ORIGINAL ARTICLE

Dichlorvos-induced oxidative stress in rat brain: Protective effects of the ethanolic extract of *Alstonia boonei* stem bark

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Organophosphorous pesticides, commonly used in agriculture for achieving better-quality products, are toxic substances that have harmful effects on human health. Recent research on pesticides, especially pesticide mixtures, has shown that they are one of the key environmental health issues. The aim of the present study was to investigate the protective effects of *Alstonia boonei* ethanolic extract in dichlorvos-induced neurotoxicity in Wistar rats. Dichlorvos (50 mg/kg body weight) was orally administered in Wistar rats for 14 days followed by the treatment of *Alstonia boonei* (200 and 400 mg/kg body weight) for 14 days. The activities of lipid peroxidation (LPO), reduced glutathione (GSH) and activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level were measured to evaluate the toxicity of these pesticides in the brain. Histological examinations of the brain were monitored. Under the influence of dichlorvos, there was significant decrease in the activities of SOD, CAT, GPx, GSH, ALT and AST and significant increase in malondialdehyde. *Alstonia boonei* showed a significant brain-protective effect by decreasing the level of lipid peroxidation and elevating the activities of antioxidative enzymes and the level of GSH. Furthermore, histological alterations in the brain were observed in dichlorvos-untreated rats and were ameliorated in dichlorvos-induced treated rats with *Alstonia boonei*. The observations presented lead us to conclude the harmful effects of dichlorovos during the exposure and the protective role of *Alstonia boonei* in minimizing these effects.

Key words: *Alstonia boonei, brain, dichlorvos, lipid peroxidation, organophosphate, oxidative stress, reactive oxygen species*

INTRODUCTION

Dichlorvos (DDVP-2,2-dichlorovinyl dimethyl phosphate) is a pesticide commonly used for the protection of stored products and grains for controlling ecto- and endoparasites of farm animals and for combating indoor and outdoor pests. Its annual world production reaches 4000 tonnes.[1] Being an organophosphorous compound, its principal mechanism of toxicity is through the inhibition of acetylcholinesterase (AChE) and/or neuropathy target esterase (NTE), which acts on the central and peripheral nervous systems.[2] In addition, dichlorvos toxicity seems to induce oxidative stress,[3,4] whose product peroxynitrite[5] may react with various amino acid residues in proteins.[6] Excess production of Reactive Oxygen Species (ROS) can cause oxidative modification of proteins, DNA and lipids. Endogenous non-enzymatic (glutathione, GSH) and enzymatic (superoxide dismutase [SOD], catalase [CAT] and glutathione peroxidase [GPx]) antioxidants detoxify these ROS and protect cells. Because of the continuous exposure of pesticides, the level of these endogenous antioxidants decreases, leading to accelerated cell death. Use of cytoprotective agents in the form of exogenous antioxidants may help in scavenging excess ROS, helping in cell survival and longevity. A number of medicinal plants are reported to possess ROS scavenging and cytoprotective activities.[7-10]

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Alstonia boonei De Wild is a large, deciduous, evergreen tree usually up to 45 m tall and 1.2 m in diameter, belonging to the family Apocynaceae consisting of about 40-60 species. It is a native of tropical and subtropical Africa, Southeast Asia, Central America and Australia. "Alstonia" is named after Dr. C. Alston (1685-1760), a Professor of Botany at Edinburgh University. It is reported for the treatment of malaria, intestinal helminthes, rheumatism, muscular pain, insomnia and hypertension. It contains phytochemicals such as saponin, alkaloids, tannins and steroids.[11,12] Although the medicinal importance of the stem bark extract of *A. boonei* in ameliorating some disease conditions is reported by several authors, to the best of our knowledge, there is lack of information on the effect of this plant on its neuroprotective potentials. *A. boonei* has been shown to possess many pharmacological and physiological activities such as antioxidants.[13] In this study, we evaluated the effect of a fresh *A. boonei* in dichlorvos-induced neurotoxicity in male Wistar rats.

MATERIALS AND METHODS

Chemicals

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate - DDVP) was bought from a local chemist in Ibadan, Nigeria. Thiobarbituric acids (TBA) were bought from Aldrich Chemical Co. (Milwaukee, WI, USA). GSH, hydrogen peroxide, 5,5'-dithios-bis-2-nitrobenzoic acid (DNTB) and Epinephrine were bought from Sigma Chemical Co., Saint Louis, MO, USA. Trichloroacetic acid (TCA) and TBA was bought from British Drug House (BDH) Chemical Ltd., Poole, UK. Other reagents were of analytical grade and the purest quality available.

Collection and extraction of *A. boonei* stem bark

The stem bark of *A. boonei* was procured from local suppliers in Ado-Ekiti (Ekiti State) and authenticated at the Department of Plant Science, Ekiti State University. The stem bark of *A. boonei* was air-dried and crushed into a fine powder. The powdered part was extracted with ethanol using maceration and the extract was concentrated in vacuum at 40°C with a rotary evaporator and water bath to dryness. The yield of the extraction was 5.01%.

Preliminary phytochemical screening

The preliminary phytochemical screening was carried out with ethanolic extracts of the *A. boonei* stem bark for the detection of various phytochemicals. Tests for common phytochemicals were carried out by standard methods.[14]

Animals

Male Wistar rats (*Rattus norvegicus*) weighing between 80 and 120 g were bought from the animal house of the Department of Chemical Sciences, Biochemistry Unit, Afe Babalola University, Nigeria. Animals were kept in aired cages at room temperature (28-30°C) and preserved on normal laboratory chow (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum*.

Ethical approval

Rats handling and treatments conform to the guidelines of the National Institute of Health (NIH publication 85-23, 1985) for laboratory animal care and use. The ethical committee of the Afe Babalola University approved this study. All animals in this study followed the institutional Animal Ethical Committee according to guidelines given by the Committee for Control and Supervision of Experiments on Animals (CPCSEA).

Induction of experimental animal

Dichlorvos was induced in groups II, III and IV. Briefly, dichlorvos was dissolved in distilled water and then managed by intravenous injection (through the tail vein) at a dose of 50 mg/kg body weight.

Study design

Twenty male rats were divided into four groups of five rats each. Group I - Control (distilled water); Group II - Dichlorvos (50 mg/kg b.w.); Group III - *A. boonei* (200 mg/kg b.w.) (14 days) + DDVP (50 mg/kg b.w.); Group IV - *A. boonei* (400 mg/kg b.w.) (14 days) + DDVP (50 mg/kg b.w.).

Preparation of brain tissues

Animals were sacrificed by cervical decapitation. The brain was removed and washed with normal saline and all the extraneous materials were removed before weighing. The rat brain tissue was minced and homogenized in 500 µL of buffer A (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 220 mM mannitol, 68 mM sucrose, 1 mM leupeptin, 5 µg/mL pepstatin A, 5 µg/mL aprotonin, 0.5 mM PMSF). The brain was kept at ice-cooled conditions all the time.

Preparation of serum

Blood was collected from the heart of the animals into plain centrifuge tubes and allowed to stand for 1 h. Serum was prepared by centrifugation at 3000 g for 15 min in a Beckman bench centrifuge. The clear supernatant was used for estimating the serum lipid profile and enzymes.

Biochemical tests

Protein contents of the samples were tested by the method of Lowry *et al*.[15] using bovine serum albumin as the standard. Alanine and aspartate aminotransferases (ALT and AST) were tested by the combined methods of Mohun and Cook[16] and Reitman and Frankel.[17] The lipid peroxidation level was tested by the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA), a product of lipid peroxides as described by Buege and Aust.[18] The tissue SOD was measured by the nitro blue tetrazolium (NBT) decrease method of McCord and Fridovich.[19] CAT was tested spectrophotometrically by measuring the rate of decomposition of hydrogen peroxide at 240 nm as described by.[20] Reduced GSH level was measured by the method of Beutler *et al*.[21] This method is on developing a stable (yellow) color when 5',5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) mix to sulfhydryl compounds. The chromophoric

product resulting from Ellman's reagent with the reduced GSH (2-nitro-5-thiobenzoic acid) holds a molar absorption at 412 nm, which is part to the reduced GSH in the test sample. The GPx was tested by the method of Rotruck *et al*.[22] When this substance is mixed with reduced GSH, its absorption shifts to a longer wavelength (340 nm), and increase at this wavelength provides a direct measurement of the enzymatic reaction.

Histopathology of tissues

The brains from the control and experimental groups were fixed with 10% formalin and embedded in paraffin wax and cut into longitudinal section of 5 μm thickness. The sections were stained with hemotoxylin and eosin dye for histopathological observation.

Statistical analysis

All the data are expressed as mean ± SEM. The significance of difference in means between the control and treated animals was determined by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test for analysis of biochemical data using SPSS (20.0). Values were considered statistically significant at $P < 0.05$.

RESULTS

Phytochemicals investigation

It was found that the ethanolic extract contained compounds known to have antioxidant activities, like tannins, phlobatannins, flavonoids, anthocyanin, cardiac glycosides and alkaloids [Table 1].

Table 1: Phytochemical screening of the ethanolic extract of *Alstonia boonei* stem bark

Phytochemical	Extract content
Alkaloids	+++
Tannin	++
Phlobatannins	++
Saponin	+
Flavonoids	+++
Anthraquinones	++
Phenol	+++
Cardiac glycosides	++

+: Trace amount present, ++: Moderate amount present, +++: Noticeable amount present

Effects of *A. boonei* stem bark on body weight and relative weight of organs of dichlorvos-induced oxidative damage in rats

In Table 2, there were significant increases ($P < 0.05$) in the relative weight of the brain of dichlorvos-untreated rats when compared with the control, while treatment with the *A. boonei* stem bark (100 and 200 mg/kg) significantly decreased the relative weight of the brain of dichlorvos-induced rats to values that were statistically similar ($P > 0.05$) to the control. All these changes induced by dichlorvos intoxication significantly ($P < 0.05$) restored to near-normal levels on administration of *A. boonei* stem bark.

Effects of *A. boonei* stem bark on antioxidant parameters and marker enzymes in dichlorvos-induced toxicity in rats

Administration of dichlorvos significantly increased ($P < 0.05$) the serum and brain lipid peroxidation (LPO) products measured as TBA-reactive substances [Table 3]. However, treatment with the *A. boonei* extract completely ameliorated dichlorvos-induced increase in LPO. In dichlorvos-induced rats, the activities of brain GSH, SOD and CAT as well as GPx decreased significantly relative to the control [Table 4]. Excellent performance of the extract at (400 mg/kg) reversed the adverse effect of dichlorvos by normalizing this enzymic antioxidant. *A. boonei* treatment to the dichlorvos-treated groups caused a significant increase in the GPx activities as well as a noticeable increase in the GSH level. In dichlorvos-induced rats, serum ALT and AST were significantly increased [Table 5] relative to the control. Treatment with *A. boonei* resulted in significant protection of the brain, as indicated by reductions in the elevated levels of ALT and AST; however, there was evidence of amelioration in the treated group.

Effects of *A. boonei* stem bark on the histology of the brain

The histology of brain slide of dichlorvos-untreated rats showed mild spongiosis, severe congestion and hemorrhage at the meninges [Figure 1]. Treatment with the ethanolic extract of the stem bark of *A. boonei* (200 and 400 mg/kg) confirmed the neuroprotective activity as a significant recovery of neuronal damage, and decreased necrosis was evident against cadmium-induced oxidative damage in the brain of the rats, which is similar to their control. The histological results further corroborated the biochemical findings, suggesting the useful effects of *A. boonei* stem bark in dichlorvos-induced toxicity in rats.

Table 2: Changes in the body weight and relative weight of organs of dichlorvos-induced oxidative damage in rats treated with the ethanolic extract of *Alstonia boonei* stem bark

Treatment	Body weight (g)		Brain	
	Initial	Final	Weight of organs (g)	Relative weight of organs
Control	100.25±0.21	117.46±5.32	6.35±0.27	0.98±0.05
Dichlorvos untreated	112.08±1.12	128.10±4.96	6.02±0.80	2.35±0.08*
Dichlorvos+200 mg/kg	121.55±2.23	149.36±7.09	6.18±0.20	1.25±0.03**
Dichlorvos+400 mg/kg	151.02±3.35	176.22±4.99	6.22±0.30	1.42±0.02**

Values are mean±S.D. of five animals per group, Dichlorvos=at 50 mg/kg, Dichlorvos treated=*Alstonia boonei* at 200 mg/kg, Dichlorvos treated=*Alstonia boonei* at 400 mg/kg, *significantly different from control ($P<0.05$), **Significantly different from dichlorvos untreated ($P<0.05$)

DISCUSSION

The phytochemical study of *A. boonei* stem bark extracts revealed the presence of polyphenol-rich compounds. Polyphenols have been suggested to decrease the oxidative stress in human. Flavonoids found in the extract may inhibit the oxidative stress by scavenging free radicals by acting as a reducing agent, hydrogen atom donating molecules or singlet oxygen quenchers; chelating metal ions and sparing other antioxidants (e.g. carotene, vitamin C and E).[23] The literature reveals that the carbonyl groups present in the flavonoids and phenolic compounds were responsible for the antioxidant activity.[24] This investigation revealed that *A. boonei* contains pharmacologically active substance(s) such as alkaloids, glycosides, saponins, tannins, flavonoids and phenolic compounds, which are responsible for the antioxidant activity. The literature survey indicates that there is no scientific evidence to support the neuroprotective effect of the ethanolic extract of *A. boonei*. Therefore, the present study is undertaken to investigate the neurotoxicity effect of dichlorvos and the effects of the *A. boonei* ethanol leaf extract in ameliorating it. The brain, compared with the lung, liver and other organs, contains relatively low levels of enzymatic and non-enzymatic antioxidants and high amounts of peroxidizable unsaturated lipids, rendering it more vulnerable to oxidative stress compared with other tissues.[25] Increasing evidences suggested that excessive production of free radicals in the brain, and the imbalance between oxidative species and antioxidant defenses, are related to the pathogenesis of neurodegenerative diseases.[26] In dichlorvos-induced rats treated with *A. boonei*, the changed body weight and liver weight parameters recovered to near-normal levels due to the antioxidant effects found in *A. boonei* stem bark. The results showed that the oral exposure of dichlorvos caused a significant increase in oxidative stress in male Wistar rats, as evident from the increased level of LPO in the serum.

Table 3: Changes in the levels of lipid peroxidation in dichlorvos-induced toxicity in rats treated with the ethanolic extract of *Alstonia boonei* stem bark

Treatments	μmol MDA/mg protein	
	Brain	Serum
Control	6.05±0.02	6.82±0.08
Dichlorvos untreated	10.62±0.03*	10.42±0.16*
Dichlorvos+200 mg/kg	4.85±0.03**	4.92±0.26**
Dichlorvos+400 mg/kg	5.85±0.02**	5.74±0.22**

Values are mean±S.E.M. of five animals per group, Dichlorvos treated=*Alstonia boonei* at 200 mg/kg, Dichlorvos treated=*Alstonia boonei* at 400 mg/kg, *Significantly different from control (P<0.05), **Significantly different from dichlorvos untreated (P<0.05). MDA: Malondialdehyde

Oxidative stress is reported to be one of the important mechanisms involved in Organophosphate (OP) toxicity.[27] Being lipophilic in nature, OP interacts with cells through biomembranes rich in polyunsaturated fatty acids thus oxidatively damaging them, known as lipid peroxidation.[28] We observed an increased TBARS level in the present study predominantly in animals given DDVP.[29] Oxidative stress occurs usually with excessive generation of free radicals, followed by a parallel depletion of antioxidant enzymes like GSH. Reduced GSH and its metabolizing enzymes provide the major defense against ROS-induced cellular damage.[30] Organophosphate appears to disturb this key cellular pathway, perhaps by disturbing mitochondrial metabolism, as suggested by.[31] In the present study, we observed depleted levels of antioxidant enzymes and elevated levels of ROS and TBARS following DDVP exposure in rats. *A. boonei* treatment improved the levels of antioxidant enzymes, mainly GSH, SOD and catalase.

Table 4: Changes in the levels of brain antioxidant parameters in dichlorvos-induced rats treated with the ethanolic extract of the *Alstonia boonei* stem bark

Treatment	Brain			
	GSH GPx (mg/g tissue)		SOD CAT (U/mg protein)	
Control	59.85±0.25	55.45±0.63	57.16±1.01	55.89±1.08
Dichlorvos untreated	14.28±0.41*	18.35±0.51*	23.53±0.88*	24.22±0.78*
Dichlorvos+200 mg/kg	43.73±0.34**	46.23±0.45**	42.88±0.03**	46.89±0.41**
Dichlorvos+400 mg/kg	50.81±0.37**	48.88±0.21**	50.34±1.00**	51.12±0.81**

Values are mean±S.E.M. of five animals per group, Dichlorvos treated=*Alstonia boonei* at 200 mg/kg, Dichlorvos treated=*Alstonia boonei* at 400 mg/kg, *Significantly different from control (P<0.05), **Significantly different from dichlorvos untreated (P<0.05). GSH: Glutathione, GPx: Glutathione peroxidase, SOD: Superoxide dismutase, CAT: Catalase

Table 5: Changes in the activities of serum and brain alanine and aspartate aminotransferases in dichlorvos-induced rats treated with the ethanolic extract of *Alstonia boonei* stem bark

Treatments	Brain (U/L)		Serum (U/L)	
	AST	ALT	AST	ALT
Control	58.65±0.02	65.56±2.24	5.54±1.77	7.37±1.46
Dichlorvos untreated	22.42±2.38*	21.28±2.04*	11.39±0.96*	14.89±2.11*
Dichlorvos+200 mg/kg	44.02±1.05**	46.52±1.54**	5.13±1.34**	5.78±1.38**
Dichlorvos+400 mg/kg	51.02±1.25**	60.12±1.82**	5.41±1.42**	6.87±1.28**

Values are mean±S.E.M. of five animals per group, Dichlorvos=at 50 mg/kg dichlorvos treated=*Alstonia boonei* at 200 mg/kg, Dichlorvos treated=*Alstonia boonei* at 400 mg/kg, *Significantly different from control (P<0.05), **Significantly different from dichlorvos untreated (P<0.05). AST: Aspartate aminotransferase, ALT: Alanine aminotransferase

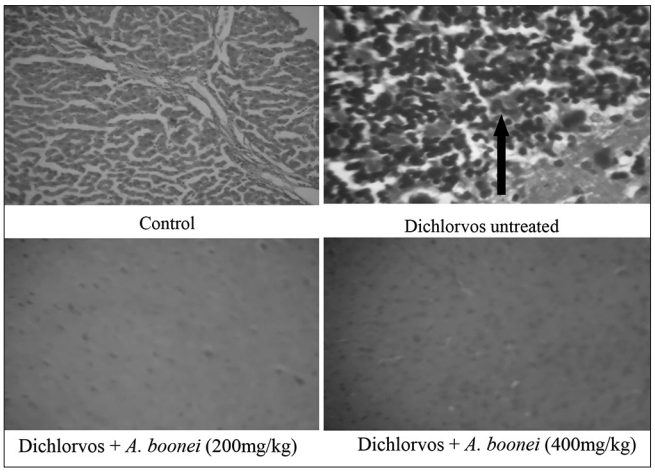

Figure 1: Changes in the histology of the brain samples of dichlorvos-induced oxidative stress in rats treated with the *Alstonia boonei* ethanolic stem bark extract. (Black arrows indicate mild spongiosis, severe congestion and hemorrhage at the meninges)

Well-known biomarkers (ALT and AST) and histological changes were examined to evaluate the neuroprotective effects of *A. boonei*. Consistent with previous studies, our study confirmed that dichlorvos exposure damaged the brain, as shown by elevation of the serum aminotransferase activities and morphological changes observed in the brain sections.[32] Interestingly, these adverse effects were significantly attenuated by *A. boonei* in the treatment groups, which indicated a prominent neuroprotective effect of *A. boonei* against dichlorvos toxicity. The increased levels of serum AST and ALT in dichlorvos-induced rats indicate an increased permeability and damage and/or neurosis of the brain. In our study, we found that the extract of *A. boonei* at a dose of 400 mg/kg caused a significant decrease in the activities of serum AST and ALT, which further supports the beneficial effects of the extract of *A. boonei* in dichlorvos-induced rats.

Histological examination of the brain tissue reveals that dichlorvos caused abnormal ultrastructural changes in the brain tissue, including spongiform necrosis, nuclear vacuolization pycnosis and lymphocytic inflammatory changes. Regarding the histopathological observation in *A. boonei*-treated dichlorvos-intoxicated rats, the observed pathological impairments by dichlorvos have been recovered to be significant, which indicates that *A. boonei* is capable of preventing the neuronal damage induced by dichlorvos.

CONCLUSION

In conclusion, the results of the present study demonstrate that *A. boonei* exhibited a significant protective action against dichlorvos-induced neurotoxicity in rats via inhibiting the lipid peroxidation and protein carbonyl formation and increasing the endogenous antioxidant defense systems in serum and brain and subsequent restoration of the normal histoarchitecture of the brain tissue. The stem bark extract also shows neuroprotective potential; it protected the rat brain against DDVP-induced neurotoxicity and enhanced the antioxidant status of the brain.

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