of the nitro blue tetrazolium dye at 560 nm [28].

2.9. Pro-inflammatory cytokine evaluation (TNF-α)

TNF-α was identified calorimetrically by ELISA using affordable kits and followed the labeled guidelines (Cat. Ab234570). In each well, 50 samples or standards were inserted. Following that, 50 antibody cocktail was added to each hole and maintained at ambient temperature for 1 h. Following that, each of the holes was aspirated and rinsed three times with washing buffers and then cultured for 10 min. Following that, 100 Stop Solutions was introduced. The corresponding wavelength readings at 450 nm were evaluated using the microplate

ELISA viewer, and TNF-levels in brain homogenization were assessed.

2.10. Gene expression analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the expression of genes nuclear factor erythroid 2-related factor 2 (NrF-2) and NF-κB. Total RNA was extracted according to the manufacturer's instructions using triazole (Life Technologies, Gaithersburg, MD, USA). The Multiscript RT enzyme kit (The Applied Biosystems, Foster City, CA, USA) was then used to swiftly synthesize cDNA. In triplicate, the cDNA was tested using real-time PCR. Real-time PCR reactions were carried out using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, CA, USA). The PCR temperature phase was 95 °C for 9 min, followed by 40 cycles of denaturation, which included denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s in NrF-2 and Nf-κB, and annealing at 55 °C for 30 s in B-actin with a 10 s extension at 72 °C. The β-actin gene was used as a housekeeping control gene. The gene accession numbers and primer sequences are shown in Table 1.

2.11. GABA neurotransmitter analysis

Concentrations of GABA within the brain tissues were calculated using the GABA Elisa kit following the manufacturer's instructions (Catalog No. MBS722440, MyBioSource Inc.).

2.12. Histopathological evaluation

Brain tissue samples were kept in a neutral formalin mixture of 7.4 pH for 48 h after the rats were killed. Fixed specimens were treated with the paraffin-embedding technique. The photographs were shot using a Leica DM500 microscope and Leica EC3 digital camera (Germany). Four-meter-thick slices were stained with hematoxylin and eosin (H and E) on a regular basis. The brains of each group were evaluated and photographed for pathologic abnormalities using an Olympus CX 31 microscope [29].

2.13. Evaluation of immunohistochemistry expression of caspase 3

Four wide portions were hydrated again in varying concentrations of ethanol after deparaffinization. Antigen was eliminated from slices by boiling them at 105 °C for 10–20 min in a 10 mM citrate buffer with 6.0 pH, the following 20 min were spent at ambient temperature. Endogenous peroxidase was inhibited for 5 min at room temperature (PBS) by 3% H_2O_2 in the presence of 100%. After washing with phosphate-buffered saline, soak in methanol. To decrease nonspecific reactivity, a 60 min incubation with 10% normal goat serum was utilized. Following that, rabbit polyclonal anti-caspase-3 antibodies were incubated overnight at 4 °C on brain slices. In addition, rabbit polyclonal anti-cleaved caspase-3 antibody (1:200, Abcam, Cat. ab7260, Cambridge, UK) and rabbit polyclonal anti-cleaved caspase-3 antibody (1:200, Abcam, Cat. ab7260, Cambridge, UK). (1:200, Abcam, Cambridge, UK, Cat. ab7260) (1:100, BioCare Medical, Concord, CA, USA, Cat. CP229C) were used. After washing in PBS, sections were incubated for a minute with biotin-conjugated goat anti-rabbit IgG antiserum, then for 30 min with streptavidin–peroxidase conjugate. Histofine kit (Nichirei Corporation, Japan) was used. The streptavidin–biotin combination was visible for 3 min in a pH 7.0 solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2. After that, the sections were counterstained using Mayer's hematoxylin solution [30].

2.14. Statistical analysis

Data is displayed as means ± the standard error of the mean. One-way ANOVA and the post hoc Duncan's numerous comparative tests were used to analyze the data. *P* values of less than 0.05 showed significant differences among groups.

3. Results

3.1. Concentration of total polyphenolic and flavonoid content

(a) The total flavonoid content for NS expressed as Naringin 1.69, Kaempferol 0.88, Luteolin

Table 1. Primer sequences used in RT-PCR for detection of NRF-2 (nuclear factor erythroid 2-related factor 2) and NF-κB (nuclear factor-κB).

Name	Accession Number	Forward-Primer (5′—3′)	Reverse-Primer (5′—3′)	Reference
β-actin	NM_031144.3	f5,- GGCATGTGCAAGGCCGGCTT -3	r5,- TAGGAGTCCTTCTGACCCATA- 3,	[3]
Nrf-2	NM_031144.3	f5, - GTCCACCCGCGAGTACAACCT-3,	r5, - GGAGCCGTTGTCGACGACGA-3	[3]
NF-κB	AF079314.2	f5,- TGGACGATCTGTTTCCCCTC - 3,	r5,- CCCTCGCACTTGTAACGGAA-3	[3]

9.36, Hesperidin 11.42, Catechin 2.09, Chrysoeriol 3.10 (Table 2) [31].

(b) The total polyphenolic content for NS found expressed as Syringic 8.06, Benzoic 3.26, Caffeic 5.33, Gallic 7.98, Cinnamic 6.54, Ellagic 1.77 (Table 3) [31].

3.2. Impact of FPN/NS on neurobehavioral observations

3.2.1. Open-field test

Animals given FPN spend more time in the peripheral than the middle part of the box and show a considerable rise in all investigated categories in comparison to the placebo population in the OPF test ($P < 0.05$). Rats given NS exhibited a significant reduction ($P < 0.05$) in all behavioral measurement categories (rearing, locomotion, and grooming) when compared with the control group, whereas rats given NS 100 mg/kg + FPN and NS 200 mg/kg + FPN had a decreased time spent in peripheral than in middle area and revealed a substantial reduction in all behavioral pattern than rats given FPN as summarized in Table 4.

3.2.2. Elevated plus maze test

Rats co-treated with FPN showed a substantial increase in the total time spent in the closed arm than the open arm, which means increased anxiety ($P<0.05$). Rats within the NS group alone exhibited no change in behavior in comparison to the untreated population, whereas rats in the NS 100 mg/kg + FPN class and the NS 200 mg/kg + FPN group when compared with FPN-treated rats demonstrated a significant ($P<0.05$) decrease within the total number of entries and the amount of time spent on the closed side of the device than the open one as summarized in Table 4.

Table 2. Total flavonoid content of Nigella sativa extract.

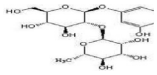
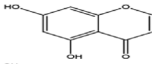
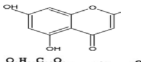
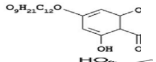
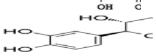
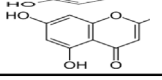
RT#	Compound	Concentration µg/ml	Chemical formula	Molecular weight	Chemical structure
4.6	Naringin	1.69	$C_{27}H_{32}O_{14}$	580.54 g/mol	
8.1	Kaempferol	0.88	$C_{15}H_{10}O_6$	286.23 g/mol	
9.0	Luteolin	9.36	$C_{15}H_{10}O_6$	286.24 g/mol	
10.0	Hesperidin	11.42	$C_{28}H_{34}O_{15}$	610.1898 g/mol	
12.0	Catechin	2.09	$C_{15}H_{14}O_6$	290.26 g/mol	
15.0	Chrysoeriol	3.10	$C_{16}H_{12}O_6$	300.26 g/mol	

Table 3. Total polyphenolic content of Nigella sativa extract.

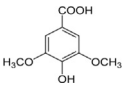
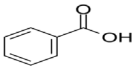
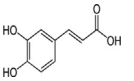
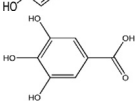
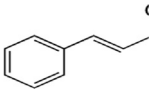
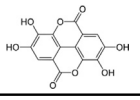
RT#	Compound	Concentration µg/m	Chemical formula	Molecular weight	Chemical structure
5.2 1	Syringic	8.06	$C_9H_{10}O_5$	198.17 g/mol	
7.0	Benzoic	3.26	$C_7H_6O_2$	122.12 g/mol	
8.1	Caffeic	5.33	$C_9H_8O_4$	180.16 g/mol	
10.0	Gallic	7.98	$C_7H_6O_5$	170.12 g/mol	
13.0	Cinnamic	6.54	$C_9H_8O_2$	148.1586 g/ml	
15.6	Ellagic	1.77	C14H6O8	302.197 g/mol.	

Table 4. Effect of Nigella sativa extract and fipronil, and their combination on open-field test, elevated plus-maze test, and dark/light test behaviors of rats (mean ± SE).

Treatment	Groups					*P* value
	CTR	FPN	NS	FPN + NS/100	FPN/NS 200	
1-open-field test						
Time in middle	157 ± 33.1^{b}	55.66 ± 4.7^{c}	148 ± 19.7^{b}	208 ± 12.7^{a}	171.3 ± 12.50^{b}	0.001
Time in peripheral	143 ± 6.99^{b}	244 ± 4.76^{a}	151.3 ± 19^{b}	92.0 ± 12.7^{c}	128.6 ± 12.50^{b}	0.000
Rearing	1.33 ± 0.21^{c}	13.0 ± 0.96^{a}	4.66 ± .210^{b}	3.66 ± .557^{b}	4.33 ± .557^{b}	0.000
Grooming	1.33 ± 0.21^{d}	7.00 ± 0.36^{a}	2.33 ± .210^{c}	2.333 ± .210^{b}	3.66 ± .2108^{b}	0.001
Boli	1.33 ± 0.21^{b}	3.66 ± 0.21^{a}	1.66 ± .210^{b}	2.00 ± .3651^{b}	1.33 ± .2108^{b}	0.000
2-EPM						
Time in openarm	158 ± 13.7^{c}	108 ± 25.1^{d}	130 ± 18.3^{b}	225 ± 13.2^{b}	237 ± 3.19^{a}	0.00
Frequency	2.66 ± 0.42^{b}	3.33 ± 0.57^{b}	4.66 ± 0.57^{a}	4.33 ± 0.42^{a}	4.66 ± 0.21^{a}	0.01
Time in closed arm	1.51 ± 14.8^{b}	2.36 ± 4.35^{a}	1.28 ± 2.63^{b}	81.33 ± 17.91^{c}	1.1233 ± 22.0^{b}	0.00
Frequency	3.00 ± 0.00^{c}	8.3 ± 0.557^{a}	4.66 ± .557^{b}	4.333 ± .210^{b}	3.333 ± .210^{c}	0.00
Boli	2.00 ± 0.36^{b}	5.66 ± 210^{a}	2.00 ± .365^{b}	2.666 ± .5577^{b}	1.666 ± .2108^{b}	0.01
3-DL test						
Time in the dark area	249 ± 10.07^{b}	246.3 ± 4.7^{a}	251.6 ± 2.4^{b}	259 ± 11.8^{c}	253.3 ± 5.32^{d}	0.03
Time in light area	110 ± 10.95^{d}	163 ± 1.28^{e}	118 ± 16.7^{c}	201 ± 7.8514^{b}	238.6 ± 5.846^{a}	0.00
Boli	1.33 ± 0.210^{c}	4.33 ± 0.21^{a}	2.00 ± 0.365^{b}	1.333 ± .2108^{c}	1.000 ± 0.000^{d}	0.00

Means with different letters within the same raw differ substantially (P <0.05). Frequency of rearing: the number of times the animal stood on its hind legs; grooming duration: the total amount of time spent grooming by the animal.

3.2.3. Dark/light test

As demonstrated in Table 4, rats receiving FPN spend significantly extended periods in the dark area in the DL test (P<0.05) than the untreated group. When compared with the control population, animals given NS alone exhibited no change in behavior. However, rats given NS 100 mg/kg + FPN and NS 200 mg/kg + FPN revealed significant declines (P<0.05) in the period spent in the dark and spent more time in the light area.

3.3. Impact of FPN/NS on GABA neurotransmitters

GABA level activity was considerably (P<0.05) reduced in rats treated with FPN when compared with the placebo group, although their actions were significantly (P<0.05) changed with different NS doses in plant-treated rats (NS 100 mg/kg and NS/200 mg/kg). Their actions were significantly (P<0.05) elevated by the administration of NS alone (NS 200 mg/kg) when compared with the toxin group as shown in Figs. 1–3.

3.4. Impact of FPN/NS on oxidative and antioxidative parameters

In this investigation, we looked at the levels of antioxidant molecules (SOD, CAT, GPx, and GSH) and other oxidants (MDA) in the brain homogenate 28 days following FPN and NS treatments. In FPN-treated rats, significant reductions in SOD and CAT activities were observed (P < 0.05). These changes were significantly counteracted with NS used at different doses (NS 100 mg/kg, 200 mg/kg), which

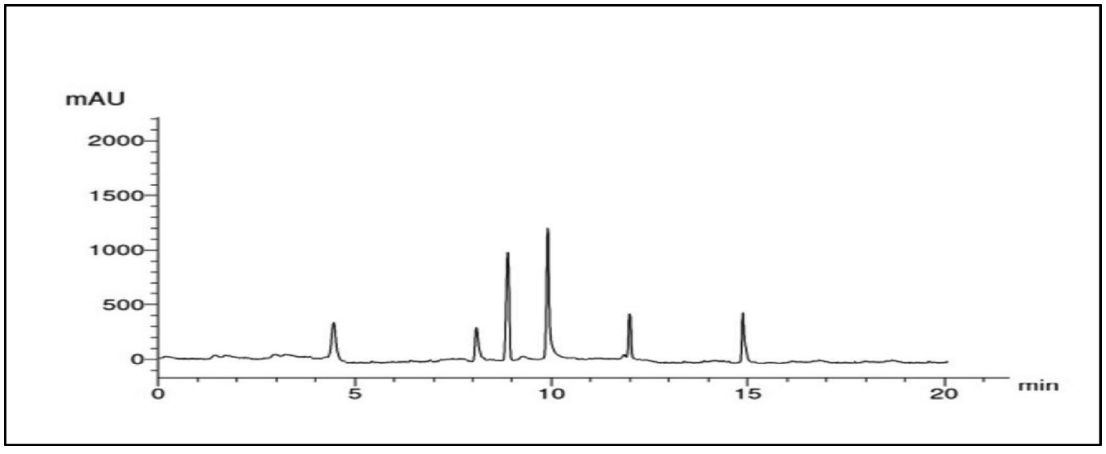


Fig. 1. Chromatograph report for Nigella sativa extract (total flavonoid content for NS).

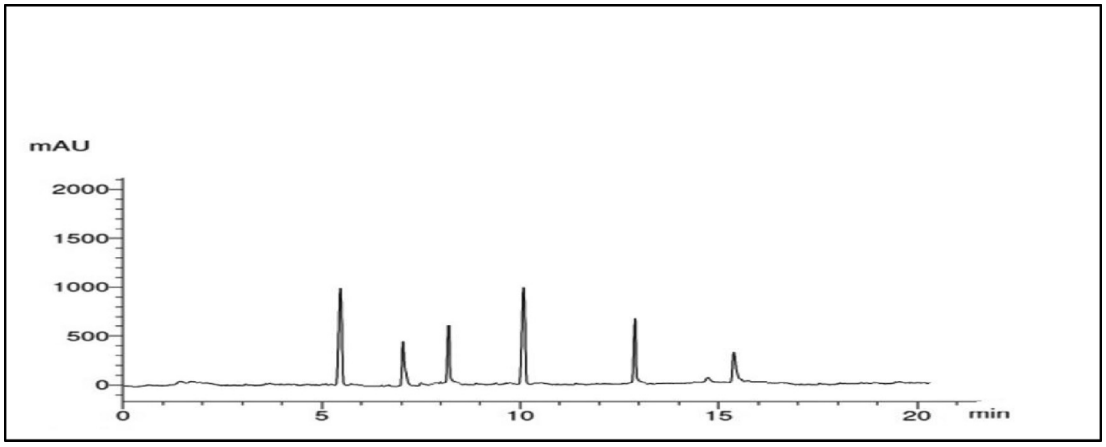


Fig. 2. Chromatograph report for Nigella sativa extract (total polyphenolic content for NS).

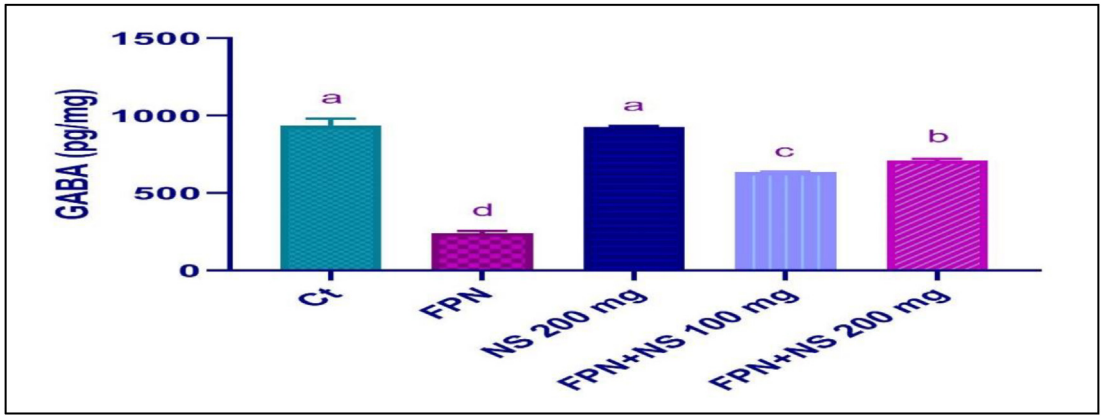


Fig. 3. Effect of fipronil on gamma amino butyric acid (GABA) neurotransmitter and the neuroprotective effect of NS plant against it. The different letters represent the statistical significance relative to that of the control group at P less than 0.05.

was administered with FPN as their levels significantly increased ($P < 0.05$) the antioxidant state of the brain tissue. GPX activity significantly increased with different doses of NS (NS 100 mg/kg, 200 mg/kg) administered with FPN, but there was a reduction in activity with no significance in the group that took FPN only (Fig. 4). Also at the oxidation level, compared with the control group, significant reductions were observed in GSH levels, and FPN treatment resulted in a significant increase ($P < 0.05$) in MDA levels. When NS was given before the FPN, these effects were significantly reversed ($P < 0.05$), and the expression of Nrf2 regulates the activity of anti-inflammatory action of TNF-α in the brain tissue. RT-PCR results revealed a significant ($P < 0.05$) decrease in Nrf-2 expression after FPN intoxication as compared with the control group. Meanwhile, NS-treated rats had increased levels, also in the NS + FPN-treated group compared with the FPN-exposed group, which were related to the downregulation of their gene expression compared with the control group (Fig. 5).

3.5. Impact of FPN/NS on inflammatory cytokines

TNF-α level was evaluated in neural tissue following FPN and/or NS exposure. In contrast to control rats, FPN-treated animals had a significant ($P < 0.05$) rise in TNF-α levels in the brain tissue. In contrast, rats in the (FPN + NS/100 mg/kg) and (FPN + NS/200 mg/kg) groups had significantly lower TNF-α levels ($P < 0.05$) than the FPN-treated group as presented in Fig. 6. The expression of NF-κB, which monitors and regulates the activity of inflammatory molecules revealed a significant ($P < 0.05$) elevation after FPN intoxication as compared with the control group (Fig. 6).

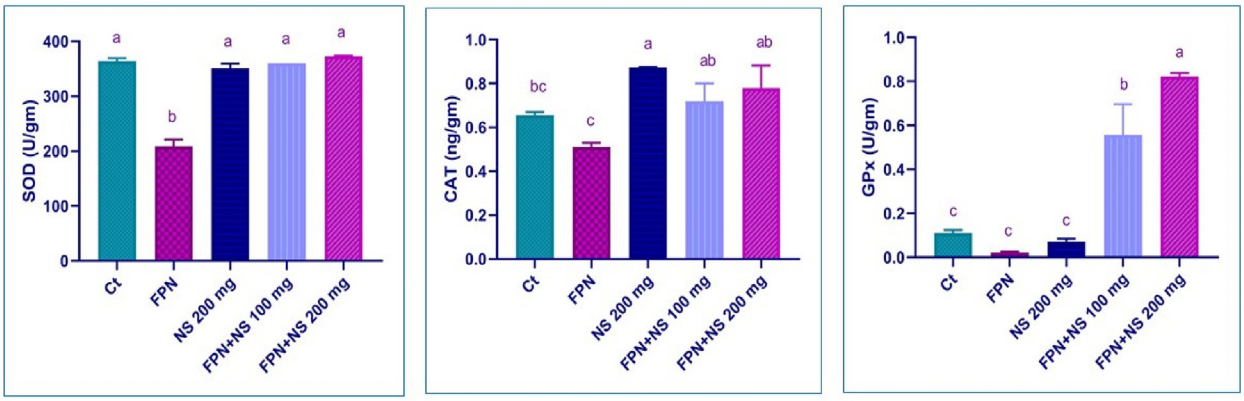


Fig. 4. The effect of fipronil on oxidative–antioxidative enzymatic parameters and protective effect of NS plant extract in each group with different dosages. All values are expressed as the mean ± SE and are significantly different at P < 0.05 following fipronil (FPN 12.6 mg/kg, orally) and/or NS (100 200 mg/kg, orally) exposure in male rats recorded as the mean ± SE in triplicate and β-actin was the housekeeping gene. The different letters represent the statistical significance relative to that of the control group at P less than 0.05. SOD (superoxide dismutase), GPX (glutathione peroxidase), and CAT (catalase).

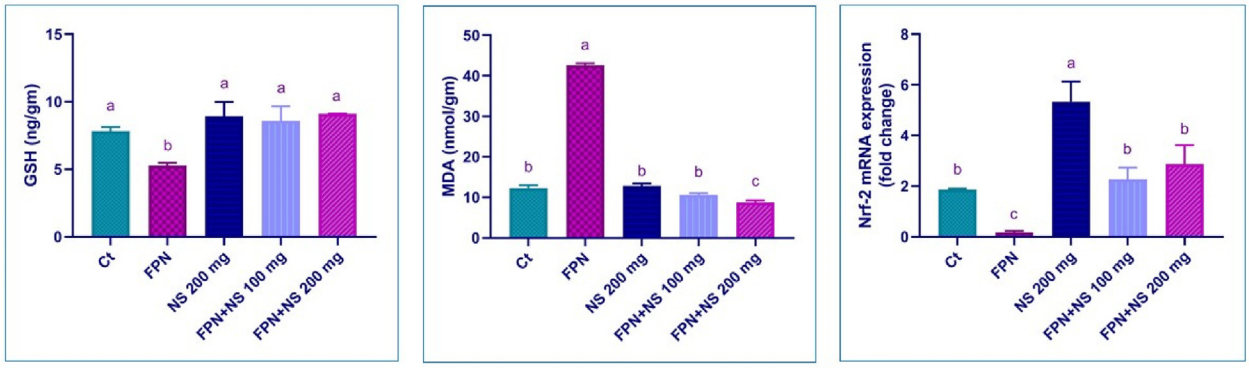


Fig. 5. The effect of fipronil on oxidative–antioxidative nonenzymatic parameters, and the protective effect of NS plant extract in each group with different dosages. All values are expressed as the mean ± SE and are significantly different at P < 0.05. Brain mRNA gene expression levels of Nrf-2 following fipronil (FPN 12.6 mg/kg, orally) and/or NS (100,200 mg/kg, orally) exposure in male rats recorded as the mean ± SE in triplicate and β-actin was the housekeeping gene. The different letters represent the statistical significance relative to that of the control group at P less than 0.05. MDA, malondialdehyde; GSH, glutathione reduced, and Nrf-2 nuclear factor erythroid 2-related factor 2.

3.6. Impact of FPN/NS on brain histopathological finding

Histopathological examination of the brain is shown in Fig. 7. The control and NS groups revealed that the cerebellum displays normal Purkinje cell, and the cerebral cortex displays normal neurons with a large round vesicular nucleus, prominent nucleoli, normal neuropil, and normal blood capillary. However, rats treated with FPN revealed that the cerebral cortex s displays congestion of the cerebral blood vessels. Notably, rats treated with NS 100 mg/kg + FPN revealed mild degenerative changes in the Purkinje cells. Moreover, rats treated with NS 200 mg/kg + FPN show that the cerebral cortex displays normal neurons with normal rounded vesicular nucleus and prominent nucleoli, normal neuropil, normal glial cells, and normal blood capillary.

3.7. Impact of FPN/NS on brain caspase-3 immunostaining

As shown in Fig. 8, the brain displays negative immunostaining against caspase-3 in control and NS-alone groups; meanwhile, the brain in rats taking FPN displays strong positive brown immunostaining in the cytoplasm of neuronal cells against caspase-3. Moreover, the brain in rats treated with NS 100 mg/kg + FPN shows moderate positive immunostaining against caspase-3 staining brown the cytoplasm of the neuronal cells. Moreover, the brain displays negative immunostaining against caspase-3 in the group treated with NS 200 mg/kg + FPN.

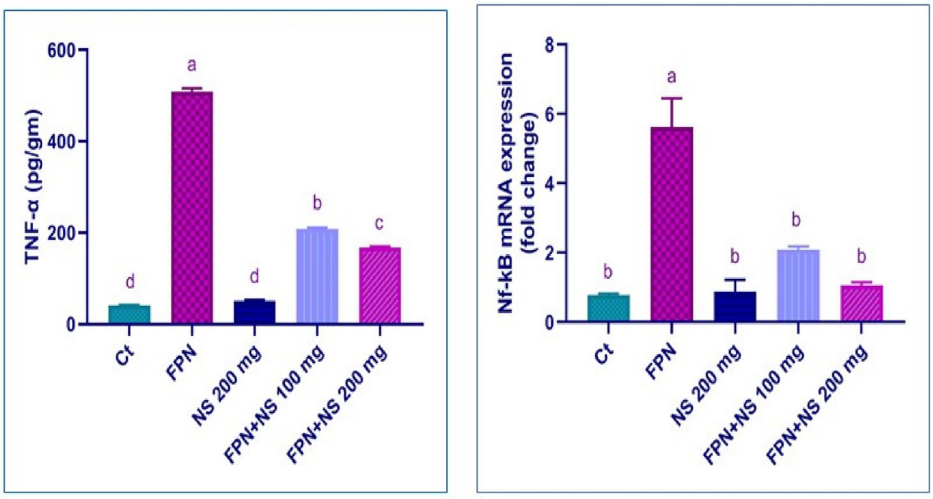


Fig. 6. The effect of fipronil and NS on proinflammatory cytokines TNF-α, brain mRNA gene expression levels of NF-κB following fipronil (FPN, 12.6 mg/kg, orally), and/or NS (100 200 mg/kg, orally) exposure in male rats. Results of mRNA were recorded as the mean ± SE in triplicate and β-actin was the housekeeping gene. The different letters represent the statistical significance relative to that of the control group at P less than 0.05. NF-Kb, nuclear factor-κB and TNF-α, tumor necrosis factor alpha.

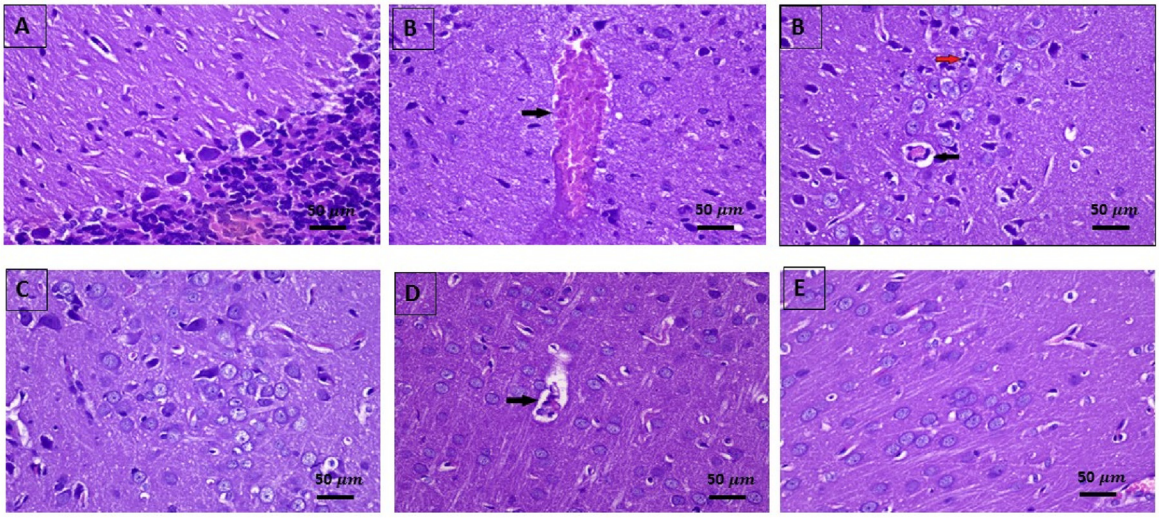


Fig. 7. Representative photomicrographs of the rat brain (HE, 400x), specimens from the (A) control group, and (C) group treated with NS alone show normal histological structure. In group (B) fipronil (FPN)-treated specimens show cerebral cortex displaying congestion of the cerebral blood vessels (arrow), neuronal degeneration, and satellitosis of perineural oligodendroglia (red arrow) and perivascular edema (black arrow), as well as necrosis of Purkinje cells (arrow). Rats treated with NS 100 mg/kg + FPN in group (D) show cytotoxic edema in neurons of the dentate gyrus in the hippocampus (arrow). The cerebral cortex is perivascular edema (black arrow) and mild degenerative changes of Purkinje cells (arrow), compared with rats treated with NS 200 mg/kg + FPN in group (E) with the cerebral cortex displaying normal neurons with normal rounded vesicular nucleus and prominent nucleoli, normal neuropil, normal glial cells, and normal blood capillary (arrow) and the cerebellum displaying normal Purkinje cells (arrow), normal granular and molecular cell layers.

4. Discussion

Recent investigations have shown that FPN and its metabolites cause neurotoxicity in different animal models [32,33]. It has a neurotoxic action that has been connected to oxidative stress and neuro-inflammation. The selectivity of FPN sulfone to GABA receptors is six times greater than that of FPN. In this study, severe reductions in GABA levels were observed in the brain with FPN-exposed rats. Such alterations were reversed in rats given varied dosages of NS. The considerable variation in GABA might be attributable to neurodegeneration and necrosis in the brain that affect its generation, distribution, as well as accumulation which translated

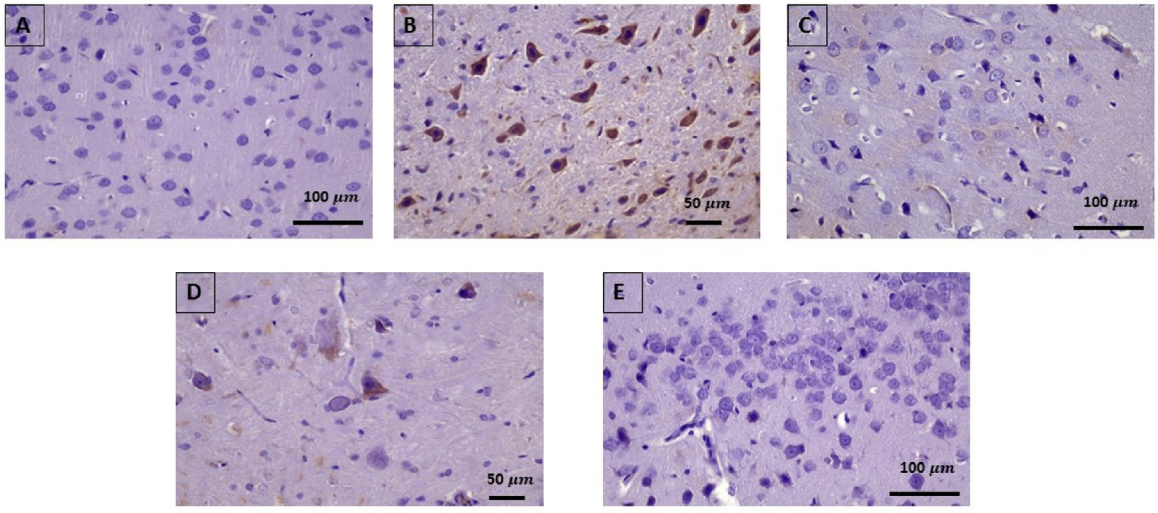


Fig. 8. Representative photomicrographs of immunohistochemistry of rat brain revealed negative immunostaining against caspase-3 in the control group (A) (IHC, DAB immunostain, hematoxylin as counterstain, 100x). Some was in group (C) which received NS alone; meanwhile, brain of rats taken FPN group (B) (IHC, DAB immunostain, hematoxylin as counterstain, 400x) displays strong positive brown immunostaining in the cytoplasm of the neuronal cells against caspase. Brain shows moderate positive brown immunostaining in the cytoplasm of Purkinje cells against caspase-3 in rats treated with NS 100 mg/kg + FPN group (D) compared with rats treated with NS 200 mg/kg + FPN group (E) showed negative immunostaining against caspase- 3.

to fast change in rat behavioral patterns, which was first noticed as clinical signs by applying some behavioral tests before the internal changes were detected. This current findings were supported by a previous investigation [9]. The effects of NS on numerous neurotransmitters, including 5-hydroxytryptamine and GABA may be connected to the actions of its constituents. NS raises the amount of 5-hydroxytryptamine, which reduces anxiety. Thymoquinone in the nervous system may also reduce nitric oxide and reverse the decreased brain GABA concentration, providing an anxiolytic effect [34].

Behavioral tests for small animals had been developed as screening tests for understanding the neurotoxic effect of chemicals as well as the general safety of new drugs. The current study discovered that the groups that received NS performed better in the OFT, DL test, and elevated plus-maze test in comparison with FPN. Animals exposed to FPN showed substantial elevation in rearing and grooming behaviors, which were used as indications of anxiety, exploration, and fear in this study. These results are in accordance with previous researchers [21]. However, the use of NS was found to reverse the negative influence of FPN on these evaluated behaviors. OPF test is considered an indicator of the emotional state and anxiety of the animal and is commonly used for the pharmacological selection of drugs that act on the central nervous system. In this test, rearing behavior is considered an indicator of locomotor and exploratory activities, whereas grooming and freezing are positively correlated with fear. The increased time spent in the peripheral part of the box indicates the anxiety or emotionality, which increased with the FPN-intoxicated group, and is reversed with NStreated groups [23,35]. In the present study, animals receiving FPN presented increased grooming and rearing behaviors, suggesting that the insecticide (FPN) increases emotionality and exploratory behaviors. These results agree with that discovered by Terçariol *et al*. [21].

In the DL test, FPN-treated rats showed greater latency or required more time to place forelimbs or all limbs on the floor of the dark box than normal rats. This result was similar to that reported by Arrant *et al*. [36], who used the DL test to evaluate behavioral differences between adolescent and adult male rats. Using factor analysis and multiple regression, they evaluated whether DL behavioral measurements indicate anxiety-like behavior equally in each age range. In each age group, duration in the light compartment, percent distance in the light, rearing, and latency to emerge into the light compartment were indicators of anxiety-like behavior, but the total distance traveled and distance in the dark compartment were indicators of locomotor activity. Adolescent rats entered the light compartment faster than adults and made fewer pokes into the light compartment. These age disparities might reflect little age rats taking more risks and less risk evaluation than adults. The anxiogenic effects of the benzodiazepine inverse agonist N-methyl-carboline-3-carboxamide (FG-7142) and the two adrenergic antagonists on anxiety-like

behaviors verified by component analysis were less sensitive in adolescents than in adults, while locomotor variables were equally impacted.

Furthermore, in the EPM test the high number of entries to the closed arm and time spent within it reflects a high level of animal anxiety. This research suggests that rats given FPN suffer anxiety. Rats exposed to FPN had a higher number of entries and spent more time in closed arms. Concurrent therapy with NS, however, resulted in lower levels of anxiety, indicating that NS has a strong anxiolytic effect and can be used as an alternate antianxiety medication. These results agree with those discovered by Benkermiche *et al*. [23], who tested ginger extract (500 mg/kg/day) and NS oil (2 ml/k/day) in mercuric chloride-treated rats for 4 weeks. On the 21st day of the experiment, each rat was put for 5 min in the middle area facing an open arm with a greater time spent on the open arms and a shorter time in the closed arms than rats treated with mercuric chloride. Then, it was found that the benefits of herbal compounds may be connected to the effects of herbs in alleviating sadness and anxiety.

In this study, the most significant findings have included the production of oxidative brain damage caused by FPN, as well as the subsequent modification of cell membrane activities, which resulted in excessive inflammatory cytokine release. Together with oxidative stress, the generated cytokines may have reduced neurotransmitter synthesis thus inducing apoptotic and necrotic neuronal death. Such activities cause neurobehavioral changes in FPN-exposed rats. In consistence with the findings of Badgujar *et al*. [9], FPN significantly increased oxidative stress markers (MDA). Due to the high lipid content of brain tissue membranes, which are rich in oxidizable polyunsaturated fatty acids, brain tissues are extremely sensitive to oxidative stress caused by FPN, finally affecting cellular membrane both structure and function as well as inhibiting membrane-bound enzymes [37]. A significant decrease in Nrf-2 level was found in the brain tissues of the FPN-treated group, which may refer to neuronal injury and an increase in oxidative enzymes in the brain, causing oxidative stress. These findings support those of Seydi *et al*. [38], who proposed that FPN causes oxidative damage through increased ROS generation. NS administration found significant dose-dependent alterations in Nrf-2 gene expression. Nrf-2 is a critical method for cells to protect against oxidative stress [39–41]. These positive results obtained for Nrf-2 gene production indicate that NS exerts its antioxidant activity against FPN neural toxicity.

The current study's findings show a substantial rise in antioxidant markers with NS administration in the two tested doses (100 200 mg/kg). The antioxidant properties of NS have already been documented [42,43]. Most of the consequences of those activities were overcome by presupplementation with NS. NS is widely recognized for its high antioxidant qualities as previously found by Leong *et al*. [44]. In the same context, Mosbah *et al*. [45] reported that NS oil was effective against acetamiprid-induced testicular oxidative damage in male rats. Thymoquinone is the major active ingredient in seed-protected organs against oxidative damage caused by a range of free radical-producing stimuli. The administration of TQ suppressed diazinon-induced cardiotoxicity and enhanced the activity of cholinesterase in challenged rats through its powerful ROS-scavenging activity [46].

Significant increases were found in the inflammatory mediator (NF-κB) gene and pro-inflammatory cytokines (TNF- α) in the brain tissues of the FPN-treated group. These increased levels might be the result of neuronal injury and brain inflammation. These findings support those of Seydi *et al*. [38], who proposed that FPN causes tissue inflammation through increased free-radical generation. Different dosages of NS administration resulted in significant dose-dependent alterations in gene expression. Inhibition of NF-κB and TNF-α are proposed methods for polyphenols to protect against neurodegenerative disorders. These negative results obtained for NF-κB gene production indicate that NS exerts its anti-inflammatory activity against FPN toxicity through the inhibition of NF-κB, which is consistent with former findings [43,47]. TQ-rich black cumin oil attenuated the levels of inflammatory mediators (TNF-α and NF-κB) in ibotenic acid-induced excitotoxicity and neuroinflammation in rats.

The current data revealed that FPN administration causes significant histopathological alteration in the brain of FPN-treated rats. These alterations include congestion of the cerebral blood vessels, neuronal degeneration, and satellitosis of perineural oligodendroglia and perivascular edema, as well as necrosis of Purkinje cells. This change is directly related to FPN-induced oxidative damage and inflammation, which cause mitochondrial and cytoskeletal dysfunction and modification, where free radicals can react with cellular components, causing significant imbalances in vital cellular activities. The disintegrated membrane loses permeability, leading to vacuolization and brain edema [48].

FPN residues were detected at high concentrations within rat brains, which imply the movement

of FPN and/or its derivative through the blood–brain barrier and through the neuronal membrane. The process of astroglia proliferation and hypertrophy is referred to as 'reactive astrogliosis.' Glial cells emit ROS and inflammatory cytokines during this process, causing brain inflammation and neuronal death. The excessive production of ROS by FPN could activate the reactive astrogliosis process and the associated inflammatory and apoptotic responses which cause ROS proliferation and mitochondrial malfunction, which leads to the generation of apoptotic proteins, mainly caspase-3 [49]. As consistent with the findings of Zhang *et al*. [50], who concluded that FPN stimulated the apoptotic signal through mitochondrial damage, the recent study results show that the brains of rats given FPN display strong positive brown immunostaining in the cytoplasm of the neuronal cells against caspase- 3. In contrast, brains show moderate positive brown immunostaining against caspase-3 in the cytoplasm of Purkinje cells in rats treated with NS100 mg/kg with FPN compared with rats treated with NS200 mg/kg + FPN, which show negative immunostaining against caspase-3. So the antioxidant as well as anti-inflammatory qualities of NS may explain their antiapoptotic effects, as seen by the negative to weak immune expression of caspase-3 in rats given FPN and NS simultaneously as previously documented [51]. According to Mittal *et al*. [52], necrosis of neurons might be linked with decreased regenerated ROS causing vascular malfunction. Throughout this cycle, glial cells emit ROS and inflammatory cytokines, which promote brain inflammation and neuronal death. FPN's excessive production of ROS may trigger the reactive astrogliosis process as well as the associated inflammatory and apoptotic responses.

5. Conclusion

The available results show that FPN induces rat brain damage by activating signaling routes of oxidative stress, inflammation, and apoptosis. NS at the two tested doses of 100, and 200 mg/kg minimize its effect. It has been shown to improve neuroprotective properties by activating GABA and improving histopathological imaging, neurobehavior, and relief of oxidative stress caused by FPN accumulation within rats' brain tissues.

Ethics approval

The current research was permitted to be executed according to the standards of the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University (Number of approvals: M/20).

Availability of data and materials

The data present in this study are not publicly available but are available on reasonable request.

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Authors' contributions

This work is part of the MSc thesis performed by N.M.M and supervised by Professor Dr. F.R.A.S. and Dr. O.A.H.

Conflict of interest

There is no conflicts of interest.

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