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Polygonum minus ethanolic extracts attenuate cisplatin-induced oxidative stress in the cerebral cortex of rats *via* its antioxidant properties

NR. Adib Ridzuan[1], SL. Teoh[1], N. Abdul Rashid[1], F. Othman[1], SN. Baharum[2], F. Hussan[3]✉

[1]*Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia, Malaysia*

[2]*Institute of Systems Biology (INBIOSIS) , Universiti Kebangsaan Malaysia, Malaysia*

[3]*Human Biology Division, School of Medicine, International Medical University, Malaysia*

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ABSTRACT

Objective: To explore the protective effect of *Polygonum minus* ethanolic extract on cisplatin-induced neurotoxicity.

Methods: *In vitro* test, total phenolic content assay and DPPH assay were performed to determine the antioxidant activity of *Polygonum minus*. For *in vivo* test, 30 male Sprague-Dawley rats were randomly divided into 5 groups: the control group, cisplatin 10 mg/kg, *Polygonum minus* 100 mg/kg, *Polygonum minus* 200 mg/kg and *Polygonum minus* 400 mg/kg. The control group and the cisplatin group were given distilled water whereas *Polygonum minus* groups received the respective dose of *Polygonum minus* extract orally for 14 d. On day 15, a single intraperitoneal administration of normal saline was given to the control group; while 10 mg/kg of cisplatin was given to the cisplatin group and *Polygonum minus* groups. Body weight, signs of illness, daily activity and mortality were observed at least once daily throughout the experimental period. On day 18, the anterior part of the brain was collected and processed for histological and ultrastructural analyses (right hemisphere). The remaining part (left hemisphere) of the brain was assayed to determine malondialdehyde and catalase levels for oxidative stress analyses.

Results: *Polygonum minus* ethanolic extract possessed high phenolic content (977.6 mg GAE/g) and 95.9% DPPH radical scavenging activities. No mortality was observed in all groups. Rats in the cisplatin group were weak and less active compared to *Polygonum minus* treated rats. In the cisplatin group, disorganised cellular layers of the cerebral cortex were observed whereas rats treated with low and mid doses of *Polygonum minus* extract had normal cerebral cortex as in the control group. Mild ultrastructural changes were observed in rats treated with low and mid doses of *Polygonum minus* extract. Meanwhile, low and mid doses of *Polygonum minus* extract significantly reduced malondialdehyde level whereas low and mid doses of *Polygonum minus* extracts groups significantly increased catalase activity compared to the cisplatin group.

Conclusions: *Polygonum minus* ethanolic extract at 100 and 200 mg/kg attenuates cisplatin-induced oxidative stress in the cerebral cortex *via* its antioxidant activity.

1. Introduction

Polygonum minus (Pm) or 'kesum' in Malaysia, is used as a flavouring ingredient in local dishes such as 'laksa' and 'asam pedas'[1]. It is originated from Southeast Asia countries especially Malaysia, Indonesia and Thailand[2]. It grows wildly in damp and

✉Corresponding author: Assoc. Prof. Dr. Farida Hussan, Human Biology Division, School of Medicine, International Medical University, No. 126, Jalan Jalil Perkasa 19, 57000 Bukit Jalil, Wilayah, Persekutuan Kuala Lumpur, Malaysia.
Tel: +60327317026
Fax: +60386567229
E-mail: faridahussan@imu.edu.my, khinpapah@gmail.com

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wet areas near river banks, drains and lakes. It also grows well on high hills and cool areas[3]. It has been traditionally used as an alternative medicine to treat indigestion, kidney stones, rheumatism and hair dandruff problem[2,3]. It has large medical spectrum and possesses medicinal values including antioxidant, antiulcer, anti-inflammatory, anticancer, antimicrobial and antiviral activities[4]. The compounds attributed to its medicinal properties are flavonoid, terpenoid, alkaloids, coumarins and tannins[5,6]. Due to its medicinal properties especially antioxidant, Pm is considered as a natural medicinal source.

Cisplatin (*cis*-diamminedichloroplatinum Ⅱ, CP) is the first platinum-based chemotherapeutic drug[7] which is effective for various human cancers such as lung, testicular, cervical, ovarian and head and neck cancers[8,9]. However, during CP treatment, the patients might suffer from its severe adverse effects such as nephrotoxicity, hepatotoxicity, and neurotoxicity[10]. CP-induced central nervous system toxicity had been least studied compared to CP-induced peripheral neurotoxicity. During chemotherapy, CP is intended to destroy cancerous cells; however, non-cancerous (normal and healthy) cells are also being attacked by CP. It does not have a selective effect on cancer cells. Therefore, drug-induced toxicities are the main concern in CP chemotherapy. CP-induced toxicity is dose-dependent where CP accumulation in the affected organs triggers prominent necrotic changes[11].

The mechanism of action of CP-induced neurotoxicity is uncertain; however, previous studies showed that oxidative stress (OS) due to overproduction of reactive oxygen species (ROS), high level of lipid peroxidation and decrease in the activity of antioxidant defence enzymes are the main causes of CP-induced neurotoxicity[12-14]. Increased level of malondialdehyde (MDA) and reduced catalase (CAT) level were observed in CP-induced neurotoxicity[15]. Other possible mechanisms of CP-induced neurotoxicity are inflammation, DNA damage and apoptotic cell death in the nervous system[15]. These changes can alter neuronal function, and cause nerve degeneration and loss of axons.

To the best of our knowledge, limited research had been performed on effect of Pm extract against CP-induced neurotoxicity or on its cognitive enhancing effect[16]. Thus, Pm was chosen in the present study against cisplatin-induced neurotoxicity. Given the anti-oxidant property of Pm, it is of high importance for treating the CP-induced neurotoxicity. This study will enlighten the beneficial effect of Pm and will be useful for the development of pharmaceutical products which are able to reduce the CP-induced central neurotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

CP was purchased from Cayman Chemical® (Ann Arbor, MI, USA). All chemicals and reagents used in this study were of analytical grades.

2.2. Plant collection and extraction of Pm ethanolic extract

Fresh matured Pm leaves were harvested from the experimental plot, Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia. The identification and authentication process was carried out and the leaves sample was deposited at the Herbarium of Faculty of Science and Technology, Universiti Kebangsaan Malaysia with the voucher numbered PM-2014-1. The leaves were air-dried at room temperature for two weeks free of direct sunlight. Dried leaves were processed to powdered-form using a commercial grinder. Pm powder was soaked in a mixture of ethanol: water (70:30) solvent for 72 h and was filtered using Whatman™ filtered paper Number 1. The extraction process was repeated three times to fully extract the potential compounds from the plant. The collected filtrate was dried using Eyela N-1000® rotary evaporator at 50 ℃ to obtain the ethanolic extract. The evaporation method was performed 4 to 5 times to acquire the ethanol-free concentrated ethanolic extract. Lastly, the extract was freeze-dried for easy-dilution preparation and administration to the rats *via* oral gavage.

2.3. In vitro antioxidant test

2.3.1. Total phenolic content (TPC) assay

TPC was determined using Folin-Ciocalteu reagent based on the method of Ahmad *et al*[2] by using gallic acid as a standard. Briefly, 0.2 mL of ethanolic solution of extract (1 mg/mL) and 1.5 mL of 10% Folin-Ciocalteu reagent were dissolved in water. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 mL of 6% natrium carbonate solution. After 90 min incubation at room temperature in the dark, the absorbance of the mixture was read at 725 nm using a spectrophotometer. The blank was prepared with ethanol solvent. All sample and control procedures were measured in triplicate and mean absorbance was obtained. TPC was calculated as mg of gallic acid equivalent per gram of extract (mg GAE/g of extract) using an equation obtained from the standard gallic acid calibration graph.

2.3.2. 1-1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The principle of the test is to detect the ability of extract to reduce DPPH free radicals. The method was performed according to the method by Ahmad *et al*[2]. Briefly, DPPH was dissolved in 50% ethanol and mixed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and 0.9% natrium chloride in 50 mL distilled water. The mixture solution was adjusted to pH 7.4. The Pm extract was dissolved in methanol. A total of 5 μL of the

dissolved extract was added to 195 μL of ice-cold DPPH solution and was allowed to stand at room temperature for 30 min. The test samples were then transferred to clear and transparent 96-well plates and the values were read at 517 nm using a microplate reader. Samples were measured in triplicates and gallic acid was used as a positive control. The percentage of scavenging effect was calculated as % DPPH scavenging activity = $(A_c - A_s/A_c)\times100$ where 'A_c' is absorbance of the control and 'A_s' is absorbance of the sample[2].

2.4. In vivo test

2.4.1. Animals and grouping

Healthy male Sprague Dawley rats aged between 6-8 weeks (n = 30) and weighing between 180-220 g were obtained from the institutional animal unit. The rats were acclimatized for a week prior to the experiment. They were individually housed at 12 h light-dark cycle, with an ambient temperature of 21 ℃ and relative humidity at (65 ± 10)%. Standard normal rat pellets and clean drinking water were given *ad libitum* to the rats. All the experiments were carried out according to the guidelines of the institutional animal ethical committee with the approval number, FP/ANAT/2014/FAIZAH/26-NOV./632-NOV.2014-SEPT.2017. The rats were randomly divided into five groups as follows: Control group, CP group, Pm 100 mg/kg (PmL), Pm 200 mg/kg (PmM) and Pm 400 mg/kg (PmH). Group control and CP were given distilled water 1 mL per 100 g body weight whereas the Pm groups (PmL, PmM and PmH) were given the respective dose of extract for 14 d *via* oral gavage. On day 15, the rats in CP and Pm groups were injected with a single dose of CP (Cayman Chemical, USA, 10 mg/kg) whereas control group received normal saline intraperitoneally. CP was freshly prepared as 1 mg/mL in 0.9% normal saline. Any signs of sickness including weakness, abnormality and mortality were closely monitored hourly for the first 6 h following CP injection. All rats were sacrificed on day 18. The whole brain was excised, rinsed in ice-cold saline and weighed. The anterior part of the brain was kept for histological and ultrastructural analyses (right hemisphere) in 10% formalin and 3% glutaraldehyde. The remaining part (left hemisphere) of the brain was kept in –80 ℃ for OS markers analysis.

2.4.2. General observation of the rats

Body weight, signs of illness, daily activity and mortality were observed at least once daily throughout the experimental period.

2.4.3. Histological analysis

For histological analysis, the brain was collected, rinsed in cold saline and immediately fixed in freshly prepared 10% neutral buffered formalin for 48 h. The samples were dehydrated in ascending alcohol series and embedded in paraffin wax block. Coronal sections of brain tissue (4 μm thickness) were cut with a microtome and stained with haematoxylin and eosin (H&E). The stained slides were examined under a light microscope at 200× magnification. All slides were visualized using a Leica® DMRXA2 Image Analysis system (Leica Micros Imaging Solutions Ltd, Cambridge, UK) for qualitative histological analysis.

2.4.4. Ultrastructural analysis

The anterior part of the right hemisphere of the brain was cut in 1 mm^3 size by using an ultra diamond knife and fixed in 3% glutaraldehyde, sucrose and 2.5% glutaraldehyde with phosphate saline. Then, it was post-fixed in 1% osmium tetroxide with phosphate buffer for 1 h. The brain tissue samples were dehydrated in serial alcohol and then embedded in araldite. Semi-thin sections were stained with toluidine blue and examined under Olympus® light microscope to determine the area of interest. After selecting the appropriate specimens, 50 nm thin sections were obtained and stained with uranyl acetate and lead citrate. The final slides were examined as qualitative analysis under a transmission electron microscope (Technai™, Netherlands).

2.4.5. Measurement of lipid peroxidation

Lipid peroxidation was determined by measuring the MDA levels. The brain tissues (10 mg) were lysed in 300 μL MDA lysis buffer and homogenized on ice. The mixtures were centrifuged at 13 000×g for 10 min and the supernatant was collected for analysis. In accordance with the protocol of Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit (BioVision, Cat No: K739-100, CA, USA), the standard was prepared. TBA reagent (600 μL) was added to all wells containing the standards and the samples. The plate was incubated at 95 ℃ for 60 min followed by cooling to room temperature in an ice bath for 10 min. The absorbance was read at 532 nm using an ELISA plate reader.

2.4.6. Measurement of brain CAT levels

The brain tissue was homogenized to a final concentration of 100 mg: 250 μL in cold buffer (50 mM, containing 1 mM EDTA) and then centrifuged at 10 000×g for 15 min at 4 ℃. The supernatant was removed and assayed with commercially available ELISA, Cayman's Catalase Assay Kit (Cat No: 707002, Cayman Chemical, USA). All wells were added with 100 μL assay buffer, 30 μL methanol and 20 μL of standard, positive control and samples accordingly. The reactions were initiated by adding 20 μL of diluted hydrogen peroxide to all wells. The plate was incubated on a shaker for 20 min, followed by addition of 30 μL potassium hydroxide to terminate the reaction. Later, 30 μL of catalase purpald (Chromogen) was added into the wells and incubated for 10 min. Finally, 10 μL of catalase potassium periodate was added and incubation was continued for another 5 min. The absorbance for the plate was read at 540 nm using a plate reader. CAT levels were calculated from the

standard calibration curve and expressed as nmol/min/mL.

2.5. Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). Differences among means were analysed using one-way ANOVA, followed by Tukey's *post-hoc* test. Statistical significance was considered at $P<0.05$. All computations were performed using SPSS software package version 24.

3. Results

3.1. Yield of Pm ethanolic extract

Total yield of Pm ethanolic extract collected from 800 g of wet weight of Pm leaves was 78.7 g. It is approximately about 10% of the initial weight of Pm leaves.

3.2. Antioxidant activities of Pm

The ethanolic extract of Pm contained high phenolic content [(977.6 ± 42.0) mg GAE/g of extract]. The DPPH scavenging activity of the Pm ethanolic extract showed (95.9 ± 1.9)% which was considered as high activity.

3.3. General observation results

No mortality was observed in all groups throughout the experiment. Although behavioural tests have not been used, our daily observations of treated animals showed less activity and reduced alertness compared to the control and Pm treated groups. The body weight was significantly reduced in the CP, PmL and PmM compared to the control group ($P<0.05$) (Figure 1). The brain weight showed no significant difference (data not shown).

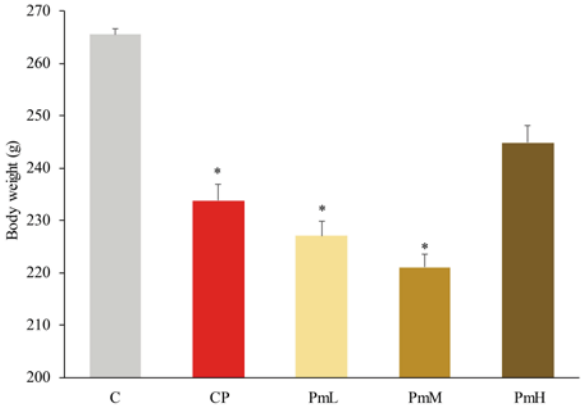


Figure 1. Body weight of rats following 72 hours after cisplatin administration. *compared with the control group ($P<0.05$). C: Control, CP: Cisplatin 10 mg/kg, PmL: *Polygonum minus* 100 mg/kg, PmM: *Polygonum minus* 200 mg/kg, and PmH: *Polygonum minus* 400 mg/kg.

3.4. Histopathological changes in brain

Control group showed features of normal cellular structures of cerebral cortex (Figure 2A). In the cerebral cortex of the control group, neurons including pyramidal cells (blue arrows) and neuroglial cells were clearly identified. However, in the CP group, disorganized layers of cerebral cortex and increased cell population (yellow arrows) were observed (Figure 2B). The PmL (Figure 2C) and PmM (Figure 2D) groups showed similar features as in the control group. We also observed increased cell population and cellular derangement in PmH group (Figure 2E).

3.5. Ultrastructure changes of neurons

Neurons in control group exhibited large, round nucleus with uniformly distributed chromatins. The nuclear membrane was smooth and clear, and various organelles were finely arranged around the nucleus. The mitochondria and endoplasmic reticulum were intact with abundant ribosomes (Figure 3A). However, the CP group exhibited poorly defined irregular nuclear membranes and mitochondrial swelling with ruptured cristae, broken rough endoplasmic reticulum and unidentified ribosomes (Figure 3B). The PmL (Figure 3C) and PmM (Figure 3D) groups showed a round nucleus with uniformly distributed chromatins. The nuclear membrane was smooth and clear. In Group PmH (Figure 3E), the nucleus was intact with unclear appearance of surrounding organelles.

3.6. Brain MDA and CAT levels

Following microscopic analyses findings, PmL and PmM were chosen and proceeded for measurement of MDA and CAT analyses. The CP group showed a significant increase of MDA levels compared to the control group [(5.08 ± 0.22) *versus* (2.66 ± 0.24) nmol/mg; $P<0.05$] which indicated the development of lipid peroxidation by CP administration. The PmL and PmM significantly reduced the MDA levels in a dose-dependent manner [(3.95 ± 0.44) nmol/mg and (3.35 ± 0.54) nmol/mg respectively].

The CP group showed significantly reduced CAT levels compared to the control group [(12.94 ± 0.43) *versus* (16.64 ± 0.64) nmol/min/mL; $P<0.05$] which indicated the decreased levels of antioxidant enzymes by CP injection. The PmL and PmM significantly increased the CAT levels [(16.70 ± 1.45) and (14.58 ± 0.52) nmol/min/mL respectively; $P<0.05$] compared to the CP group.

4. Discussion

CP-induced neurotoxicity is one of the adverse effects following CP administration. There are less studies on CP-induced central nervous system toxicity compared to CP-induced peripheral neurotoxicity.

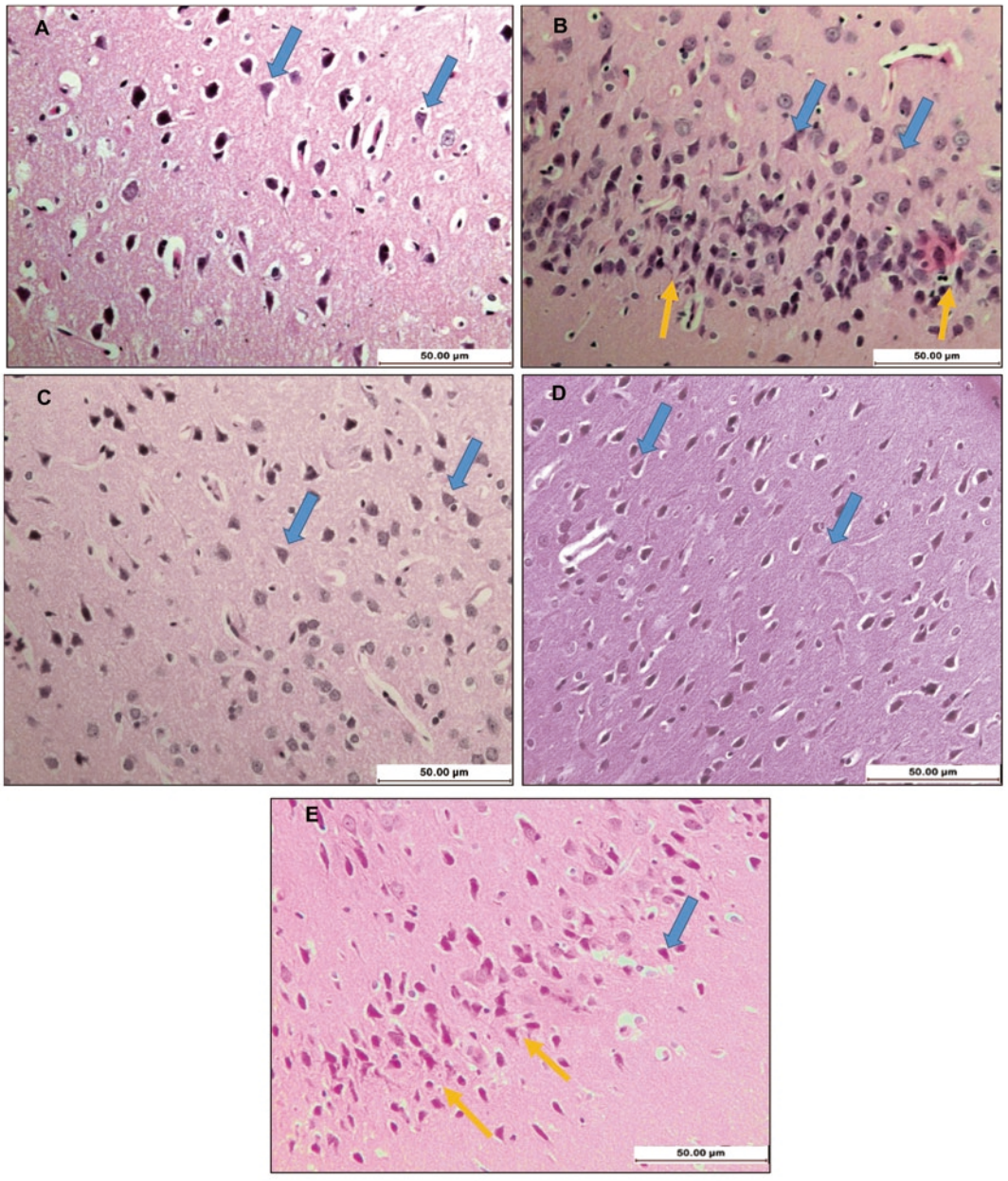


Figure 2. Photomicrograph of the cerebral cortex of rats (A) the control group; (B) the cisplatin group; the low (C), mid (D) and high (E) dose group with *Polygonum minus* extract. Pyramidal cells (blue arrows); increased cell population (yellow arrows) (H&E staining, 200×). The control group shows features of normal cellular structures of the cerebral cortex; Moreover, low and mid dose groups with *Polygonum minus* extract show similar features as in the control group. Disorganized layers of cerebral cortex and increased cell population are observed in the high dose group with *Polygonum minus* extract.

Till date, to the best of our knowledge, no specific treatment has been developed and limited study has been conducted on Pm in order to prevent CP-induced neurotoxicity. Thus, the present study aimed to investigate the protective effects of Pm ethanolic extract against CP-induced OS in the brain. One possible way to reduce OS is by supplementing the exogenous antioxidant. The plants are natural sources of antioxidants. The plant with higher levels of phenolic compounds has higher antioxidant activity which becomes a good candidate in prevention of OS-induced cell injury[17].

CP elicits neurotoxic effect in treating cancer patients. Its therapeutic dose needs to be adjusted or the treatment cycle should be stopped when the toxicity occurs. Peripheral neuropathy is more common than that of the central nervous system (CNS). It could be due to the function of blood-brain-barrier in CNS which prevents the entry of CP to reduce the exposure to nervous tissue[18]. However, the CNS toxicity does occur even within the recommended dose[19]. This might be owing to the direct toxic effect of CP or the consequences of electrolytes imbalance[20]. The signs of CNS toxicity are ranging from cognitive impairment to unconsciousness[21].

The postulated mechanism of CNS toxicity is OS[14,15]. The mitochondria of the CP-exposed cells either normal or cancerous cells are the source of production of ROS[20]. The ROS has a high affinity to bind the cell membrane lipid and subsequently cause lipid peroxidation. The MDA is one of the end products of lipid peroxidation. Excessive formation of ROS causes depletion of innate antioxidants, leading to OS[22]. Our findings on MDA and

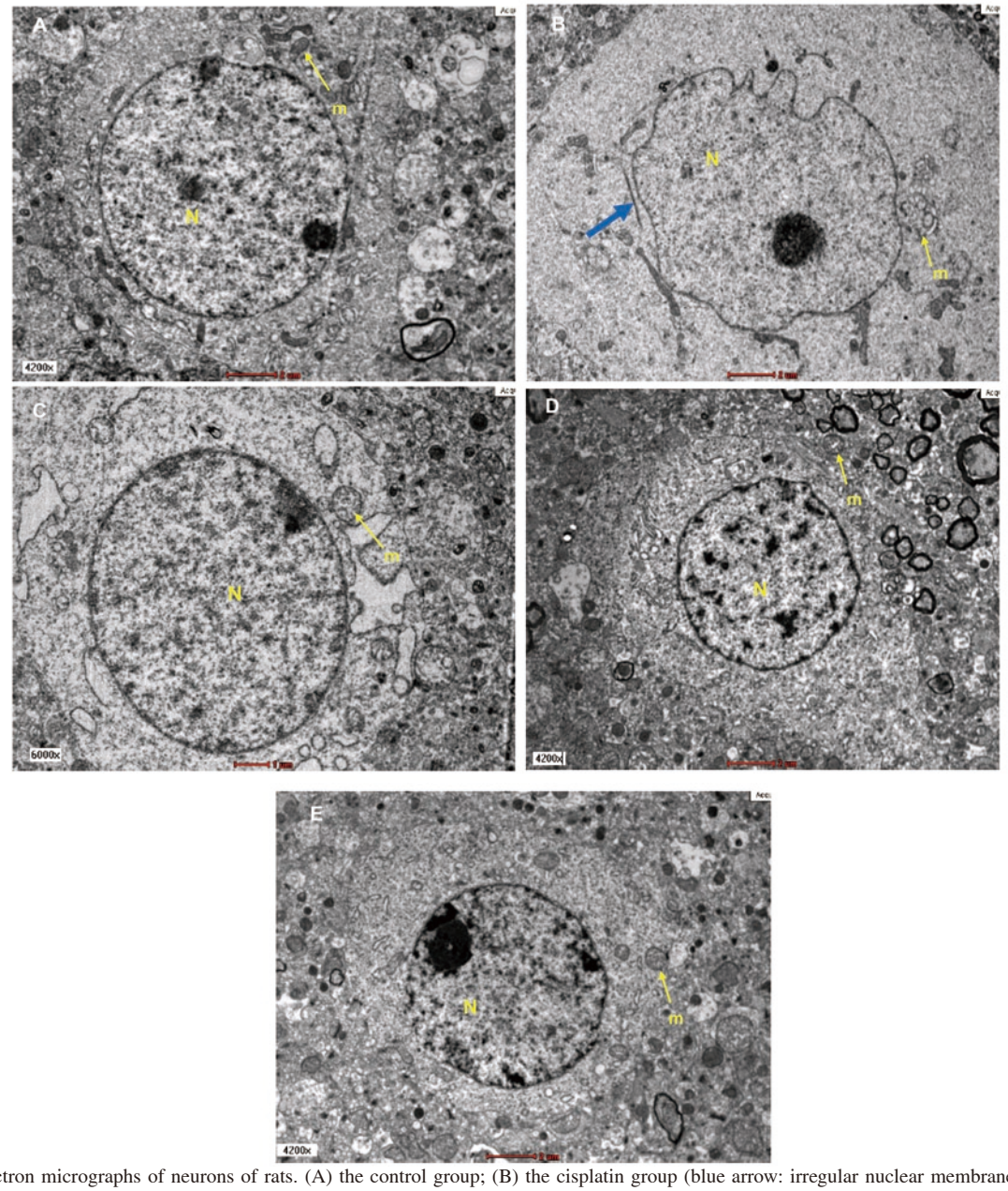


Figure 3. Electron micrographs of neurons of rats. (A) the control group; (B) the cisplatin group (blue arrow: irregular nuclear membrane); (C) the low dose group, (D) the mid dose group and (E) the high dose group of *Polygonum minus*. Nucleus: N, mitochondria: m (yellow arrow) (4 200×). Neurons in the control group exhibit large, round nucleus with uniformly distributed chromatins. The cisplatin group exhibits poorly defined irregular nuclear membranes and mitochondrial swelling with ruptured cristae, broken rough endoplasmic reticulum and unidentified ribosomes. The low dose group and the mid dose group of *Polygonum minus* show a round nucleus with uniformly distributed chromatins. The nuclear membrane is smooth and clear. In the high dose group of *Polygonum minus*, the nucleus is intact with unclear appearance of surrounding organelles.

CAT suggest that the cells in the brain exposed to CP succumb to OS. CP causes OS by generating ROS which can alter normal cell physiology[23,24]. This is due to the brain consumes more than 20% of the oxygen utilized by the other organs during the mitochondrial respiration, it possesses high probability to be challenged by ROS compared to other tissues. Moreover, brain neurotransmitter also autoxidized and produced their own ROS, thus automatically increased the generation of the ROS in the brain[14]. In the present study, the highest OS level was observed in the CP group without the Pm supplementation. The nervous tissue is most sensitive against OS because it contains abundant polyunsaturated fatty acids which are highly susceptible to the lipid peroxidation[25]. The ROS such as superoxide and hydrogen peroxide are broken down by innate antioxidant enzymes, SOD and CAT, respectively. The CAT is one of the supportive enzyme systems among superoxide dismutase and glutathione peroxidase which act as the first line cellular defence against ROS by decomposing O_2 and H_2O_2 prior to the formation of more harmful hydroxyl and alkoxyl radicals. Excessive formation of ROS causes depletion of innate antioxidants, leading to OS[26]. MDA reacts with nucleic acid bases that impair the normal cell function. These findings proved that even short administration of CP formed ROS, imbalanced the ROS and antioxidant defence system which

leads to neuronal injury.

One possible way to reduce OS is the use of supplementation with exogenous antioxidants like those present in plants. The plant such as Pm with a high level of phenolic compounds has higher antioxidant activity which becomes a good candidate in prevention of OS-induced cell injury[26]. The Pm ethanolic extract used in the present study has high total phenolic contents and was able to attribute the 96% reduction of DPPH free radicals. However, further detailed analysis of compounds present in the Pm extract is recommended to determine the exact mechanism of protection. In the literature, the phenolic acids, flavonoids and non-flavonoids compounds comprise in the total phenolic contents and these compounds exhibit antioxidant activity[17]. The flavonoids that normally attribute this activity are gallic acid, rutin, quercetin and coumaric acid[27], which are soluble based on the polarity, thus the compounds are able to pass through the blood brain barrier[28].

The mechanism of reduction of OS by the phenolic compound passes *via* removal of free radicals or by reduction of hydroperoxides. However, the removal of these radicals is unlikely to happen *in vivo*, thus one of the postulated protective mechanisms is to prevent the formation of these radicals by stimulating the endogenous antioxidant system *via* nuclear factor erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap 1) signalling pathway[29]. Our finding suggested that the phenolic contents of Pm prevented the formation of free radicals as lipid peroxidation was reduced and the endogenous antioxidant enzyme activity remained the same as in the control group. This could be owing to the properties of phenolic compounds of Pm such as hydrogen donating ability, ability to interrupt lipid peroxidation and ability to act as a metal chelator[27]. Further investigation is required to understand the exact mechanism of protection by Pm.

Throughout the alteration of metabolism of the brain, CP indirectly impaired the brain morphology. In the present study, CP produced microscopic alterations to the brain cells. Dark pyknotic nuclei were also observed in the CP group which was similar to the finding by Moneim[14]. The present findings demonstrated that alteration in brain morphology might be due to the accumulation of platinum compound in the cerebral cortex layers, thus leading to high levels of platinum-DNA binding in the cerebral layers. It is postulated that CP caused damage in brain vasculature, interfered integrity of blood brain barrier, and disturbed brain homeostasis, leading to damaging cerebral neurons and surrounding organelles[25]. The histological and ultrastructural findings in our study showed that the protective effect of Pm was only observed at low (100 mg/kg) and medium dose (200 mg/kg) whereas the high dose (400 mg/kg) showed a detrimental effect. This effect could be explained by the facts that the high dose consumption of phenolic compound produced the paradoxical effect because the proxidants, such as semiquinones and quinones are formed due to interaction between the phenolic compounds and metal ions[17,29]. Therefore, the supplementation of high dose of Pm extract should be cautious as it might produce the unwanted effect.

In conclusion, the present study suggested that OS is the main concern in CP-induced neurotoxicity. However, the supplementation of Pm extract with low and medium doses prevented the CP-induced neurotoxicity in rats without interfering with antitumour efficacy of CP. The protective effect is due to antioxidant property of Pm which prevents the formation of free radicals. Further dose adjustment and calculation are needed for the human usage.

Conflict of interest statement

Authors declare that there are no competing interests.

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