Exploration of Aqueous *Phyllanthus amarus* Leaf Extract as a Protective Agent in Mercury Chloride-Exposed Wistar Rats: A Neurobehavioural Study

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**ABSTRACT:** Mercury (Hg) exposure is known to damage vital components of the central nervous system primarily through oxidative stress. Although *Phyllanthus amarus* (PA) is reported to be rich in antioxidants, there is a dearth of relevant research evidence to show its protective activity against Mercuric chloride (HgCl₂)-induced neurotoxicity. Accordingly, this study was designed to investigate the possible protective activity of aqueous *Phyllanthus amarus* leaf extract (APALE) in HgCl₂-exposed rats using standard neurobehavioural assessments. Thirty adult Wistar rats were randomized into six groups of five rats each viz: A: Control, distilled water; B: HgCl₂ (4 mg/kg body wt.); C: APALE (200 mg/kg body wt.) + HgCl₂ (4 mg/kg); D: APALE (400 mg/kg body wt.) + HgCl₂ (4 mg/kg); E: APALE (200 mg/kg body wt.); F: APALE (400 mg/kg body wt.). All administrations were through an oesogastric tube for 14 days. Thereafter, neurobehavioural tests (open field, novel object and Y-maze) were done and experimental rats were sacrificed by cervical dislocation. Body, brain and cerebellar weights were examined across all groups. HgCl₂ exposure significantly (p<0.005) decreased whole body weight across experimental groups. Similarly, a reduction in brain and cerebellar weight was observed. Significant neurobehavioural alterations to locomotion, discrimination index and cognition/memory, induced by HgCl₂, were observed across experimental groups. However, protection against the deleterious effects induced by HgCl₂ was observed following pretreatment with APALE. Conclusively, these findings show that APALE demonstrated potent protective activity against HgCl₂-induced neurotoxicity and may be useful in the management of Hg toxicity and other related disorders.

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Mercury (Hg), present naturally in the environment, is often considered to be a highly toxic environmental hazard. Human activities such as agriculture, mining and industrial wastewater discharge are reported to aid its dispersal throughout the environment (Clarkson and Magos, 2006). Although Hg is used in the production of dental amalgams, batteries, thermometers and cosmetics, human exposure is primarily through water, air and contaminated food (Jahan et al., 2019). Reports indicate that low or high exposure to Hg causes harmful effects such as pulmonary impairment, reproductive dysfunctions, hypertension, nephrotoxicity and neurotoxicity (Jaishankar et al., 2014). Following ingestion of Hg, mercuric ions are reported to bind to sulfhydryl groups which cause a decrease in glutathione levels and thiols as well as lipid peroxidation stimulation, thus leading to increased production of reactive oxygen species (ROS), oxidative stress and tissue damage/injury (Owoeye and Farombi, 2015). This activates an alteration in the functionality of biological membranes, eventually resulting in the development of diverse pathological disorders such as neurodegenerative diseases (Uttara et al., 2009). In Nigeria, there is an upsurge in Hg exposure and neurotoxicity due to a significant increase in gold mining activities across the country (Owoeye et al., 2019). Endogenous and exogenous antioxidants have been reported to attenuate metal and Hg-induced oxidative stress through the inhibition and scavenging of free radicals in different experimental models; some exogenous antioxidants include vitamin E, carotenoids, ascorbic acid, polyphenols (Animoku et al., 2016; Enogieru and Momodu, 2021b; Fadda et al., 2019).
2020). While the above-mentioned protective activities have been demonstrated in experimental studies, there are no effective treatments that entirely eradicate the toxic effects of Hg in clinical trials with humans (Hallal et al., 2016). To this end, necessary supportive care is done to preserve vital functions in addition to the administration of chelator agents in a bid to aid the body’s natural machinery to eliminate Hg from tissues. Unfortunately, these drugs are known to have harmful side effects and are also ineffective in the complete removal of Hg from tissues (Tchounwou et al., 2003). In recent times, the clinical importance of plant-based naturally occurring antioxidants has received substantial attention due to the adverse effects of synthetic antioxidants (Engelhart et al., 2002; Enogieru and Momodu, 2021a). Several plant products and extracts have been demonstrated to have protective activity against Hg-induced neurotoxicity in experimental studies (Anyanwu et al., 2020; Hallal et al., 2016). Phyllanthus amarus, a member of the Euphorbiaceae family, is conventionally utilized for gonorrhoea, dysentery, kidney ailments, pain and diabetes. Over the years, increased focus has been directed towards this plant due to its numerous pharmacological properties; these include anti-inflammatory, antibacterial, antioxidant, immunomodulatory, anticancer, antinoceptive, antiulcer, antifungal, antiplasmodic, antilipidemic, nephroprotective, antiviral and hepatoxprotective effects (Alagan et al., 2019; Eweka and Enogieru, 2011). This wide range of pharmacological activities is unified primarily by the antioxidant properties of the plant. For instance, Phyllanthus amarus contains such principal constituents as phyllanthin, hypophyllanthin, corilagin, geranin, amarin, repandusinic acid, phyllanthusin D, rutin and quercetin 3-O-glucoside; all of which are reported to potently scavenge free radicals in a range of systems (Londhe et al., 2008).

Although the protective activity of Phyllanthus amarus against Lipopolysaccharide-induced neurotoxicity has been reported (Alagan et al., 2019), there is a dearth of relevant research evidence to demonstrate a similar protective activity against Hg-induced neurotoxicity. Therefore, the present study sought to examine the protective activity of aqueous Phyllanthus amarus leaf extract against Hg-induced neurotoxicity using neurobehavioural assessments and changes to the body, brain and cerebellar weights in adult Wistar rats.

MATERIALS AND METHODS

**Extraction of the Aqueous Extract of Phyllanthus amarus:** Fresh leaves of Phyllanthus amarus were collected from the University of Benin environs and identified at the Department of Plant Biology and Biotechnology, University of Benin, Edo state with herbarium number UBH-P347. The leaves were air-dried, pulverized and weighed. Thereafter, 1kg of the powdered leaves was extracted with 1.2 litres of distilled water for 24 hours. The mixture was filtered on Whatman filtered paper and the filtrate evaporated at 60°C using a vacuum rotary evaporator (Buchi, Switzerland). The crude extract, gotten from the filtrate through freeze-drying, was weighed and kept in a refrigerator at 4°C. Afterwards, the crude extract was dissolved in an appropriate volume of distilled water to make a concentration of 100 mg/ml from which different doses of 200 and 400 mg/kg body weight by oral route were reconstituted and administered to experimental animals.

**Preliminary phytochemical screening:** Phytochemical screenings were performed using standard procedures (Sofoiwora, 1993; Trease and Evans, 1983). Glycosides, flavonoids, tannins, phenols, saponins, terpenoids, steroids, alkaloids and phyllobotanin were screened for their presence in the plant material.

**Chemicals and reagents:** Normal saline was manufactured by Unique Pharmaceuticals, Sango-Otta, Nigeria and Mercury Chloride (HgCl₂, 99% purity) by Loba Chemie Pvt. Ltd, Mumbai, India. Other reagents were all of the analytical grades.

**Ethical approval and care of Animals:** This study was submitted for review and approval was granted by the Research Ethics Committee of the College of Medical Sciences, University of Benin, with the number CMS[REC]2021165. The experiments were carried out at the Anatomy Department, School of Basic Medical Sciences, University of Benin. Animals were obtained from the breeding colony of the Department of Anatomy and were fed with standard rat chow (Bendel livestock feed, Edo state, Nigeria) and water throughout the entire study period. They were weighed weekly before commencement and throughout the experiment using a digital weighing scale calibrated in grams and recorded to the nearest whole number. The animals received humane care following the principle of humane care and the use of laboratory animals.

**Research design:** Following acclimatization, thirty (30) adult Wistar rats, weighing between 120g and 180g, were divided into six groups of five animals each as shown in Table 1.

**Neurobehavioural Tests:** Neurobehavioural tests were utilized to assess how HgCl₂ administration affects locomotor, learning and memory across experimental groups. Also, these tests were done to evaluate the protective ability of Phyllanthus amarus against the
possible neurobehavioural alterations induced by HgCl₂. On experimental day 15, rats in the experimental groups were weighed and assessed for neurobehavioural alterations. The following behavioural tests were used: open field test (OFT), Novel object recognition test (NORT) and Y-maze test.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Treatment details</th>
</tr>
</thead>
<tbody>
<tr>
<td>A - Control</td>
<td>served as control, received distilled water only</td>
</tr>
<tr>
<td>B - Hg</td>
<td>Received HgCl₂ (4 mg/kg body wt.)</td>
</tr>
<tr>
<td>C - Hg + PA1</td>
<td>Received 200 mg/kg body wt. of aqueous Phyllanthus amarus leaf extract + HgCl₂ (4 mg/kg body wt.)</td>
</tr>
<tr>
<td>D - Hg + PA2</td>
<td>Received 400 mg/kg body wt. of aqueous Phyllanthus amarus leaf extract + HgCl₂ (4 mg/kg body wt.)</td>
</tr>
<tr>
<td>E - PA1</td>
<td>Received 200 mg/kg body wt. of aqueous Phyllanthus amarus leaf extract</td>
</tr>
<tr>
<td>F - PA2</td>
<td>received 400 mg/kg body wt. of aqueous Phyllanthus amarus leaf extract</td>
</tr>
</tbody>
</table>

Rats received the plant extract one hour before the administration of HgCl₂. Treatments were administered to the rats for 14 days through an orogastric tube and were sacrificed on day 15 by cervical dislocation after anaesthesia.

**Open Field Test (OFT):** The OFT is considered a suitable measure of locomotion and anxiety in experimental animals (Millan, 2003). The open field utilized in this study was a square wooden arena (72 cm × 72 cm × 20 cm) with lines on its floor dividing it into 18 cm by 18 cm square (Olopade et al., 2012). The open field apparatus was cleaned with alcohol between experimental animals in neuroscience (Ennaceur et al., 2009). The NOR was performed in a wooden open box apparatus measuring 80x60x40cm as previously described (Malik et al., 2013). The objects to be differentiated were of two different shapes and colours and were heavy enough to prevent displacement by the animals during the test.

A day before the test, rats were given free access to explore the box (devoid of any object) for 3 minutes. Exploration was measured as a direction of the nose of each rat at a distance less than 2 cm to the object and/or touching with the nose. On the day of the test, two similar objects (FO1 and FO2) were placed in two opposite corners of the box, and the time spent by each rat exploring the objects was recorded and classified as the first trial (T1). For the real test (T2), a novel object (NO) was switched with one of the objects (FO2) used in T1, and each rat was left in the box to explore for 5 minutes.

The time spent exploring the familiar (FO1) and the new (NO) object was recorded separately, and the discrimination index (D) was calculated as D = NO−FO1/NO + FO1. The apparatus was cleaned with alcohol and the position of the two objects during T2 was changed randomly to avoid place preference and the influence of olfactory stimuli

**Y-Maze test:** The Y-maze test is often considered a reliable, non-invasive test to evaluate cognitive changes in experimental animals through the measurement of spontaneous alternation behaviour (Hritcu et al., 2012). The Y-maze utilized in this study consisted of three identical arms (33×11×12cm each) which are symmetrically separated at 120° with an equilateral triangular central area. The rats were placed at the end of one arm and allowed to move freely through the maze for 5 minutes after which every session was stopped. An arm entry was recorded when the hind paws of the rat were completely within the arm and spontaneous alternation behaviour was defined as three consecutive entries in three different arms (i.e. A, B, C or A, C, B etc.). An alternation was defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as the total of alternations / total arm entries – 2×100). After each session, the maze was cleaned with 10% ethanol to remove residual odour.

**Determination of relative brain weight:** Following the assessment of neurobehavioral activities, rats were sacrificed by cervical dislocation. Thereafter, the brains were accessed through a longitudinal cranial incision, weighed and dissected into the cerebellum, cerebrum and hippocampus. To mitigate the individual bodyweight differences, the relative brain weight (%) was expressed as a percentage of the final body weight at sacrifice (Kim et al., 2008).

**Statistical Analysis:** Statistical analysis was performed using GraphPad Prism statistical package (version 7) and data expressed was as the standard error of mean (SEM) using a one-way analysis of variance (ANOVA) followed by Turkey’s multiple comparisons post-hoc test.

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RESULTS AND DISCUSSION

Phytochemical screening: Results of the phytochemical screening are shown in Table 2. The aqueous *Phyllanthus amarus* leaf extract was found to contain glycosides, flavonoids, tannins, phenols, saponins, terpenoids, steroids and alkaloids. However, phylobotanin was observed to be absent.

![Image](https://example.com/image.png)

**Table 2**: Qualitative phytochemical analysis of *Phyllanthus amarus* leaves

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Phylobotanin</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: + = Present; - = Absent

Effect of treatment on body and brain weights: Table 3 shows the changes in body, brain and cerebellar weight across experimental groups. A significant decrease (p< 0.05) was observed in the final body weight of rats treated with HgCl\(_2\) alone when compared to control. Also, there was a significant (p< 0.05) increase in the final body weight of rats pretreated with 200mg/kg and 400mg/kg body weight of *Phyllanthus amarus* (Hg + PA1 & Hg + PA2) when compared to the HgCl\(_2\) alone group.

Effect of treatment on Neurobehavioural activity: Findings from the OFT evaluation are presented in Figure 1. A significant decrease (p< 0.05) was observed in rearing activity in rats treated with HgCl\(_2\) alone when compared to control and a significant increase (p< 0.05) in rearing activity in pretreated rats (Hg + PA2) when compared to HgCl\(_2\). For grooming, a significant increase (p< 0.05) was observed in rats treated with HgCl\(_2\) when compared to control and a significant decrease (p< 0.05) in pretreated rats (Hg + PA2) when compared to HgCl\(_2\). For ambulation, a significant decrease (p< 0.05) was observed in rats treated with HgCl\(_2\) when compared to control and a significant increase (p< 0.05) in pretreated rats (Hg + PA2) when compared to HgCl\(_2\). For immobility, a significant increase (p< 0.05) was observed in rats treated with HgCl\(_2\) when compared to control and a significant decrease (p< 0.05) in pretreated rats (Hg + PA1 & Hg + PA2) when compared to HgCl\(_2\).

The findings from the NOR evaluation are presented in Figures 2-5. For the test T2, a significant decrease (p< 0.05) in mean exploration times for the novel object (NO) was observed in rats treated with HgCl\(_2\) alone when compared to control and a significant increase (p< 0.05) was observed in pretreated rats (Hg + PA1 & Hg + PA2) when compared to HgCl\(_2\) (Figure 3). For the total exploration times (T1 and T2), a significant decrease (p< 0.05) was observed in rats treated with HgCl\(_2\) alone when compared to control, however, a significant increase (p< 0.05) was observed in pretreated rats (Hg + PA1 & Hg + PA2 - for T2) when compared to HgCl\(_2\) (Figure 4). The enhancement in discrimination capacity (by *Phyllanthus amarus* at 200 and 400 mg/kg), in contrast to HgCl\(_2\), was demonstrated by the findings shown in Figure 5. There was a significant decrease (p< 0.05) in rats treated with HgCl\(_2\) alone when compared to control and a significant increase (p< 0.05) in pretreated rats (Hg + PA1 & Hg + PA2) when compared to HgCl\(_2\) alone.

In the Y-maze task, the significant reduction (p< 0.05) in spontaneous alternation when compared to control demonstrates that HgCl\(_2\) significantly induced cognitive deficits in the rats. Conversely, pretreatment with *Phyllanthus amarus* significantly attenuated the cognitive deficits induced by HgCl\(_2\) after fourteen days’ administration (Figure 6).

![Image](https://example.com/image.png)

**Table 3**: Body weight, absolute whole brain and cerebellar weight, relative brain/cerebellar weight and cerebellum/brain weight ratio of control and treatment groups after 14 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial Body weight (g)</th>
<th>Final Body weight (g)</th>
<th>Absolute whole brain weight (g)</th>
<th>Cerebellar weight (g)</th>
<th>Relative brain weight (%)</th>
<th>Relative cerebellar weight (%)</th>
<th>Cerebellum/Brain weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>156.8 ± 7.004</td>
<td>179.7 ± 8.090</td>
<td>1.667 ± 0.033</td>
<td>0.570 ± 0.029</td>
<td>0.927 ± 0.047</td>
<td>0.333 ± 0.012</td>
<td>0.342 ± 0.012</td>
</tr>
<tr>
<td>Hg</td>
<td>151.8 ± 1.887</td>
<td>147.7 ± 7.881*</td>
<td>1.467 ± 0.088</td>
<td>0.460 ± 0.015</td>
<td>0.890 ± 0.021</td>
<td>0.313 ± 0.009</td>
<td>0.315 ± 0.012</td>
</tr>
<tr>
<td>Hg + PA1</td>
<td>166.7 ± 5.783</td>
<td>177.3 ± 3.839</td>
<td>1.633 ± 0.033</td>
<td>0.557 ± 0.020</td>
<td>0.940 ± 0.015</td>
<td>0.320 ± 0.006</td>
<td>0.341 ± 0.008</td>
</tr>
<tr>
<td>Hg + PA2</td>
<td>160.7 ± 5.207</td>
<td>177.7 ± 5.548*</td>
<td>1.700 ± 0.058</td>
<td>0.550 ± 0.023</td>
<td>0.900 ± 0.006</td>
<td>0.320 ± 0.001</td>
<td>0.324 ± 0.003</td>
</tr>
<tr>
<td>PA1</td>
<td>159.0 ± 9.644</td>
<td>177.3 ± 6.625</td>
<td>1.630 ± 0.067</td>
<td>0.577 ± 0.018</td>
<td>0.940 ± 0.017</td>
<td>0.340 ± 0.010</td>
<td>0.354 ± 0.010</td>
</tr>
<tr>
<td>PA2</td>
<td>159.0 ± 5.774</td>
<td>178.5 ± 4.330</td>
<td>1.633 ± 0.033</td>
<td>0.560 ± 0.036</td>
<td>0.947 ± 0.009</td>
<td>0.323 ± 0.015</td>
<td>0.343 ± 0.015</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM of each group. *p*<0.05 compared with the control group, †*p*<0.05 compared with the Hg-alone group.

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In this study, findings demonstrate that administration of HgCl$_2$ induced alterations in overall body and brain weights as well as neurobehavioural deficits in experimental rats. However, pre-treatment of these rats with 200mg/kg and 400mg/kg aqueous *Phyllanthus amarus* leaf extract before administration of HgCl$_2$ protected against the observed deficits. Alterations to body weight are often considered an important indication of the general health status of animals and are a useful evaluator of the adverse effect of drugs and metal toxicity (Agbon *et al.*, 2014; Salawa *et al.*, 2009). Results from this study revealed a significant reduction in body weight of rats administered with HgCl$_2$ alone together with a
decrease in the absolute whole brain and cerebellar weights. Accumulating evidence shows that metal toxicity can lead to substantial brain and body weight loss in rats if left untreated (Fiati Kenston et al., 2018; Nwokocha et al., 2012). Reports indicate that the reduction in the body and brain weight observed during metal toxicity is possibly due to the depletion of nutrients, attenuation of protein synthesis and dysregulation of metabolic activities (Oyewole and Oladele, 2017).

Nevertheless, pre-treatment of experimental rats with *Phyllanthus amarus* protected against body and brain weight loss induced by Hg. This suggests that the extract is capable of enhancing protein synthesis and improving metabolic activities in the brain. The OFT is commonly utilized to evaluate anxiety, locomotor activities and pharmacological selection of drugs that act on the central nervous system in rodents (Kuniishi et al., 2017). Ambulation and rearing behaviours are reported to be potent indicators of locomotor and exploratory activities, respectively, while grooming and immobility are positively linked to fear or emotionality (Terçariol and Godinho, 2011). In the present study, rats treated with HgCl₂ alone displayed increased immobility and grooming behaviours, suggesting that HgCl₂ increases emotionality, fear and anxiety in experimental rats. This is in agreement with other findings that demonstrate an increase in immobility following metal exposure in rats (Omeiza et al., 2021; Singh et al., 2018). Similarly, HgCl₂ decreased ambulation and rearing behaviours, thus signifying an impairment of locomotor and exploratory activities in the experimental rats. This is in agreement with the findings of other authors that reported a positive correlation between ambulation and rearing (Masood et al., 2003; Vidal, 2016). Pre-treatment of experimental rats with *Phyllanthus amarus* mitigated the alterations induced by HgCl₂ across experimental groups, thus demonstrating a protective effect. This is also in agreement with previous studies that showed the protective ability of plant extracts against metal toxicity in experimental rats (Owoeye and Gabriel, 2016; Seddik et al., 2010). The NOR test measures the natural tendency of a rat to explore a novel object against a familiar object. Although there was no significant difference in time spent exploring FO1 and FO2 in the trial phase, significant differences were observed in the test phase. Hg-treated rats exhibited less total exploration time during the trial test than control and pre-treated rats. Also, findings showed a significant reduction in the total exploration time of the novel object in rats exposed to HgCl₂. This is in agreement with earlier findings showing that Hg-treated rats exhibit a
significant reduction in discrimination index in the NOR task (Kim et al., 2019). Phyllanthus amarus pretreated rats were observed to have spent significantly more time exploring the novel object than the familiar object during T2 and had a significantly higher discrimination index in the NOR task. This indicates that the extract protects memory formation in rats exposed to HgCl₂, thus agreeing with previous studies demonstrating the potent memory-enhancing activity of Phyllanthus amarus (Alagan et al., 2019) and other plant extracts in models of cognitive impairment (Balmus and Ciobica, 2017; Enogieru and Momodu, 2021a). Spontaneous alternation behaviour using the Y-maze test has been considered an indicator of memory function in rodents (Hritcu et al., 2011). Here, rats are expected to recall the maze arm that was last visited and try to enter as many different arms as possible. The order of arm entries and the total amount of arm entries are recorded and a percentage is calculated. Animal cognition is then assessed based on the score and low scores are considered as cognitively impaired. Furthermore, exposure to Hg has been reported to alter spontaneous alternation behaviour in mice (Bourdineaud et al., 2012). In the present study, rats exposed to HgCl₂ exhibited a significantly lower sequence of spontaneous alternations in the maze when compared to control and pretreated rats, thus suggesting that these rats were unable to fully remember which arm they entered last. In contrast, pretreatment with Phyllanthus amarus protected against the cognitive deficit induced by HgCl₂ in the Y-maze task. Together, these results suggest that Phyllanthus amarus possess memory-enhancing properties. The present study showed the presence of several essential medicinal phytochemical constituents in the aqueous Phyllanthus amarus leaf extract. The phytochemicals identified in this study especially, flavonoids, phenols and alkaloids are commonly known for their potent pharmacological activities. These phytochemical constituents may be responsible for scavenging ROS and protecting against the DNA damage associated with Hg-induced toxicity. Previous reports have shown that phyllanthin, a major lignan present in Phyllanthus amarus leaves, is a potent neuroprotective agent (Tao et al., 2020; Yuan et al., 2021). In agreement with these reports, the observed neuroprotective activity of Phyllanthus amarus leaf extract in this study may be attributable to phyllanthin.

Conclusion: In conclusion, brain and body weight, as well as neurobehavioural indicators for locomotion, learning, memory and cognition, were significantly distorted following exposure to HgCl₂. However, pretreatment with aqueous Phyllanthus amarus leaf extract protected against the distortions induced by HgCl₂. Consequently, Phyllanthus amarus can be considered as a promising neuroprotective agent relevant in the management of Hg neurotoxicity and other related disorders.

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