Attenuation of Oxidative Stress and Cognitive Impairment in Cadmium Chloride-Exposed Wistar Rats Pre-treated with Ethanolic Turmeric Root Extract

Adaze Bijou Enogieru, Gabriel Osemudiamen Inegbedion

ABSTRACT

Background: Turmeric (Curcuma longa), belonging to the Zingiberaceae family, is a widely used spice in cuisines of African, Asian and other countries globally. Despite the enormous pharmacological benefits of turmeric, there is very little experimental evidence to demonstrate its protective activity against cadmium-induced neurotoxicity. Accordingly, this study is aimed at investigating such activity in Wistar rats and its possible mechanisms of action. Methods: Y-maze and Novel object recognition tests were utilized to evaluate memory impairments while antioxidants activity and lipid peroxidation were evaluated to outline the antioxidant mechanism of action following pre-treatment of rats with Ethanolic turmeric root extract (200 mg/kg body weight) 1 hour before cadmium administration for 21 consecutive days. In addition, the histology of the cerebrum and hippocampus was investigated to determine possible anatomical alterations across experimental groups. Results: The ethanolic extract of turmeric root at the dose of 200 mg/kg significantly improved the memory of rats and protected against the impairments induced by cadmium. In addition, the extract significantly increased cerebral and hippocampal antioxidant enzyme activities (SOD, GPx and CAT), decreased lipid peroxidation (MDA) and protected against the degenerative changes observed in the cerebrum and hippocampus of rats treated with cadmium alone. Conclusion: Taken together, these findings suggest that the ethanolic extract of turmeric root protected against the cognitive impairments induced by cadmium possibly through the attenuation of the oxidative damaging activity of cadmium.

Keywords: Turmeric, Cadmium, Antioxidant, Memory, Oxidative Damage.

INTRODUCTION

Cadmium is often considered an extremely lethal element that affects human health following long term exposure even at low concentrations [1]. The toxicity of cadmium is validated by its inclusion in the top ten chemicals of primary public health concern by the World Health Organization [2]. This has led to public anxiety and increased research activities targeted at finding therapeutic strategies capable of mitigating its adverse effects. When cadmium is absorbed into the body, it is transported through the blood and binds to red blood cells and plasma proteins and is thereafter dispersed all through the organs and tissues with a half-life of about 17-30 years [3]. Extensive cadmium exposure may lead to chronic damage to the brain, kidney, testes, lung and bone [3]. In the cells, cadmium affects apoptosis, cell proliferation and differentiation as well as other cellular activities. Following cadmium exposure, its metabolism and elimination from the body are subject to the presence of free radical scavenging antioxidants and the activity of antioxidant enzymes. [4]. The pathophysiology of cadmium is mainly dependent on the induction of oxidative stress, which is typified by (i) excessive generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS); (ii) significant reduction of endogenous antioxidants and free-radical scavengers; and (iii) inactivation of enzymes that aid in the detoxification of ROS [4].

In the body, there are a moderate number of antioxidant protection machinery against free radicals and ROS. Chelation techniques have also been utilized in the mitigation of cadmium-induced toxicity [5]. Numerous thiol-containing compounds have been exploited as treatments for heavy metal intoxications due to their ability to scavenge free radicals, reinitiate cellular thiol pools, and form steady complexes with heavy metals [6]. However, due to the possible side effects and adverse health risks linked to the chelation therapy and synthetic thiol-containing compounds in the treatment of cadmium toxicity, natural exogenous antioxidants from dietary sources in form of medicinal plants have been encouraged. Reports indicate that some of these medicinal plants possess more beneficial pharmacological activities than their synthetic equivalents in addition to being harmless, adequate, cheaper, culturally acceptable and appropriate for treatment of heavy metal disorders [7]. Also, several medicinal plants such as turmeric, Sutherlandia frutescens, Carpobrotus edulis, Crossyne guttata and their isolated bioactive compounds/molecules are well known internationally for their potency [8-11].
Curcuma longa (Zingiberaceae) is a widely studied functional food belonging to the Zingiberaceae family. Since ancient times, turmeric has been used as a spice in cuisines of Africa, Asia and in other countries globally. Numerous communities across the world utilize turmeric to manufacture traditional medications in the treatment of human diseases and disorders. For instance, it is useful in the treatment of stomach ailments, hepatic disorders, dyslipidemia and arthritis [12]. Some of the pharmacological activities of turmeric include antioxidant, anti-inflammatory, hypolipidemic and antimicrobial effects [12, 13]. Studies show that turmeric scavenges free radicals, boost the activities of antioxidant enzymes, and attenuates lipid peroxidation [14]. The pharmacological benefits of turmeric have been attributed to its bioactive constituents; two of the most significant constituents of turmeric is its volatile oil and curcumin. Evidence shows that curcumin plays a key role against oxidative stress in dopaminergic neuronal cells and ultimately enhances neuroprotection possibly through mediation of the BDNF/TrkB signaling pathway [13]. Other neuropharmacological benefits of turmeric include its protective activity against traumatic brain injury, depression, anxiety, Alzheimer’s disease and Parkinson’s disease [16-18].

Despite the enormous beneficial properties of turmeric, there is very little experimental evidence to demonstrate its protective activity against cadmium-induced neurotoxicity. Accordingly, this study is aimed at investigating such activity in Wistar rats, hence findings from this study will provide the first research evidence of the neuroprotective activity of turmeric in cadmium-exposed Wistar rats.

**MATERIALS AND METHODS**

**Plant material**

The roots of turmeric (Curcuma longa) were bought from a nearby market in Benin City, specifically in the market of Uselu. It was identified and authenticated at the Department of Plant Biology and Biotechnology, University of Benin, Edo State, and a voucher specimen was deposited with the number UBH-C397.

**Animals**

Adult Wistar rats, weighing between 130g - 180g, obtained from the breeding colony of the Department of Anatomy were used for the experiments. Animals were freely allowed to water and top feeds growers mash (manufactured by Premier feed mills Co. Ltd, 1 Eagle Flour Road, Lagos/Ibadan expressway Toll point, Ibadan, Oyo State, Nigeria). Acclimatization lasted for 14-days and animals received humane care following the principle of humane care and the use of laboratory animals. This study was reviewed and approved by the Research Ethics Committee of the College of Medical Sciences, University of Benin, with the number CMS[REC]2021[171].

**Preparation of the Ethanolic Extract**

The roots of turmeric (Curcuma longa) were washed, chopped into bits, air-dried under ambient temperature without exposure to sunlight and pulverized. 1kg of the plant material was soaked in 3litres of absolute ethanol and thoroughly extracted for 72 hours by maceration and recurrent stirring before filtering using hydrophilic cotton and Whatman filtered paper. The obtained filtrate was subsequently evaporated in a vacuum at 40°C under a pressure of 175 mbar with a rotary evaporator (SM-52 CS-1, Surgifield Medical, England) and freeze-dried to dryness with a freeze dryer (LGI-10, Searchtech, United Kingdom). The ethanolic extract was stored at 4°C until needed for further experiments.

**Phytochemical Screening**

The qualitative assessment of the chemical composition of the roots of turmeric (Curcuma longa) was done using standard methods [19, 20]. Compounds such as alkaloids, saponins, steroids, tannins, anthocyanin, phenols, flavonoids, carbohydrates, phylobotanins, terpenes and cardiac glycosides were tested.

**Acute Toxicity Study**

This study was carried out according to a previously described method with little modification [21]. Briefly, three groups, A1, B1 and C1, containing three rats each were administered with single doses of 10, 100 and 1000mg/kg bodyweight of ethanolic turmeric root extract respectively. The rats were then observed for 72 hours to monitor behavioural changes and possible mortality. Following the expiration of 72 hours, three new groups, A2, B2, and C2, containing two rats each were administered with single doses of 1600, 2900, and 5000mg/kg bodyweight of ethanolic turmeric root extract respectively. The rats were also observed for 72 hours for possible behavioural changes and mortality.

**Chemicals and Reagents**

Normal saline was manufactured by Unique Pharmaceuticals, Sango-Ota, Nigeria and Cadmium chloride (CdCl\textsubscript{2} 98% purity) by Loba Chemie Pvt. Ltd, Mumbai, India. Other reagents were all of the analytical grades.

**Grouping and Treatment Schedule**

Rats were randomly assigned into four different groups of six rats each. The experimental design was as follows:

- **Group 1 (Control):** received distilled water only for 3 weeks
- **Group 2 (Cd):** received Cadmium (5 mg/kg body wt.) for 3 weeks
- **Group 3 (Cd + Tu):** received 200 mg/kg body wt. of ethanolic turmeric root extract + Cadmium (5 mg/kg body wt.) for 3 weeks
- **Group 4 (Tu):** received 200 mg/kg body wt. of ethanolic turmeric root extract for 3 weeks

Rats were pretreated with turmeric one hour before administration of Cadmium

After 3 weeks, the rats were subjected to the novel object recognition and Y-maze tests. Following the neurobehavioral tests, biochemical evaluation of antioxidants activity and histopathological assessment of the cerebrum and hippocampus were carried out.

**Neurobehavioral tests**

**Novel object recognition (NOR) test**

This test, commonly utilized to evaluate short-term and long-term memory in rodents, was carried out as previously described [22]. Briefly, on the 21\textsuperscript{st} day of the experiment, each rat explored the apparatus for 2 minutes, while on day 22 (test day), two sessions (T1 and T2) of 3 minutes each was allowed. In T1 (trial), two similar objects (FO1 and FO2) were placed at opposite corners of the apparatus. Thereafter, rats were left to individually explore both identical objects. At the end of T1, rats were returned to their cages and a 1-hour interval was given before T2. In T2 (real test), a new object (NO) was used to replace FO2, and each rat was left to explore FO1 and NO. Thereafter, the total time spent in exploring FO1 and FO2 (in T1), and that spent in exploring FO1 and NO (in T2) was recorded.

The discrimination index (DI) was calculated as follows:

- **Time with Novel object (NO) – time with the familiar object (FO1)**
- **Time with Novel object (NO) + time with the familiar object (FO1)**
**Y-Maze test**

Studies show that the Y-maze is a consistent, non-invasive test that assesses cognitive changes in rodents via an analysis of spontaneous alternation behaviour in the Y-maze task [23]. The Y-maze utilized in this study consisted of three identical arms (33x11x12cm each) which are symmetrically separated at 120° with an equilateral triangular central area. Experimental rats, placed at the end of one arm, were allowed to move freely through the maze for 5 minutes following which each session was stopped. An arm entry (a measure of general activity) was recorded as positive when a rat’s hind paw was completely within the arm while spontaneous alternation behaviour was recorded as three successive entries in three different arms (i.e. A, B, C or A, C, B, etc.). The percentage alternation was calculated as Total alternation number/ (Total number of entries minus 2) x 100. After each session, the maze was cleaned with 10% ethanol to remove the residual odour [24].

**Biochemical Evaluation**

The cerebrum and hippocampus were homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.4), and the homogenates were thereafter centrifuged at 10,000 g for 10 min at 4°C [25]. The supernatants were collected and evaluated for Superoxide Dismutase (SOD) [26], Catalase (CAT) [27], Malondialdehyde (MDA) [28] and Glutathione Peroxidase (GPx) [29].

**Histological Examination**

After 72 hours of storage in Bouin's fluid, the cerebrum and hippocampus were processed through the paraffin wax embedding method as previously reported [30]. The Haematoxylin and Eosin staining method described by Drury and Wallington was also carried out [30]. Thereafter, sections were observed under a LABO® trinocular microscope (Labo Microsystems GmbH, Germany) with an Omax 9.0MP USB Digital Microscope Camera (Korea).

**Statistical Analysis**

Analysis of data was performed using GraphPad Prism Software V7 (www.graphpad.com/scientific-software/prism/). Values were presented as mean ± standard error of mean. Statistical significance (p<0.05) was determined by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons.

**RESULTS**

**Phytochemical screening**

The qualitative phytochemical analysis revealed that the root of turmeric contains alkaloids, saponins, steroids, tannins, anthocyanin, phenols, flavonoids, carbohydrates, phylobotanins, terpenes and cardiac glycosides. This indicates that the ethanolic extract of turmeric root is rich in phytoconstituents (Table 1).

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Results</th>
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<td>Alkaloids</td>
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<td>Saponins</td>
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<td>Steroids</td>
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<td>Phylobotanins</td>
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<td>Terpenes</td>
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**Table 1: Phytochemical analysis of the root of turmeric**

Turmeric root extract; (+): Present; (-): Absent

**Acute Toxicity**

No behavioural changes, abnormality or mortality was observed across experimental groups following administration of ethanolic turmeric root extract at doses from 10 to 5000 mg/kg bodyweight. There were no noticeable behavioural differences across experimental groups.

**Effect of Treatment in the Novel Object Recognition Test**

Findings show that control rats and turmeric alone treated rats took a longer time to explore the familiar and novel object (Figure 2). However, rats treated with cadmium alone had significantly lesser (p<0.05) time of exploration than that of control and turmeric pre-treated rats, signifying an impairment of the memory process (Figure 3). Pre-treatment of rats by turmeric (200 mg/kg) protected against the impairments induced by cadmium; this is demonstrated by a significant increase (p<0.05) in the total exploratory time of the rats in this task as compared to the cadmium alone group (Figure 3). For the discrimination index, results show a significant reduction (p<0.05) in the ability to discriminate between the novel and familiar objects following treatment with cadmium alone as compared to control. Post hoc comparisons indicated that rats pre-treated with 200 mg/kg of ethanolic turmeric root extract discriminated significantly better and higher than rats treated with cadmium alone (Figure 4).
Effect of Treatment on Cerebral Antioxidant Activity and Lipid Peroxidation

Following treatment with cadmium alone, cerebral SOD, CAT and GPx activities decreased significantly ($p<0.05$) when compared with that of control (Figure 6). Also, cerebral MDA activity was significantly increased ($p<0.05$) in cadmium alone treated rats when compared with control. Conversely, pre-treatment of rats with ethanolic turmeric root extract significantly increased ($p<0.05$) cerebral SOD, CAT and GPx activities in rats when compared to rats treated with cadmium alone. There was no significant difference ($p>0.05$) between control and rats treated with ethanolic turmeric root extract alone.

Effect of Treatment on Hippocampal Antioxidant Activity and Lipid Peroxidation

Following treatment with cadmium alone, hippocampal SOD, CAT and GPx activities decreased significantly ($p<0.05$) when compared with control (Figure 7). Also, hippocampal MDA activity was significantly increased ($p<0.05$) in cadmium alone treated rats when compared with control. Conversely, pre-treatment of rats with ethanolic turmeric root extract significantly increased ($p<0.05$) hippocampal SOD, CAT and GPx activities in rats when compared to rats treated with cadmium alone. There was no significant difference ($p>0.05$) between control and rats treated with ethanolic turmeric root extract alone.

Effect of Treatment on The Histology of the Cerebrum and Hippocampus

Figures 8 and 9 show the histology of the cerebrum and hippocampus of rats in the experimental groups following appropriate treatments. Figure 8A shows the normal histology of the cerebral cortex revealing six layers from external to internal with pyramidal and granular neuronal cells. Figure 9A shows the normal histology of the hippocampus showing the cortex narrowed into a single layer of densely packed pyramidal neurons the cornu ammonis (CA) region CA1. The histology of the cerebrum of the cadmium alone group (Figure 8B) revealed severe histological alterations in the layers of the cortex as compared to the control group. Some areas appear more cellular particularly in the outer pyramidal layer while others were less crowded with cells, particularly in the inner granular layer. Also degenerating pyramidal cells and vacuolation was observed...
particularly in the outer pyramidal and inner granular layer. The hippocampus showed the CA1 region with histological alterations to its structure (Figure 9B). This is demonstrated by severe vacuolation, pyknotic nuclei and degenerating pyramidal cells and neurons. In the pretreated group of rats, the cerebral cortex and hippocampus displayed improved cortical architecture with pyramidal and granule cells similar to that of control and no vacuolation in the cerebral and hippocampal tissue (Figure 8C & 9C). There were no observed histological differences between the cerebrum and hippocampus of rats treated with ethanolic turmeric root extract alone and control (Figure 8D & 9D).

**DISCUSSION**

In this study, we report that pretreatment with ethanolic turmeric root extract protected against impaired learning and memory behaviours induced by cadmium in adult Wistar rats. Specifically, the phytochemical screening showed that the ethanolic extract of turmeric root contains alkaloids, saponins, steroids, tannins, anthocyanin, phenols, flavonoids, carbohydrates, phylobotanins, terpenes and cardiac glycosides. This finding is in agreement with previous reports on the phytochemical composition of the plant [31, 32]. Accumulating evidence shows that the pharmacological action of plants is based on its phytochemical constituent and the presence of important phytochemicals in this extract (particularly alkaloids, flavonoids, phenols and saponins) demonstrates its pharmacological benefit and relevance for medicine and therapy. Phenols, flavonoids and saponins have been previously reported to have antioxidant and neuroprotective properties [33, 34]. The toxicological study showed that no mortality was recorded at the 5000 mg/kg limit dose; thus signifying that the LD$_{50}$ is greater than 5000 mg/kg and that the extract is not toxic. The non-toxic nature of the plant is further vindicated by reports of the use of turmeric as a food plant with a high safety margin. This is in agreement with previous findings demonstrating that the LD$_{50}$ of turmeric is greater than 5000 mg/kg [35, 36].

In this study, two standard memory tests, Y-maze and novel object recognition, were utilized to assess the protective effect of the ethanolic extract of turmeric root against cadmium-induced neurotoxicity in rats. The primary output from the Y-maze analysis is the percentage of spontaneous alternation behaviour. This is often considered as a measure of short-term spatial memory in rodents which requires them to remember the arm most recently entered in a bid to alternate the choice of next arm entry [24], thus, spontaneous alternation behaviour was established from consecutive entries into three different arms. Furthermore, cadmium administration has been reported to impair spontaneous alternation behaviour in mice [37, 38]. Our results show that spontaneous alternation behaviour in cadmium alone treated rats was significantly lower than in control rats. Conversely, rats pre-treated with ethanolic turmeric root extract had significantly higher percentages of spontaneous alternation behaviour when compared to rats treated with cadmium alone, thus protecting against the cognitive deficit induced by cadmium in the Y-maze task. The novel object recognition test has been widely explored in...
neuroscience studies of neurobehaviour, cognition, memory and brain function in rodents [24, 39]. Our findings show that exploration of the novel object was significantly reduced in rats treated with cadmium alone when compared to control. In this test, an exploratory and memory retention ability is required of the experimental rats such that each rat must sufficiently explore the familiar object during the trial phase to differentiate between it and a novel object during the real test phase [30]. Findings from this study show that rats treated with cadmium alone displayed lower total exploration times during the real test than control rats. The discrimination index was observed to be significantly reduced in the group of rats treated with cadmium alone when compared to control, suggesting an impairment of the learning and recognition process. This is in agreement with previous neurobehavioral reports demonstrating that cadmium treated rats display significantly reduced exploratory behaviour and discrimination index in the novel object recognition test [40, 41]. Following pre-treatment of rats with ethanolic turmeric root extract, a significant increase was observed in the exploration and discrimination index of the rats when compared to those treated with cadmium alone, thus indicating that the extract protects against memory impairments and cognitive dysfunction in rats treated with cadmium.

Memory disorders induced by cadmium are linked to elevated oxidative stress in the brain [42, 43]. Reports indicate that cadmium induces its neurotoxicity via the reduction of enzymatic antioxidants and an ensuing elevation in lipid peroxidation. Also, thiol status modulation, ion transport changes and DNA damage are other reported mechanisms [44]. During the induction of oxidative stress, lipid peroxidation is reported to be a key player with an important role in the toxicity of several heavy metals [45–46]. This agrees with the findings from this study showing that cadmium increased the MDA level, an oxidative product of lipid peroxidation, in the brain. In a bid to counteract the harmful effects of reactive oxygen species and free radicals, cells are fortified with potent antioxidant defence mechanisms. Findings from this study reveal that the cadmium-induced increase in lipid peroxidation corresponded with a significant decrease in the activities of the cellular enzymatic antioxidants SOD, CAT and GPX. This is in agreement with several reports showing that cadmium deactivates several enzymes and proteins involved in the regulation and attenuation of stress [47, 48]. However, pretreatment of rats with ethanolic turmeric root extract protected against the dysregulation of the antioxidant enzymes activity and induction of lipid peroxidation. This may be due to the extract’s free radical scavenging, anti-oxidative, metal chelating and anti-lipid peroxidative properties, as previously reported [49, 50].

Several reports show that cadmium exposure induces severe histological changes in the brain [51, 52]. From this study, administration of cadmium alone to rats induced alterations to the cerebral cortex, particularly the outer pyramidal and inner granular layers, as well as the pyramidal cells and neurons of the hippocampus. The disparity and loss of cellularity in the inner granular layer of the cerebral cortex, degenerating pyramidal neurons and cells as well as vacuolation of the CA1 region of the hippocampus are considered as hallmarks of cytoskeletal disorganization [53]. Vacuolation is often attributable to cellular shrinkage and withdrawal of their processes thereby leaving peri-cellular spaces [54]. In agreement with our study, structural changes to the neurons and cells of the cerebral cortex and hippocampus are known to cause deficits in learning, memory and cognition [51, 52, 55, 56]. The degenerative changes to the cerebral and hippocampal structures may be linked to the vulnerability of rats to cadmium toxicity and its ability to induce oxidative damage [57]. Pretreatment of rats with ethanolic turmeric root extract protected against the histological alterations in the cerebrum and hippocampus of rats exposed to cadmium alone, thus demonstrating its potent protective activity.

CONCLUSION
The qualitative phytochemical screening of ethanolic turmeric root extract showed that it contained various important phytochemicals. The presence of these phytochemicals in the plant, its ability to regulate antioxidant enzymes activity and inhibit lipid peroxidation are possible explanations for the neuroprotective activity demonstrated in this study. It is therefore suggested and recommended that regular intake of turmeric in diet could be neuroprotective and that this plant could be developed as a neuroprotective agent useful against cadmium toxicity and other related disorders.

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Conflict of Interest
None declared.

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REFERENCES

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