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# Effect of linoleic acid isolated from Ashwagandha extract on acetylcholine and Biochemical Markers in Rats with Neurodegeneration disorders

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## ABSTRACT

*This study investigated the therapeutic and preventive potential of the ethanolic extract of Withania somnifera [Ashwagandha] and its isolated fatty acid, linoleic acid, against trichloroethylene [TCE]-induced neurodegenerative disorders in male Wistar rats. A total of eight experimental groups were established: a negative control (distilled water), a positive control [TCE], and six treatment groups receiving linoleic acid, palmitic acid, the crude ethanolic extract, or combinations thereof, either prophylactically or therapeutically. Biochemical assessments were performed to determine serum and brain levels of acetylcholine, vitamin B12, and linoleic acid. Rats exposed to TCE exhibited significant reductions in vitamin B12, palmitic acid, and linoleic acid levels, alongside elevated acetylcholine compared with the negative control. In contrast, treatment with linoleic acid, palmitic acid, the ethanolic extract, or their combinations significantly restored vitamin B12 and linoleic acid levels while reducing acetylcholine concentrations. These findings indicate that Ashwagandha extract and linoleic acid exert both therapeutic and protective effects against TCE-induced neurotoxicity by improving biochemical parameters, highlighting their potential as promising candidates for managing neurodegenerative disorders.*

***Keywords:*** *Ethanolic extract; acetylcholine; neurodegeneration; trichloroethylene; Withania somnifera; linoleic acid.*

## Introduction

Neurodegenerative disorders create a major worldwide health problem because they impact millions of people across the globe. Research studies demonstrate that genetic predisposition interacts with environmental factors to elevate the chances of developing these disorders [1]. The combination of motor and cognitive deterioration in these conditions prevents people from carrying out their daily responsibilities which leads to decreased life quality. The medical field has made substantial progress in treating neurodegenerative diseases yet most of these conditions remain without a definitive treatment solution. The current therapeutic methods primarily provide symptom relief and pain management while attempting to restore some level of functional equilibrium [1]. Neurodegenerative disorders present with neurological symptoms that include mild paralysis and sensory neuropathy and cognitive decline and psychological impairments [2]. The spectrum includes three main diseases which are Alzheimer's disease [3], multiple sclerosis [4] and Parkinson's disease. Neuronal damage occurs through multiple mechanisms which include central nervous system dysfunction and demyelination and oxidative stress and progressive neurological deterioration [5]. Human health care has depended on medicinal plants as its fundamental base since ancient times because these plants contain bioactive compounds which help prevent and manage chronic diseases [6]. Traditional medicine recognizes *Withania somnifera* as Ashwagandha or Indian ginseng because it contains various pharmacological properties. The antioxidant and anti-inflammatory compounds in Ashwagandha [7] protect neuronal cells by blocking oxidative damage and neuroinflammation. People have used the substance in powdered form with milk or water to boost their mental and physical abilities according to numerous reports [8].

The neuroprotective compounds of Ashwagandha have been identified through phytochemical studies which show that withanolides and withaferin A are the main active components [9]. The extensive history of Ashwagandha consumption by humans along with scientific evidence about its nervous system benefits makes it a promising substance for neurodegenerative disease research [10]. Based on this rationale, the present study was designed to extract an ethanolic preparation of *Withania somnifera* roots and to isolate its major fatty acid, linoleic acid. The researchers aimed to determine how the extract and linoleic acid separately and together affected specific biochemical markers in male rats who received trichloroethylene to induce neurodegeneration. The research method used this approach to study how these compounds work as treatments and preventatives for neurotoxic damage.

## Materials and Methods

### Plant material and preparation of ethanolic extract

Roots of *Withania somnifera* were purchased from local markets in Nineveh Governorate, Iraq. They were thoroughly washed with distilled water to remove dust and impurities, then air-dried at room temperature away from direct sunlight. After drying, the roots were ground into a fine powder using an electric grinder and stored in airtight glass containers under dark, dry conditions until further use. The extraction process required 20 g of powdered roots to soak in 100 mL of 70% ethanol solution while stirring at room temperature for 24 hours before filtering through sterile filter paper. The filtrate was centrifuged at 2500 rpm for 10 min, concentrated under reduced pressure using a rotary evaporator, and stored at 2–4 °C in sterile glass containers until use. This method was adapted from previous studies [11,12].

**Isolation and quantification of linoleic acid**

Linoleic acid was isolated and quantified by reversed-phase HPLC using a Shim-pack C18 column [250 × 4.6 mm]. Samples were derivatized with 9-fluorenylmethylchloroformate and sodium phosphate buffer [0.05 M, pH 9.3] prior to injection. The column was maintained at 50 °C, with acetonitrile–water as the mobile phase at a flow rate of 1.5 mL/min. Detection was performed using fluorescence at excitation and emission wavelengths of 265 and 315 nm. Quantification was achieved by comparison with pure linoleic acid standards [13].

**Chemicals and reagents**

Trichloroethylene (TCE) was administered orally (gavage) at a dose of 200 mg/kg body weight for six consecutive weeks to induce neurodegeneration, as previously described [14]. Diagnostic kits were used for biochemical analyses: linoleic acid kits [Sun Long, China], serum vitamin B12 assay with a Cobas e411 analyzer [Roche Diagnostics], and acetylcholine measured by colorimetric spectrophotometry.

**Experimental animals**

A total of 48 adult male Wistar rats (220–350 g; 11 ± weeks old) were obtained from the Animal House, College of Veterinary Medicine, University of Mosul. Animals were housed under controlled lighting and temperature conditions, with ad libitum access to food and water. They were acclimatized for one week prior to experimental procedures.

**Experimental design**

Animals were randomly divided into eight groups [n = 6 per group]:

- **Group 1 [Negative control]:** Received distilled water and standard diet.
- **Group 2 [Positive control]:** Received TCE [200 mg/kg] daily for 6 weeks.
- **Group 3:** Received TCE for 6 weeks, then linoleic acid [150 µg/kg] for 4 weeks.
- **Group 4:** Received TCE for 6 weeks, then palmitic acid [0.24 mg/kg] for 4 weeks.
- **Group 5:** Received TCE for 6 weeks, then ethanolic extract of *W. somnifera* [300 mg/kg] for 4 weeks.
- **Group 6:** Received TCE for 6 weeks, then ethanolic extract [300 mg/kg] + linoleic acid [150 µg/kg] for 4 weeks.
- **Group 7:** Received TCE for 6 weeks, then ethanolic extract + palmitic acid for 4 weeks.
- **Group 8 [Preventive group]:** Received linoleic acid [150 µg/kg] for 4 weeks prior to TCE administration for 6 weeks.

All treatments were administered orally by gavage.

## Results

**Biochemical parameters**

Table 1 summarizes the biochemical alterations observed across the experimental groups. Exposure to trichloroethylene [TCE] in the positive control group resulted in a significant increase in serum acetylcholine levels [10.50 ± 1.53 nmol/mL] compared with the negative control group [7.46 ± 0.47 nmol/mL; $p < 0.05$]. Conversely, vitamin B12 and linoleic acid concentrations in both serum and brain tissues were markedly reduced following TCE administration.

**Acetylcholine levels**

Rats in the positive control group exhibited significantly elevated acetylcholine levels compared with the negative control, confirming the neurotoxic effect of TCE. Treatment with linoleic acid, palmitic acid, or the ethanolic extract of *Withania somnifera* either alone or in combination resulted in a reduction of acetylcholine levels compared with the positive control. The most notable decrease was observed in the group treated with linoleic acid and ethanolic extract together [8.05 ± 0.41 nmol/mL], which approached values closer to the negative control. The preventive group [linoleic acid prior to TCE exposure] also demonstrated a partial protective effect, though acetylcholine remained higher than in the negative control.

**Vitamin B12 levels**

Vitamin B12 levels decreased significantly in the positive control group [1307.24 ± 4.14 pg/mL] compared with the negative control [1339.84 ± 3.91 pg/mL; $p < 0.05$]. Administration of linoleic acid, palmitic acid, or ethanolic extract either individually or in combined regimens significantly restored vitamin B12 levels. The highest recovery was observed in the linoleic acid group [1341.14 ± 5.45 pg/mL] and the combined ethanolic extract + linoleic acid group [1346.34 ± 3.90 pg/mL], both of which exceeded the baseline levels of the negative control.

**Serum linoleic acid**

A significant reduction in serum linoleic acid was detected in rats exposed to TCE [2.16 ± 1.14 mg/L] compared with the negative control [6.91 ± 1.91 mg/L; $p < 0.05$]. Treatment with linoleic acid [6.97 ± 1.29 mg/L] and the combined ethanolic extract + linoleic acid [6.87 ± 0.62 mg/L] restored serum levels close to those of the negative control. Palmitic acid and ethanolic extract alone showed moderate improvements, whereas the preventive linoleic acid group displayed the highest serum levels [7.89 ± 1.49 mg/L].

**Brain tissue linoleic acid**

TCE exposure significantly depleted brain linoleic acid [123.20 ± 17.03 mg/L] compared with the negative control [196.84 ± 13.35 mg/L]. Rats treated with linoleic acid [171.0 ± 10.48 mg/L] or the combined ethanolic extract + linoleic acid [155.8 ± 6.27 mg/L] demonstrated significant restoration of brain linoleic acid. The preventive linoleic acid group achieved the greatest recovery [177.44 ± 16.19 mg/L], highlighting its protective efficacy. In contrast, palmitic acid alone produced only marginal improvement, while the ethanolic extract alone yielded moderate but non-significant increases.

*Table 1. Biochemical parameters across experimental groups [mean ± SD]*

| Parameter | Negative control | Positive control [TCE] | Linoleic acid | Palmitic acid | Ethanolic extract | Extract + Linoleic | Extract + Palmitic | Preventive Linoleic |
|---|---|---|---|---|---|---|---|---|
| Acetylcholine [nmol/mL] | 7.46 ± 0.47$^{a}$ | 10.50 ± 1.53$^{b}$ | 9.12 ± 0.38$^{b}$ | 9.69 ± 0.48$^{b}$ | 9.17 ± 0.55$^{b}$ | 8.05 ± 0.41$^{a}$ | 9.10 ± 0.72$^{b}$ | 10.17 ± 0.63$^{b}$ |
| Vitamin B12 [pg/mL] | 1339.84 ± 3.91$^{cd}$ | 1307.24 ± 4.14$^{a}$ | 1341.14 ± 5.45$^{cd}$ | 1331.02 ± 5.08$^{b}$ | 1338.30 ± 4.99 | 1346.34 ± 3.90$^{d}$ | 1330.10 ± 4.27$^{b}$ | 1326.66 ± 5.45$^{b}$ |
| Serum linoleic acid [mg/L] | 6.91 ± 1.91$^{c}$ | 2.16 ± 1.14$^{a}$ | 6.97 ± 1.29$^{c}$ | 3.74 ± 1.15$^{b}$ | 5.11 ± 0.92$^{b}$ | 6.87 ± 0.62$^{c}$ | 4.97 ± 1.17$^{b}$ | 7.89 ± 1.49$^{c}$ |
| Brain linoleic acid [mg/L] | 196.84 ± 13.35$^{d}$ | 123.20 ± 17.03$^{a}$ | 171.0 ± 10.48$^{c}$ | 119.62 ± 4.48$^{a}$ | 129.45 ± 7.33$^{a}$ | 155.84 ± 6.27$^{b}$ | 128.22 ± 5.06$^{a}$ | 177.44 ± 16.19$^{c}$ |

*Different superscript letters indicate significant differences at $p < 0.05$*

## Discussion

### Acetylcholine levels

The present findings demonstrated that rats exposed to trichloroethylene [TCE] exhibited a significant elevation in serum acetylcholine compared with the negative control. This abnormal increase can be attributed to the inhibitory effect of TCE on acetylcholinesterase activity, leading to excessive accumulation of acetylcholine at synaptic clefts and subsequent cholinergic dysfunction [15]. Such accumulation has been previously associated with impaired neurotransmission and neuronal injury in models of organic solvent exposure [16].

Treatment with *Withania somnifera* ethanolic extract and isolated linoleic acid significantly reduced acetylcholine levels compared with the positive control. This neuroprotective effect may be linked to bioactive compounds such as withaferin A, known to attenuate neuroinflammation by suppressing NF-κB signaling and inflammatory cytokines [17]. Similarly, linoleic acid has been reported to modulate hippocampal inflammation through downregulation of TNF-α, IL-1β, and IL-6, thereby preserving neuronal integrity [18]. These outcomes align with our findings, where combined administration of extract and linoleic acid produced the greatest reduction in acetylcholine, highlighting a potential synergistic interaction.

### Vitamin B12 levels

A significant decline in vitamin B12 was observed in TCE-exposed rats, consistent with the hypothesis that organochlorine compounds impair gastrointestinal absorption of cobalamin and disrupt intrinsic factor function [19]. Vitamin B12 deficiency is considered an early biomarker of neurotoxicity, given its essential role in myelin synthesis and neuronal signaling [20].

Therapeutic administration of linoleic acid, palmitic acid, or Ashwagandha extract significantly restored vitamin B12 levels, with linoleic acid and the extract + linoleic acid combination achieving the most pronounced effects. Essential fatty acids such as linoleic acid are known to mitigate oxidative stress and inflammation, indirectly reducing vitamin B12 depletion [21]. Moreover, withanolides from Ashwagandha promote neuronal survival and reduce homocysteine accumulation, which spares vitamin B12 consumption in methionine metabolism [22,23]. These observations suggest that both linoleic acid and Ashwagandha not only act as neuroprotectants but also support metabolic processes critical for maintaining cobalamin homeostasis.

### Serum linoleic acid

The marked reduction in serum linoleic acid following TCE exposure is consistent with reports that TCE metabolism via cytochrome P450 enzymes generates reactive intermediates, including trichloroacetic acid and chloral hydrate, which promote lipid peroxidation and fatty acid depletion [24,25]. Restoration of serum linoleic acid after treatment with linoleic acid and Ashwagandha extract confirms their capacity to counteract oxidative degradation. Linoleic acid supplementation enhanced absorption through fatty acid transporters such as CD36, thereby increasing systemic availability [26]. Ashwagandha extract, on the other hand, contains steroidal lactones capable of activating PPAR-α and PPAR-γ, transcription factors that regulate fatty acid uptake and storage [27].

Interestingly, palmitic acid treatment also improved serum linoleic acid, possibly due to competitive interactions in fatty acid metabolism. Palmitic acid can influence the activity of desaturase enzymes [Δ5- and Δ6-desaturase], thereby modulating unsaturated fatty acid stability [28]. This suggests an indirect protective role in preventing linoleic acid catabolism under oxidative stress conditions.

### Brain tissue linoleic acid

A significant decline in brain tissue linoleic acid was evident in the TCE group, corroborating earlier studies linking solvent exposure to lipid oxidation, membrane damage, and altered fatty acid metabolism in neural tissues [29]. Supplementation with linoleic acid restored brain concentrations, reflecting its ability to cross the blood–brain barrier and support neuronal membrane integrity. Conjugated linoleic acid derivatives have been shown to stimulate anti-inflammatory mediators such as oleoylethanolamide [OEA] and palmitoylethanolamide [PEA], which aid in cellular repair and synaptic stabilization [30].

Preventive administration of linoleic acid yielded the most pronounced neuroprotection, suggesting that prophylactic supplementation may preserve brain lipid composition before the onset of toxic injury. Ashwagandha extract alone produced moderate improvements, consistent with reports that its phytoconstituents enhance neuronal regeneration and reduce oxidative stress [31,32,33]. Although palmitic acid alone failed to significantly improve brain linoleic acid, its combination with Ashwagandha extract produced partial benefits, likely reflecting complementary antioxidant mechanisms.

### Overall interpretation

Taken together, the findings underscore that both *Withania somnifera* extract and linoleic acid exert therapeutic and preventive effects against TCE-induced neurodegeneration. Their mechanisms appear to converge on reducing neuroinflammation, enhancing antioxidant defenses, stabilizing fatty acid metabolism, and preserving vitamin B12 homeostasis. These synergistic interactions support the potential of Ashwagandha-derived compounds as promising candidates for future neuroprotective therapies.

## Conclusion

The study shows that TCE exposure causes severe neurotoxic damage which produces elevated acetylcholine levels and reduced vitamin B12 levels and major decreases in linoleic acid content in blood and brain tissue. The administration of *Withania somnifera* ethanolic extract with isolated linoleic acid and their combination proved effective in treating these biochemical changes. The combination of extract and linoleic acid treatment resulted in better neuroprotective effects than using either substance alone because the two compounds work together in a synergistic manner.

The administration of linoleic acid before toxic exposure proved to be an effective preventive measure against TCE-induced neurodegeneration which suggests its potential use as a protective treatment. The research indicates that linoleic acid and *Withania somnifera* extract work as treatments and preventatives through their ability to control oxidative stress and decrease neuroinflammation and maintain metabolic stability.

The safety profile and availability of these agents make them suitable candidates for future preclinical and clinical research into natural neuroprotective strategies with cost-effective potential. Future research needs to study the biological mechanisms of these compounds and perform tissue tests and clinical trials to confirm their effectiveness in treating neurodegenerative diseases.

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