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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2017.05.013

Bougainvillea spectabilis flowers extract protects against the rotenone-induced toxicity

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ARTICLE INFO

Article history:
Received 21 Feb 2017
Received in revised form 20 Mar 2017
Accepted 19 Apr 2017
Available online 18 May 2017

Keywords:
Bougainvillea spectabilis
Flowers
Extract
Rotenone
Oxidative stress
Parkinson's disease

ABSTRACT

Objective: To investigate the effect of two extracts of *Bougainvillea spectabilis* (*B. spectabilis*) flowers with yellow and pink/purple on brain oxidative stress and neuronal damage caused in rats by systemic rotenone injection.

Methods: Rotenone 1.5 mg/kg was given three times per week alone or in combination with *B. spectabilis* flowers extracts (25 mg or 50 mg) via the subcutaneous route for 2 weeks. Brain concentrations of the lipid peroxidation marker malondialdehyde (MDA), reduced glutathione, nitric oxide (nitrite), the pro-inflammatory cytokine interleukin-1beta (Il-1β) as well as butyrylcholinesterase, and paraoxonase-1 (PON-1) activities, were determined. Histopathology and caspase-3 immunohistochemistry were also performed.

Results: Rotenone resulted in significant increases of brain MDA (the product of lipid peroxidation), and nitric oxide content along with decreased brain reduced glutathione. There were also marked and significant inhibition of brain PON-1 and BChE activities and increased Il-1β in brain of rotenone-treated rats. *B. spectabilis* flowers extract itself resulted in brain oxidative stress increasing both lipid peroxidation and nitrite content whilst inhibiting PON-1 activity. The yellow flowers extract inhibited BChE activity and increased brain Il-1β. When given to rotenone-treated rats, *B. spectabilis* extracts, however, decreased lipid peroxidation while their low administered doses increased brain GSH. Brain nitrite decreased by the pink extract but showed further increase by the yellow extract. Either extract, however, caused further inhibition of PON-1 activity while the yellow extract resulted in further inhibition of BChE activity. Histopathological studies indicated that both extracts protected against brain, liver and kidney damage caused by the toxicant.

Conclusions: These data indicate that *B. spectabilis* flowers extracts exert protective effect against the toxic effects of rotenone on brain, liver and kidney. *B. spectabilis* flowers extracts decreased brain lipid peroxidation and prevented neuronal death due to rotenone and might thus prove the value in treatment of Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder of progressive nature for which there is no curative remedy [1]. In PD there is selective loss of the dopamine containing neurons of the substantia nigra pars compactica (SNPc) that project to the striatum [2]. This results in disordered voluntary motor activity causing slowing and difficulty in initiating movements, muscular rigidity, postural instability, and hand tremor [3]. In the course of the disease, other neuronal systems including cholinergic, noradrenergic, and glutamatergic neurons are also affected as well by the degenerative process with emergence of non-motor manifestations such as depression, and cognitive decline [4,5]. The mechanisms underlying cell death in PD are not clear but there is accumulating evidence which suggests a leading role for oxidative stress and neuroinflammation [6,7]. In the treatment of PD, replacement

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Peer review under responsibility of Hainan Medical University.

therapy with L-dopa, the precursor of dopamine and dopamine receptor agonists remain first line agents [8,9].

Parkinson's disease is an idiopathic disorder in the majority of instances with only of 5% of cases being of a genetic origin [10]. The aetiology of idiopathic PD presumably involves the exposure to an environmental toxin and a genetic component as well [11]. Recently interest has focused on the role of pesticides in relation to PD being driven by epidemiological studies that associates between pesticide exposure and PD [12,13]. Rotenone is a pesticide of plant origin [14] that when injected into rodents replicates many features of human idiopathic PD including motor impairment and pathologic brain changes such as nigrostriatal degeneration and α-synuclein inclusions [15,16]. In recent years, the rotenone model of PD has been widely used to explore the mechanisms of cell death in PD and possible therapeutic interventions [17–19].

In this study, we aimed to explore the utility of an extract from the flowers of *Bougainvillea spectabilis* (*B. spectabilis*as) a possible agent in the treatment of PD. *Bougainvillea* (family: Nyctaginaceae) is a popular ornamental plant grown in many parts of the world in tropical and subtropical gardens for its colorful flower bracts of red, pink, yellow, and white verities [20]. *Bougainvillea* spp. also showed medicinal properties for a methanolic extract of the leaves of *B. spectabilis* displayed an anti-inflammatory activity [20] while an aqueous extract exerted hypoglycaemic and hyoplipidaemic activity in diabetic rats [21].

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats from the Animal House of the National Research Centre, Cairo weighing (180–200) g were used in the study. Rats were group-housed under temperature- and light-controlled conditions and allowed standard laboratory rodent chow and water *ad libitum*. Animal procedures followed the recommendations of the Ethics Committee of the National Research Centre and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and chemicals

Rotenone was purchased from Sigma–Aldrich (St Louis, MO, USA) and dissolved in 100% dimethyl sulfoxide. The flowers of *B. spectabilis* (yellow and pink) were obtained from local garden in Giza province. The plant was identified and authenticated by an expert in the Orman garden. The flowers of *B. spectabilis* (yellow and pink) (100 g for each) were crushed and extracted with 70% methanol by soaking at room temperature and the methanol extract was evaporated under reduced pressure and lyophilized (8 g for each).

2.3. Study design

Rats were randomly divided into ten groups, with six rats in each group. The first set of the experiment comprised five groups and aimed to investigate the effect of the *B. spectabilis* pink flowers extract. The second set of the experiment also comprised five groups and aimed to investigate the effect of the *B. spectabilis* yellow flowers extract. The groups in each set were as follows: group 1 received saline; group 2 received the extract only at a dose of 50 mg/kg subcutaneously; group 3 received subcutaneously injection of rotenone at a dose of 1.5 mg/kg; groups 4 and 5 received rotenone 1.5 mg/kg subcutaneously along with the extract at doses of 25 mg/kg or 50 mg/kg. Treatments were given every other day for 2 weeks. Rats were then euthanized by decapitation for tissue collection; their brains were quickly removed out on an ice-cold plate, washed with ice-cold phosphate-buffered saline (pH 7.4), weighed, and stored at −80 °C until further biochemical studies. The tissues were homogenized with 0.1 M phosphate-buffered saline (pH 7.4) to give a final concentration of 10% weight/volume for the biochemical assays.

2.4. Determination of total phenolic content

The concentration of total phenolics of the plant extract was determined according to the method described by Kumar *et al.* [22]. Gallic acid was used as standard. Briefly, a mixture of 100 µL of plant extract (100 µg/mL), 500 µL of Folin-Ciocalteu reagent and 1.5 mL of Na_2CO_3 (20%) was shaken and diluted up to 10 mL with water. After 2 h, the absorbance was measured at 765 nm (using a spectrophotometer). All determinations were carried out in triplicate. The total phenolic concentration was expressed as gallic acid equivalents.

2.5. Determination of total flavonoid content

Total flavonoid concentration of plant extract and fractions was determined according to the reported procedure by Kumaran and Karunakaran [23]. A total of 100 µL of plant extract (10 mg/mL) in methanol was mixed with 100 µL of 20% $AlCl_3$ in methanol and a drop of acetic acid, and then diluted to 5 mL with methanol. The absorbance was measured at 415 nm after 40 min against the blank. The blank consisted of all reagents and solvent without $AlCl_3$. All determinations were carried out in triplicate. The total flavonoid concentration was expressed as rutin equivalents.

2.6. Determination of malondialdehyde

Lipid peroxidation products were determined in brain homogenates by measuring the level of malondialdehyde (MDA) using the colorimetric method of Nair and Turne [24]. Thiobarbituric acid (TBA) reactive substances react with thiobarbituric acid to produce TBA-MDA adduct having a peak absorbance at 532 nm.

2.7. Determination of nitric oxide

Nitric oxide in the brain homogenate was measured using Griess reagent [25]. Nitrite which is a stable end product of nitric oxide radical is used as indicator for the production of nitric oxide. Concentrations were determined using a spectrophotometer by measurement of absorbance at 540 nm.

2.8. Determination of reduced glutathione

Reduced glutathione was measured in brain homogenates with the method of Ellman *et al.* [26]. The procedure is based on the reduction of Ellman's reagent [DTNB; 5, 5′-dithiobis (2-nitrobenzoic acid)] by-SH groups of reduced glutathione to

form 2-nitro-s-mercaptobenzoic acid which can be determined using spectrophotometer at 412 nm.

2.9. Determination of paraoxonase-1 activity

Arylesterase activity of PON-1 was measured using phenylacetate as a substrate and the formation of phenol was measured spectrophotometrically by monitoring the increase in absorbance at 270 nm at 25 °C. One unit of arylesterase activity is defined as 1 μM of phenol formed per minute. Enzyme activity was calculated based on the extinction coefficient of phenol of 1 310 M^{-1} cm^{-1} at 270 nm, pH 8.0 and 25 °C and expressed kilo International Unit/Liter (kU/L) [27,28].

2.10. Determination of butyrylcholinesterase activity

Butyrylcholinesterase activity was determined in brain supernatants using commercially available kit (Ben Biochemical Enterprise, Milan, Italy). In this colorimetric assay, cholinesterase catalyzes the hydrolysis of butyrylthiocholine forming butyrate and thiocholine. The thiocholine then reacts with dithiobis-nitrobenzoic acid (DTNB) to form a colored compound. The increase in the absorbance in unit time at 405 nm is proportional to the activity of the cholinesterase in the sample.

2.11. Quantification of Interleukin-1β

The level of interleukin-1β in brain tissue was determined with a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Glory Science Co, Ltd, Del Rio, TX, USA).

2.12. Histopathological and immunohistochemical studies

Brain, liver and kidney tissues were fixed in 10% buffered formalin, dehydrated in graded ethanol and embedded in paraffin using standard procedures. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) for histopathological examination under light microscope. Immunostaining of anti-caspase-3 antibodies was performed with streptavidin-biotin. In brief, sections of 4 μm thick were deparaffinized and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The specimens were then incubated with anti caspase-3 antibodies as the primer antibody at a 1: 100 dilution. The specimens were counter stained with H&E. Negative controls were prepared by substituting normal mouse serum for each primary antibody.

3. Results

3.1. Total phenolic and flavonoid content

The total phenolic content was found to be 55.05 ± 0.01 and 56.06 ± 0.00 for the yellow and pink flowers extracts, respectively. The total flavonoid content was found to be (319.33 ± 14.87) and (217.03 ± 12.87) μg/mL for the yellow and pink flowers extracts, respectively.

3.2. B. spectabilis alone

3.2.1. Oxidative stress

The administration of only *B. spectabilis* flowers extracts at 50 mg/kg elicited an increase in brain MDA and nitrite content by 24.6% and 17.4% for the pink flowers extract and 43.8% and 115.8%, for the yellow flowers extract respectively. There were also a significant and marked decrease in brain GSH by 28.4% and 31.6% by the pink and yellow flowers extracts, respectively as compared to the saline control group (Tables 1 and 2).

3.2.2. PON-1 activity

Paraoxonase-1 activity decreased by 41.4% and 72.4% by the pink and yellow flowers extracts, respectively as compared to the saline control group (Tables 1 and 2).

Table 1
Effect of *B. spectabilis* pink flowers extract on brain oxidative stress induced by systemic rotenone administration in rats.

Extracts	Saline	Only pink flowers extract 50 mg/kg	Rotenone	Rotenone + pink flowers extract 25 mg/kg	Rotenone + pink flowers extract 50 mg/kg
MDA (ng/g tissue)	21.10 ± 0.98	26.30 ± 1.10*	28.50 ± 1.30*	31.50 ± 2.10	20.80 ± 1.70+#
Nitrite (μg/g tissue)	24.80 ± 1.56	29.20 ± 1.30*	35.40 ± 3.20*	38.50 ± 2.00	24.40 ± 1.20+#
GSH (μg/g tissue)	4.72 ± 0.38	3.38 ± 0.09	3.28 ± 0.05*	3.73 ± 0.02	3.33 ± 0.04
PON-1 (kU/L)	22.40 ± 1.30+	17.61 ± 0.36*	11.94 ± 0.63*	12.44 ± 0.39	10.00 ± 1.02+#

*$P < 0.05$ compared with saline. +$P < 0.05$ compared with rotenone only treated group. #$P < 0.05$ compared with the lower dose of the extract.

Table 2
Effect of *B. spectabilis* yellow flowers extract on brain oxidative stress induced by systemic rotenone administration in rats, on brain cholinesterase activity (BChE) in rats treated with systemic rotenone and on brain IL-1β in rats treated with systemic rotenone.

Extracts	Saline	Yellow flowers extract (50 mg/kg)	Rotenone	Rotenone + yellow flowers extract (25 mg/kg)	Rotenone + yellow flowers extract (50 mg/kg)
MDA (ng/g tissue)	18.36 ± 1.00	26.40 ± 1.80*	25.40 ± 2.10*	19.40 ± 0.76+	13.53 ± 1.20+#
Nitrite (μg/g tissue)	26.00 ± 1.32	56.12 ± 3.00*	34.15 ± 1.90*	48.30 ± 2.20+	42.00 ± 2.70+
GSH (μg/g tissue)	4.81 ± 0.15	3.29 ± 0.05	2.96 ± 0.02*	3.63 ± 0.04	3.10 ± 0.07
PON-1 (kU/L)	19.84 ± 1.60+	5.48 ± 0.22*	8.40 ± 0.62*	6.10 ± 0.18+	3.56 ± 0.12+#
BChE (U/L)	283.90 ± 13.00	215.74 ± 7.42*	181.69 ± 9.30*	68.13 ± 2.10+	75.70 ± 3.90+
IL-1β (pg/mL)	40.800 ± 2.103	68.265 ± 3.510*	57.845 ± 1.860*	62.45 ± 3.93	58.57 ± 3.30

*$P < 0.05$ compared with saline. +$P < 0.05$ compared with rotenone only treated group. #$P < 0.05$ compared with the lower dose of the extract.

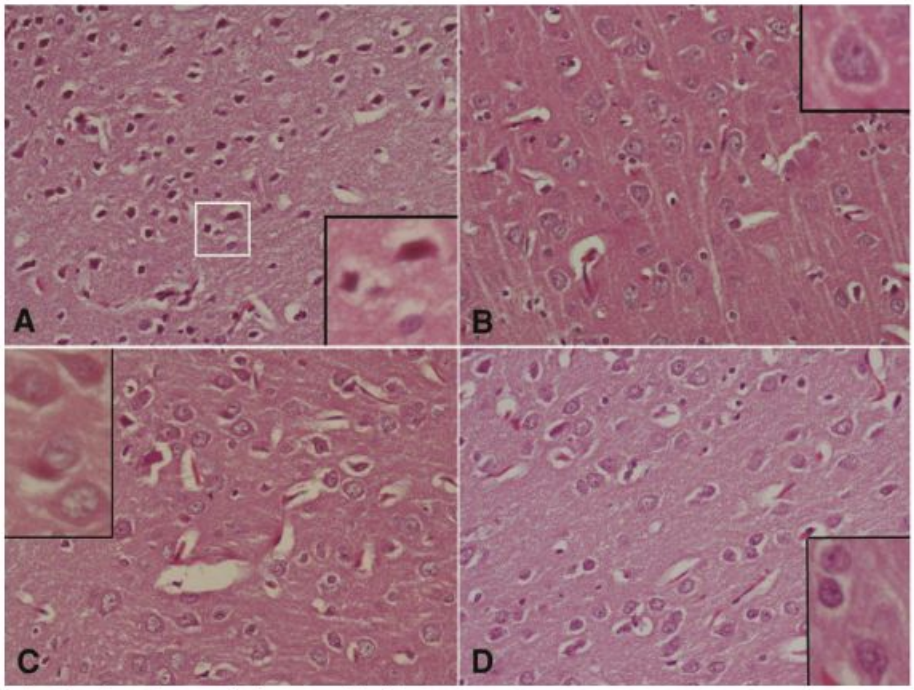

Figure 1. Photomicrograph of sections of rat cerebral cortex with pink flowers.
(A) Rotenone only treatment shows many dark neurons that appear in the magnified part with small deeply-stained nucleus and acidophilic cytoplasm. (B) Pink flowers extract only treatment shows quite normal structure of cerebral cortex tissue. (C) Rotenone and pink flowers extract (25 mg/kg) shows only a few cells with signs of degeneration and some other with dark cytoplasm with normal nuclei. (D) Rotenone and pink flowers extract (50 mg/kg) shows almost brain normal tissue (H&E ×200; ×500 for the corners).

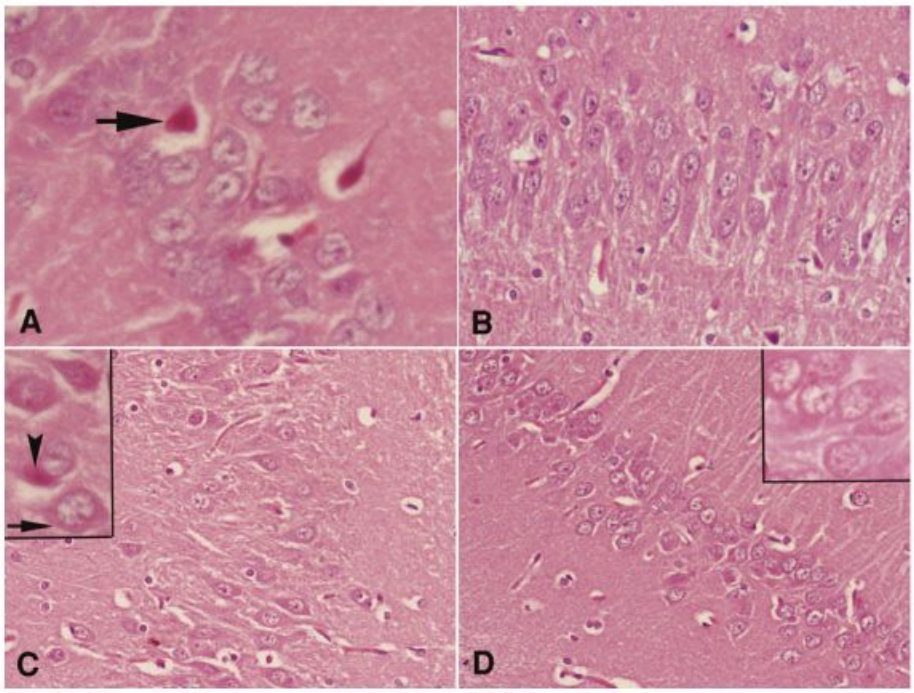

Figure 2. Photomicrograph of sections of rat hippocampus with pink *B. spectabilis* flowers extract.
(A) Rotenone only treatment shows some dark neurons (arrow) with small deeply-stained nucleus and acidophilic cytoplasm. (B) Pink flowers extract only shows quite normal structure of hippocampal tissue. (C) Rotenone and pink flowers extract (25 mg/kg) shows disarrangement of neurons with a few cells that show signs of degeneration (arrowhead) and some other with dark cytoplasm with normal nuclei (arrow). (D) Rotenone and pink flowers extract (50 mg/kg) shows almost normal shaped neurons, although cytoplasm appear slightly darker than normal (H&E ×200 for B, C and D; ×500 for A and the corners).

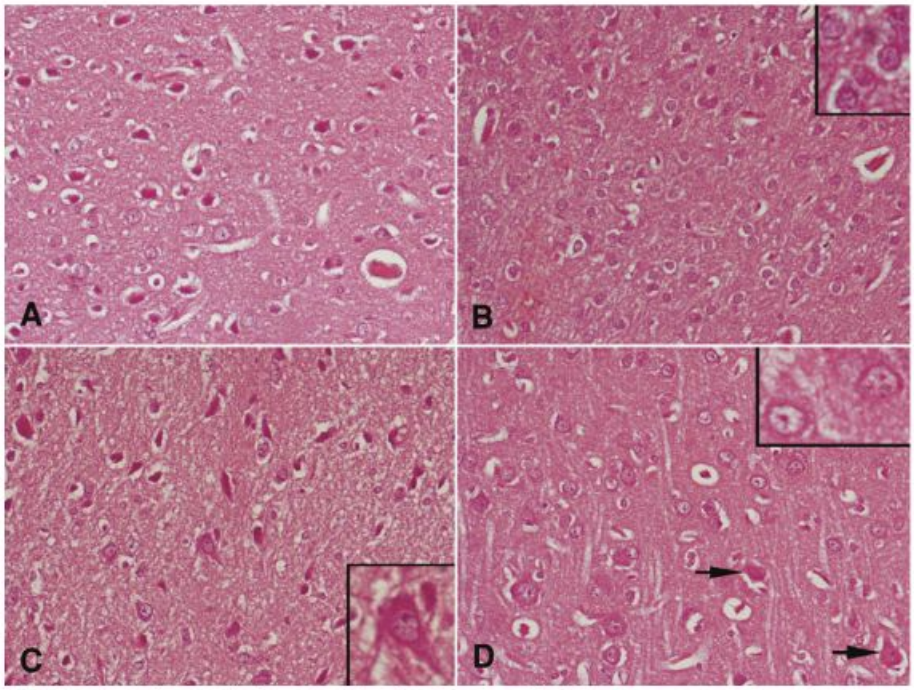

Figure 3. Photomicrograph of sections of rat cerebral cortex with yellow *B. spectabilis* flowers extract.
(A) Rotenone only treatment shows many dark neurons if compared with normal cells. (B) Yellow flowers extract only quite normal shaped neurons. (C) Rotenone and yellow flowers extract (25 mg/kg) shows some cells with signs of degeneration and some other with dark cytoplasm with normal nuclei. (D) Rotenone and yellow flowers extract (50 mg/kg) shows few cells with dark cytoplasm and normal nuclei (H&E ×200; ×500 for the corners).

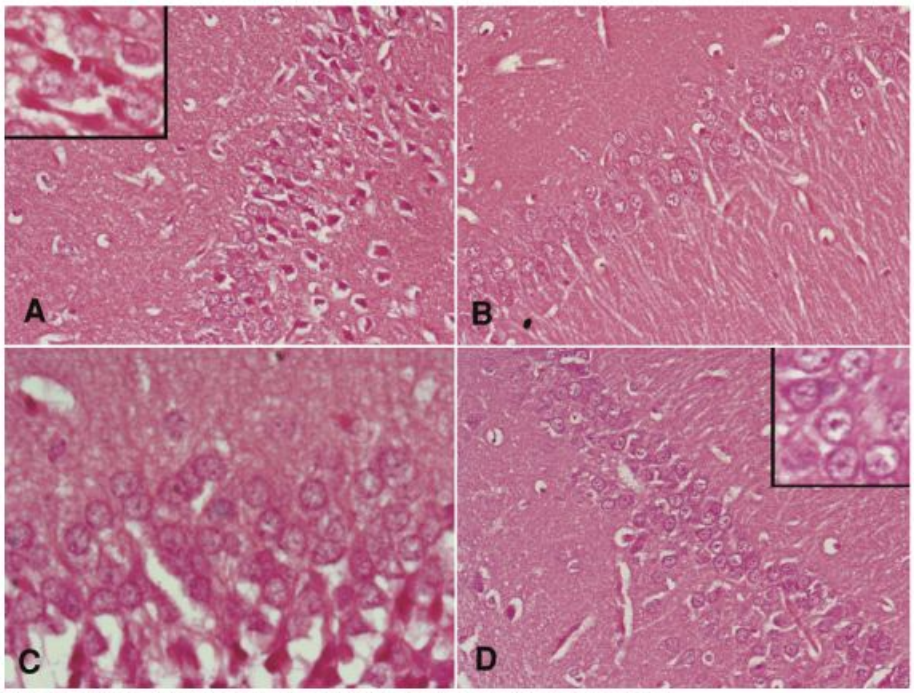

Figure 4. Photomicrograph of sections of rat hippocampus with yellow *B. spectabilis* flowers extract.
(A) Rotenone only treatment many dark neurons if compared with normal cells, they also appear disarranged. (B) Yellow flowers extract only shows quite normal shaped neurons. (C) Rotenone and yellow flowers extract (25 mg/kg) shows some cells with dark cytoplasm. (D) Rotenone and yellow flowers extract (50 mg/kg) shows few cells with dark cytoplasm and normal nuclei (H&E ×200 for A, B and D; ×500 for C and the corners).

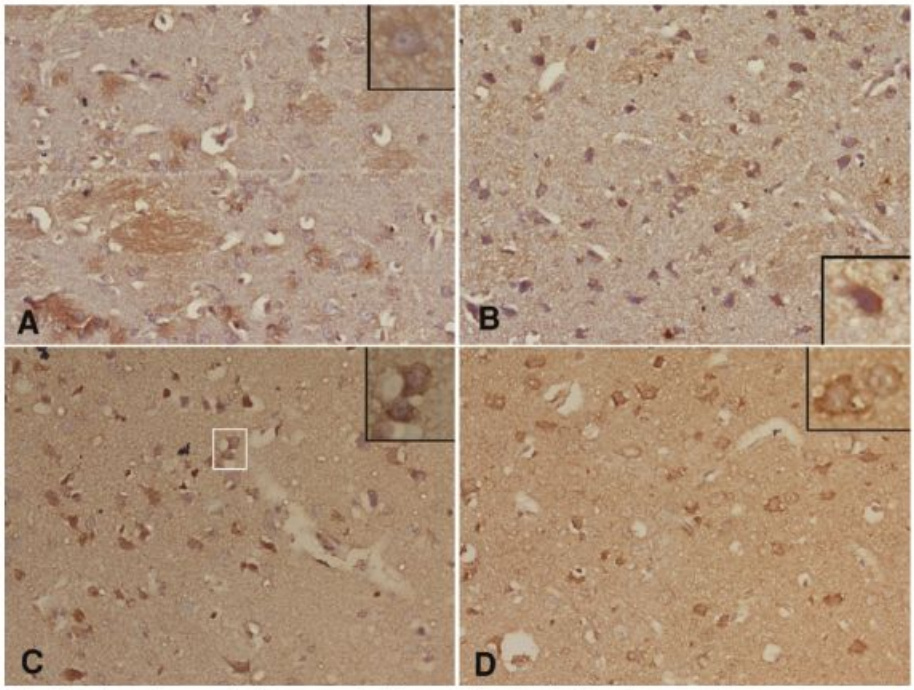

Figure 5. Photomicrograph of sections of rat cerebral cortex stained immunohistochemically with caspase-3.
(A) Vehicle shows no positively stained cells. (B) Rotenone only shows many neurons that appear positively stained. (C) Rotenone and pink flowers extract (25 mg/kg) shows some neurons that give positive reaction in their cytoplasm. (D) Rotenone and pink flowers extract (50 mg/kg) shows few cells with positive reaction to the stain (H&E ×200; ×500 for the corners).

3.2.3. BChE activity

The yellow flowers extract given at 50 mg/kg resulted in 24% inhibition of BChE activity as compared to the saline control group (Table 2).

3.2.4IL-1beta

Compared with the saline group, a significant increase in IL-1beta by 67.3% was observed in rats treated with the yellow flowers extract at 50 mg/kg (Table 2).

3.3. B. spectabilis in rotenone-treated rats

3.3.1. Oxidative stress

Rats treated with rotenone alone exhibited significant increments in brain MDA and nitrite concentrations by 35.1–38.3% and 42.7–31.3%, respectively. Meanwhile, there was a significant decrease in brain GSH by 30.5–38.5% compared with the saline control group (Tables 1 and 2).

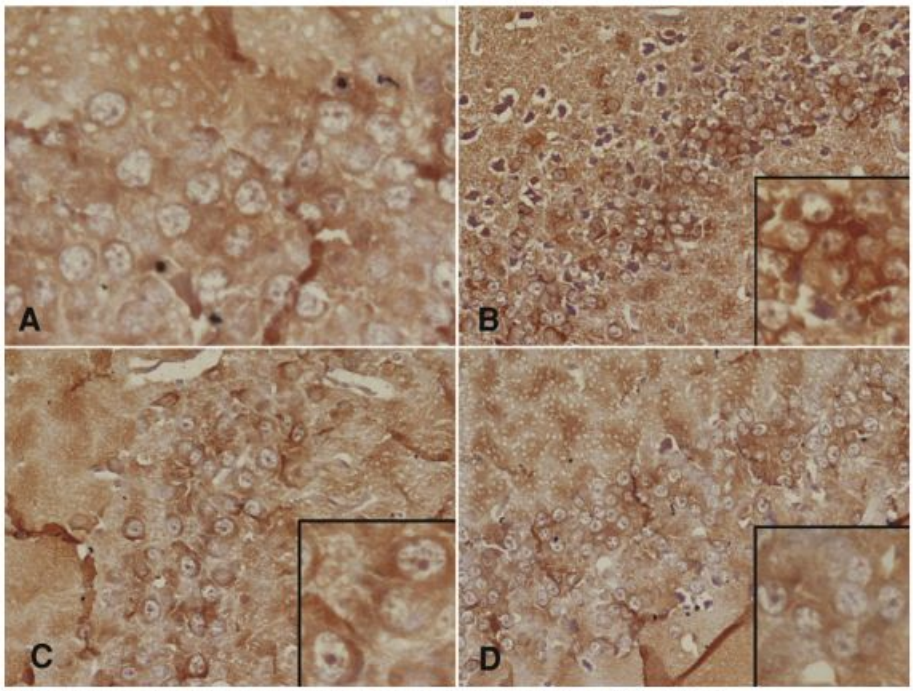

Figure 6. Photomicrograph of sections of rat hippocampus stained immunohistochemically with caspase-3.
(A) Vehicle shows no positively stained cells. (B) Only rotenone shows many neurons that appear positively stained. (C) Rotenone and pink flowers extract (25 mg/kg) shows slight reduction in the number of neurons that give positive reaction in their cytoplasm. (D) Rotenone and pink flowers extract (50 mg/kg) shows marked reduction of positively stained cells, although a few cells with positive reaction are still observed (×200 for B, C and D; ×500 for A and the corners).

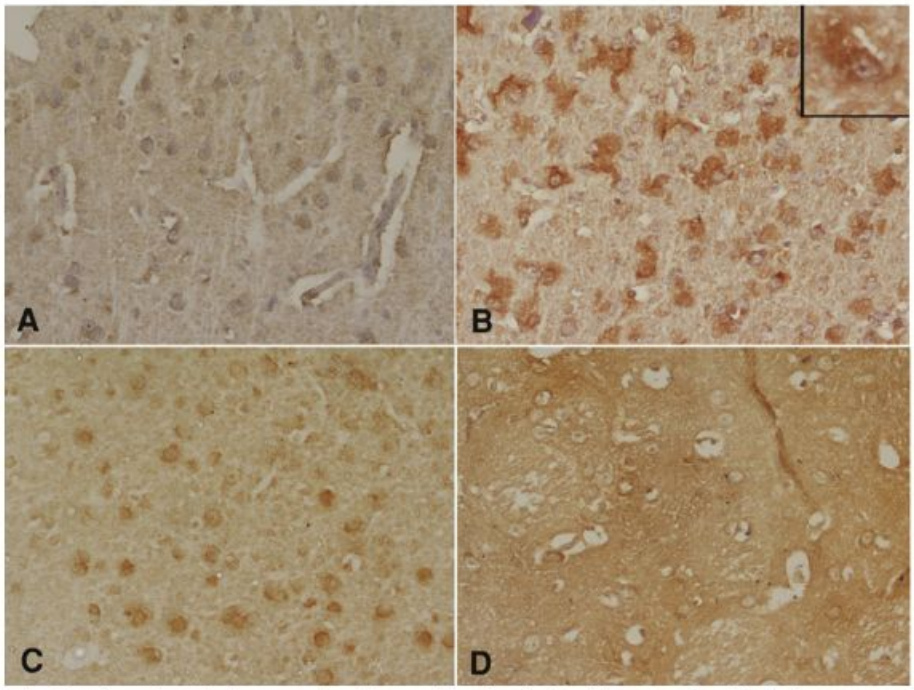

Figure 7. Photomicrograph of sections of cerebral cortex stained immunohistochemically with caspase-3 antibody from rat.
(A)Vehicle shows negative reaction to the stain in almost all the neurons. (B) Rotenone only shows many neurons that give positive reaction in their cytoplasm if compared to the negative cells in the previous section. (C) Rotenone and yellow flowers extract (25 mg/kg) shows that many positively stained cells are still observed. (D) Rotenone and yellow flowers extract (50 mg/kg) shows reduction of positively stained cells number but some cells with positive reaction are still present (H&E ×200; ×500 for the corner in B).

When given to rotenone-treated rats, *B. spectabilis* pink extract at 50 mg/kg resulted in significant decreases in brain MDA content by 27.1% compared with the rotenone only treated group. The yellow extract given at 25 and 50 mg/kg resulted in 23.6% and 46.7% decrease in brain MDA content. Brain nitrite showed 31.1% decrease after treatment with the pink extract. In contrast, 41.4% and 23% increments in brain nitrite were observed following the administration of the yellow extract at 25 and 50 mg/kg, respectively. Meanwhile, the lower dose of the pink or yellow extract increased brain GSH by 13.7% and 22.6%, respectively compared with the rotenone only treated group (Tables 1 and 2).

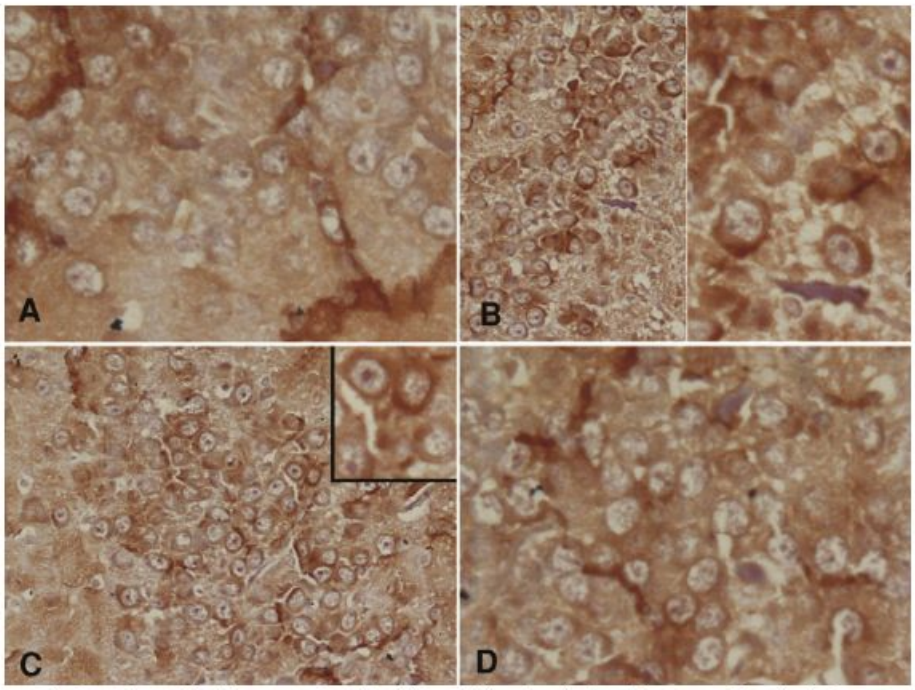

Figure 8. A photomicrograph of sections of the hippocampus stained immunohistochemically with caspase-3 antibody from rat.
(A) Vehicle shows negative reaction to the stain in almost all the neurons. (B) Rotenone only shows many neurons that give positive reaction in their cytoplasm in two parts of the photomicrograph with different magnification powers. (C) Rotenone and yellow flowers extract (25 mg/kg) a result nearly similar to that of control rat that received rotenone only. (D) Rotenone and yellow flowers extract (50 mg/kg) shows reduction of positively stained cells number, although some positively stained cells are still present (×200 & ×500).

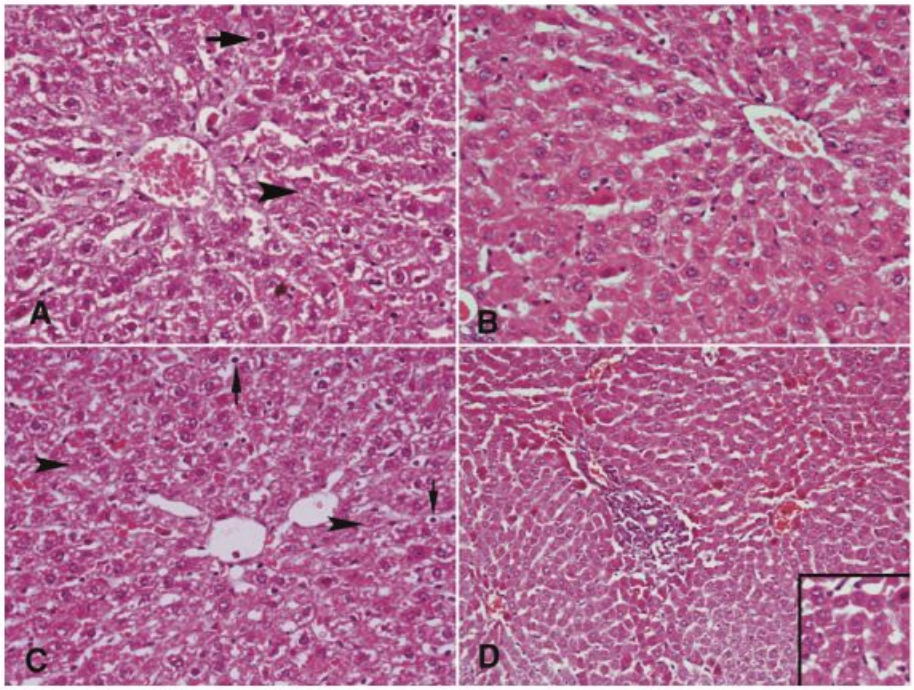

Figure 9. Photomicrograph of sections of liver tissue with pink *B. spectabilis* flowers extract.
(A) Rotenone shows vacuolar degeneration of many hepatocytes with congestion of central vein. Some cells show karrhyolysis (arrowhead), while others appear apoptotic (arrow). (B) Pink flowers extract shows quite normal structure of liver tissue. (C) Rotenone and pink flowers extract (25 mg/kg) shows amelioration of vacuolar degeneration, although some apoptotic cells are noticed. Hepatocytes with karrhyolysis are also seen. (D) Rotenone and pink flowers extract (50 mg/kg) shows almost normal structure of liver tissue except for a focal aggregation of inflammatory cells in between the lobules (H&E ×200 for A, B and C; ×100 for D; ×200 for the corner).

3.3.2. PON-1 activity

Injection of rotenone was associated with 46.7–57.7% inhibition of brain PON-1 activity compared with the saline treated group. In rats treated with rotenone, PON-1 activity showed further decreased by 16.2% in rats treated with the pink flowers extract at 50 mg/kg. It showed 27.4% and 57.6% decrements in rats treated with the yellow flowers extract at 25 and 50 mg/kg, respectively (Tables 1 and 2).

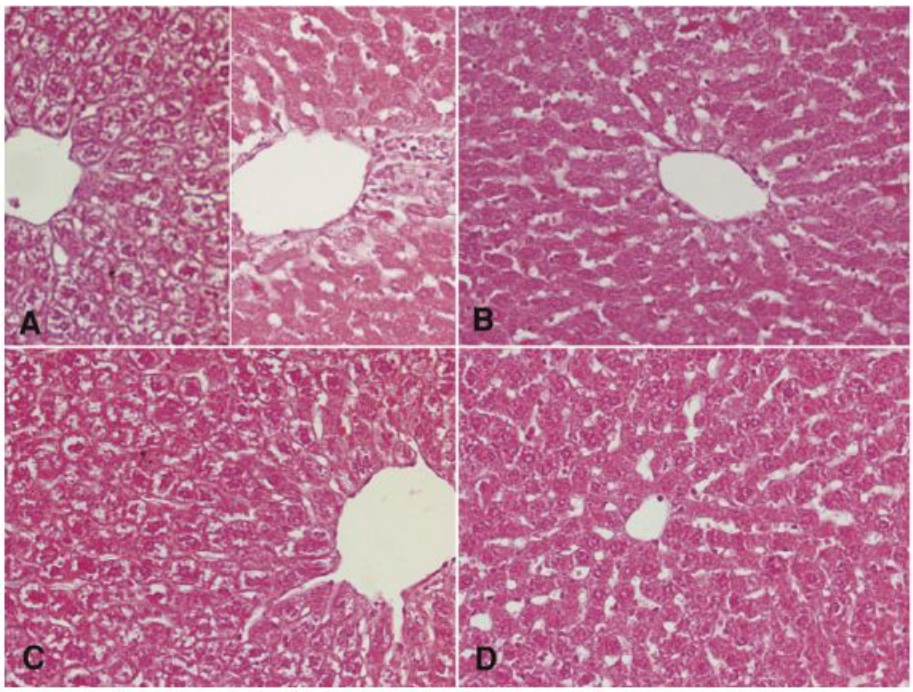

Figure 10. Photomicrograph of sections of liver tissue with yellow *B. spectabilis* flowers extract.
(A) Rotenone shows marked vacuolar degeneration in many hepatocytes (in the left part of the figure) and light inflammatory cells infiltration around blood vessels (in the right part of the figure). (B) Yellow flowers extract shows quite normal hepatic tissue. (C) Rotenone and yellow flowers extract (25 mg/kg) shows slight reduction of vacuolar degeneration in hepatocytes and no inflammatory infiltrate. (D) Rotenone and yellow flowers extract (50 mg/kg) shows quite normal structure of liver tissue (H&E ×200).

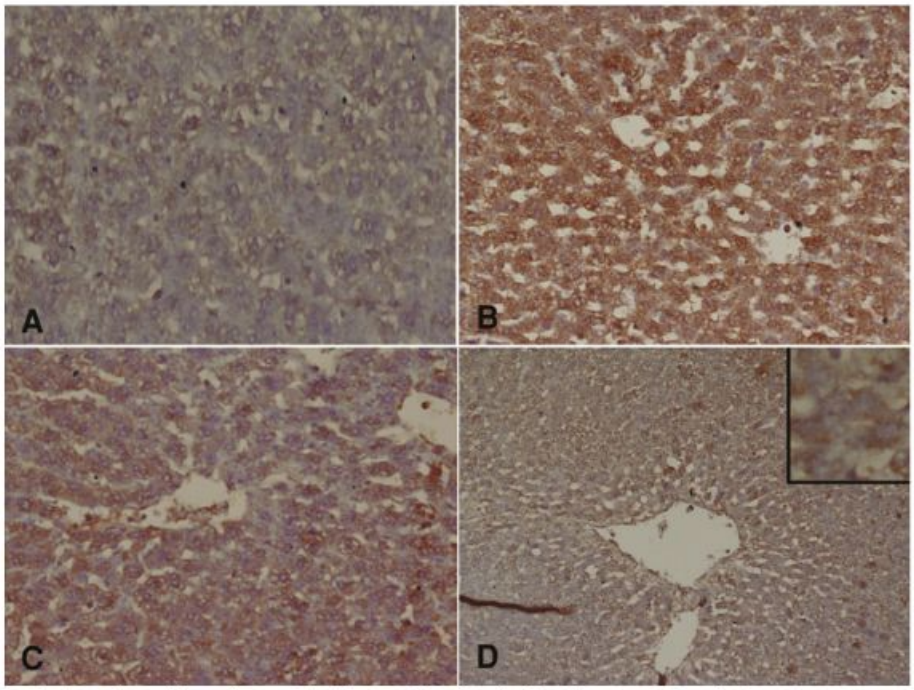

Figure 11. Photomicrograph of sections of liver tissue stained immunohistochemically with caspase-3 antibody.
(A) Vehicle shows negative reaction to the stain all over the field. (B) Rotenone shows most of hepatocytes appear with positive reaction to the stain. (C) Rotenone and yellow flowers extract (25 mg/kg) shows many positively stained cells are still noticed especially at the periphery of the lobules. (D) Rotenone and yellow flowers extract (50 mg/kg) shows reduction of positively stained cells number only a few positive cells are observed (×200 for A, B and C; ×100 for D).

3.3.3. BChE activity

The rotenone only group exhibited 36% inhibition of brain BChE activity compared with the saline group. Rats treated with both rotenone and the yellow flowers extract at 25 mg/kg and 50 mg/kg exhibited further inhibition of BChE activity by 62.5% and 58.3% as compared to the rotenone only group (Table 2).

3.3.4. IL-1beta

Rotenone injection resulted in 41.8% increase in brain IL-1beta compared with saline treated rats. The administration of the yellow flowers extract at 25 mg/kg or 50 mg/kg had no significant effect on IL-1beta in rotenone-treated rats (Table 2).

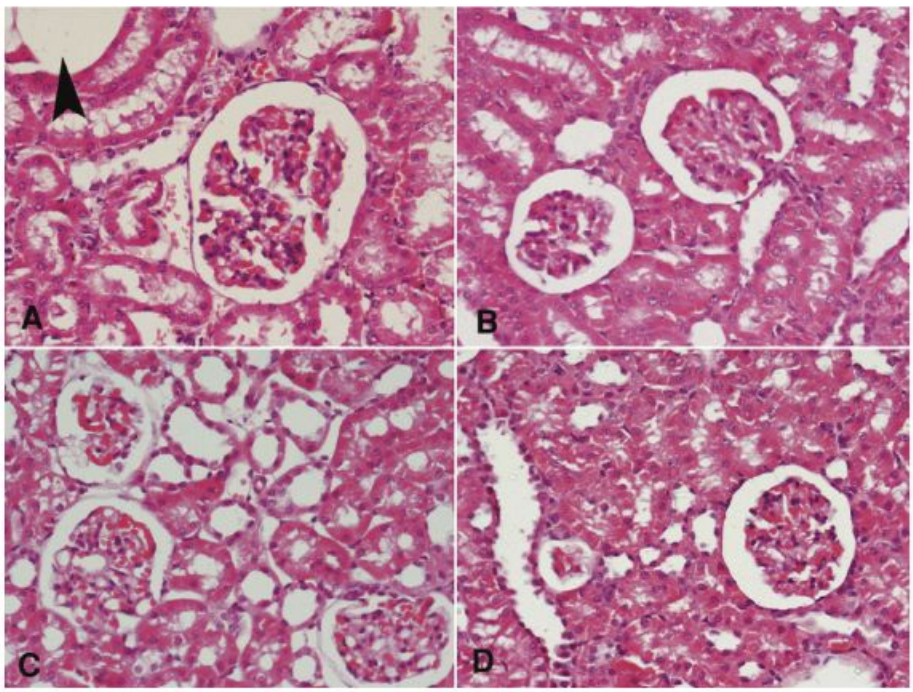

Figure 12. Photomicrograph of sections of renal tissue with pink *B. spectabilis* flowers extract.
(A) Rotenone shows signs of edema in the form of dilated tubules, atrophy of the lining epithelium and dilatation of the urinary space of glomeruli. (B) Pink flowers extract shows quite normal structure of renal tissue. (C) Rotenone and pink flowers extract (25 mg/kg) shows that signs of edema are still seen. (D) Rotenone and pink flowers extract (50 mg/kg) shows almost normal structure of renal tissue (H&E ×200).

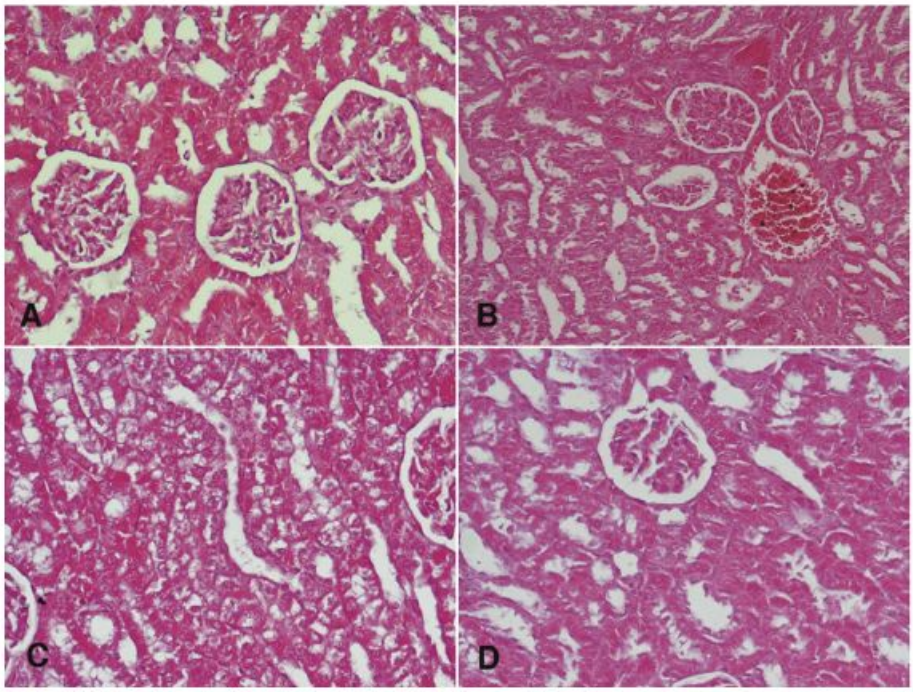

Figure 13. Photomicrograph of kidney tissue sections from rat with yellow *B. spectabilis* flowers extract.
(A) Yellow flowers extract only shows quite normal kidney tissue. (B) Rotenone only shows interstitial hemorrhage and formation of fibrous tissue in between the glomeruli. (C) Rotenone and yellow flowers extract (25 mg/kg) shows vacuolar degeneration of many epithelial cells lining the tubules. (D) Rotenone and yellow flowers extract (50 mg/kg) shows no vacuolar degeneration of epithelial cells or interstitial hemorrhage (Hx&E ×200).

3.4. Histopathological studies

3.4.1. Brain tissue

Rotenone exerted a damaging effect on brain tissue causing degeneration of neurons in the cerebral cortex (Figure 1A). Rats treated with pink flowers extract alone showed normal brain tissue (Figure 1b). On the other hand, the administration of pink flowers extract ameliorated the rotenone effects in a dose-dependent manner as small deeply-stained neurons were still observed with low dose of the extract (Figure 1C) and more reduction of damaged cells occurred with the high dose of the extract (Figure 1D). Similar results were seen in the

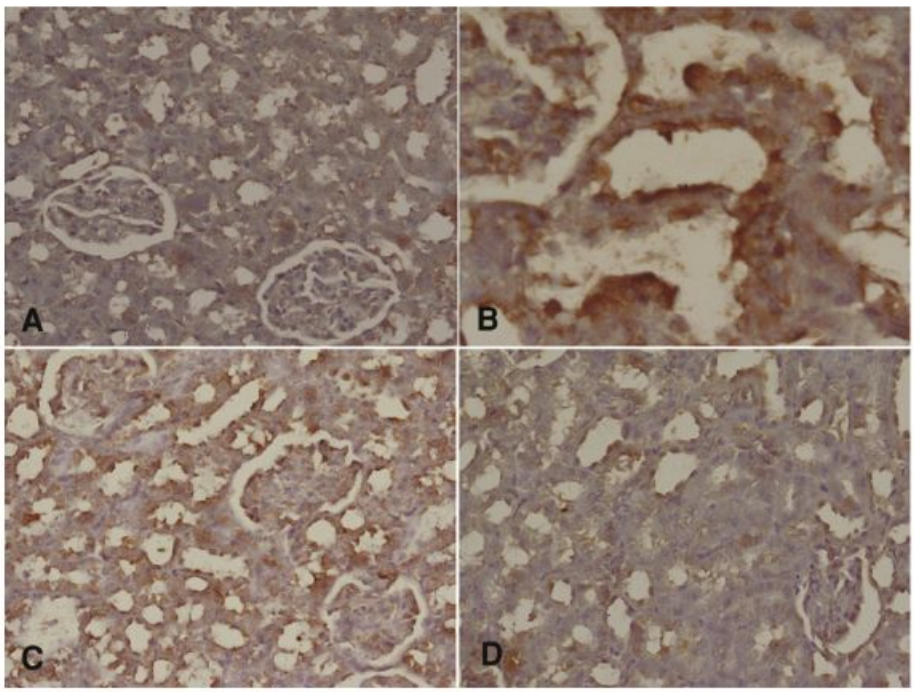

Figure 14. Photomicrograph of sections of renal tissue stained immunohistochemically with caspase-3 antibody from rat.
(A) Vehicle shows negative reaction to the stain in almost all the cells. (B) Rotenone only shows positive reaction to the stain in many of the cells lining the tubules. (C) Rotenone and yellow flowers extract (25 mg/kg) shows many positively stained cells are still observed. (D) Rotenone and yellow flowers extract (50 mg/kg) shows reduction of positively stained cells number (×200 for A, C and D; ×500 for B).

hippocampal region (Figure 2). Lesser degree of protection was obtained with the yellow extract as even with the high dose neurons with dark cytoplasm were still noticed (Figures 3 and 4). Using immunohistochemical staining for caspase-3 confirmed the above results (Figures 5–8).

3.4.2. Liver tissue

Rotenone caused marked vacuolar degeneration of many hepatocytes and nuclear changes (Figure 9A). Pink flowers extract given alone had no effect on liver tissue that appeared quite normal (Figure 9B). Pink flowers extract reduced the vacuolar degeneration in a dose-dependent manner, but cellular infiltration was still present (Figure 9C and D). Similar protective effects were also exerted with the yellow flowers extract (Figure 10) which decreased caspase-3 immunoreactivity (Figure 11).

3.4.3. Kidney tissue

Rotenone caused oedema and atrophy of epithelial lining of tubules (Figure 12A). Rats treated with only pink flowers extract showed normal renal tissue (Figure 12B). Pink flowers extract ameliorated these effects in a dose-dependent manner (Figure 12C and D). The yellow flowers extract also showed protective effects and decreased caspase-3 immunoreactivity (Figures 13 and 14).

4. Discussion

The aim of this study was to investigate the potential of two extracts of *B. spectabilis* yellow and pink/purple flowers in alleviating neurodegeneration induced in the brain of rats by systemic rotenone injection. Moreover, the effect of the extracts on liver and kidney damage caused by the pesticide extended was evaluated. Our data showed that the administration of *B. spectabilis* pink and yellow flowers extract to rotenone-treated rats was associated with a decrease in brain neuronal damage caused by the pesticide. In this respect, the pink flowers extract was more effective in ameliorating the damaging effect of rotenone with almost normal brain tissue seen after treatment with the high dose. Meanwhile, lesser degree of protection was seen in the case of the yellow flowers extract since some neurons with dark cytoplasm were still observed. *B. spectabilis* flowers extract also showed antiapoptotic effect, decreasing the positively stained neurons with anti-caspase-3 antibody. There were also clear protective effect for either extract upon the rotenone-induced liver and kidney damage.

When injected into rodents, rotenone, a pesticide of plant origin [14] has been shown to replicate the behavioral, biochemical and pathological changes of human PD [15,16]. Thus rotenone-induced nigrostriatal neurodegeneration is widely used to model human PD and to test potential therapeutic agents. In this study and in agreement with previous work [29–32], rotenone was found to cause brain oxidative stress. Increased levels of the lipid peroxidation marker malondialdehyde could be detected in the brain tissue after the administration of rotenone, and the brain nitric acid content also increased markedly after the toxicant. The occurrence of oxidative stress in the brains of rats treated with rotenone is also supported by decreased reduced glutathione content. Glutathione (γ-glutamylcysteinylglycine) which exists in the thiol-reduced and disulfide-oxidized forms is an important cellular antioxidant that functions to maintain the redox state of the cell. Glutathione protects the cell against reactive oxygen metabolites via both direct scavenging action and glutathione peroxidase-catalyzed enzymatic reactions. Hence the decrease in reduced glutathione levels could be due to the increase in oxidative stress [33]. Oxidative stress is largely thought to play a significant role in the rotenone-induced neuronal damage which could be alleviated with the use of antioxidants [29,34].

Our data also showed that rotenone increased brain interleukin-1beta (Il-1β), a proinflammatory cytokine capable of decreasing cerebral blood flow [35]. Moreover and in agreement with previous work, we observed significant inhibition of paraoxonase-1 (PON-1) activity in the brain of rats treated with the toxicant. This enzyme hydrolyzes the oxons of a number of organophosphorus insecticides [36] and a decrease in its activity has been linked to increased susceptibility to develop PD after exposure to insecticides [37]. Whether the decrease in PON1 activity is due to direct effect of rotenone or oxidative stress is not yet clear. However, there is evidence that the enzyme is sensitive to oxidative modification [38].

Previously, rotenone has been shown to decrease acetylcholinesterase activity following its systemic injection in rats [30,31]. The significance of this finding is yet to be determined but it could be relevant to the ability of the toxin to cause PD. The cholinesterase family of enzymes hydrolyzes the neurotransmitter acetylcholine and hence inhibition of enzyme activity will result in an increase level of acetylcholine in brain [39]. In PD there exists an imbalance between dopaminergic and cholinergic neurotransmission due to loss of dopaminergic neurons in the substantia nigra and striatum [40]. Moreover, anticholinergic drugs are used in mild cases and for the hand tremor [41]. The decrease in both BChE and AChE activities in the brain of rotenone treated rats could reflect loss of cholinergic neurons which was shown in previous studies [42]. AChE and/or BChE might also serve as a marker for rotenone neurotoxicity.

The mechanism by which *B. spectabilis* flowers extract prevent neuronal damage due to rotenone is unclear but could be ascribed to a lowered extent of oxidative damage due to their phenolic and flavonoid content. It was observed that the neuroprotective effect of *B. spectabilis* flowers extract was associated with a decrease in lipid peroxidation. Interestingly, however, only the lower doses of the extracts were found to increase reduced glutathione content in brains of rotenone-treated rats. Moreover, the extracts exerted differing effects on brain nitric oxide being decreased by the pink extract but increased after the yellow extract. When given in absence of rotenone, the extracts, however, showed evidence of increased oxidative stress with an increase in malondialdehyde. Notably the yellow extract increased brain nitric oxide content whilst markedly inhibiting PON-1 activity compared with the pink extract. Support for the presence of oxidative stress is also supported by the decline in PON1 activity in the brain of rats given only the extracts. Moreover, the administration of the yellow flowers extract by itself was capable of increasing Il-1β in brain. This occurred despite quite normal structure of the brain tissue on histology. These latter observations in saline treated rats suggest that the brain can tolerate moderately increased oxidative stress and inflammation without the development of overt cell injury. It is also possible that the absence of overt neuronal damage by the extracts and the neuroprotection against rotenone toxicity is due to other active constituents in the

extract. Flavonoids act as antioxidants by direct scavenging of reactive oxygen metabolites, reducing α-tocopheryl radical or chelating transition metal ions [43]. Prooxidant activity of flavonoids has also been shown eg., quercetin, a member of flavone family found in tea exhibited prooxidant actions *in vivo* [44].

Earlier work suggested a highly selective action for the pesticide on the nigrostriatal pathway [15,16]. Other researchers, however, provided data suggestive of a more widespread damage to other brain regions [30,31,45]. Liver involvement with fatty change has also been reported [46]. In this study, we demonstrated that rotenone-induced neurotoxicity involved not only the substantia nigra and striatum but also the cerebral cortex, and as well. Moreover, both the kidney and liver were affected by the pesticide. Interestingly, histopathological investigation showed that treatment with *B. spectabilis* flowers extracts exerted protective effect on the widespread organ damage caused by rotenone.

In this study, *B. spectabilis* flowers extracts were shown to reduce brain lipid peroxidation and protect against neuronal damage caused by rotenone in rat brain. Moreover, these extracts reduced liver and kidney injury in this model. These data suggest that *B. spectabilis* flowers extracts might be of value in the treatment of neurodegenerative disorders.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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