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Effect of ethanolic extract of Ashwagandha and its isolated linoleic acid on the oxidative and reductive biomarkers in rats with induced neurodegeneration

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A B S T R A C T

The purpose of this study is to investigate the effects of an alcoholic extract of Ashwagandha (Withania somnifera) and the fatty acid isolated from it on oxidative and reductive biomarkers in rats whose neurodegenerative disorders were induced by trichloroethylene (TCE). Laboratory animals were divided into eight groups. The first group (negative control) received only distilled water. The second one (positive control) had the disease induced by TCE. The third one was treated with linoleic acid, the fourth with palmitic acid, and the fifth with the alcoholic extract. The sixth group received linoleic acid along with the alcoholic extract. The seventh one received palmitic acid along with the alcoholic extract, and the eighth one received linoleic acid first, followed by the induced disease. The results showed that the TCE-treated rats had a significant decrease in GSH, GPx, and UA and a significant increase in MDA compared to the negative controls. Rats treated with linoleic acid, alcoholic extract, a combination of both, and the pre-treatment linoleic acid group showed noticeable increases in GSH, GPx, and UA and noticeable decreases in MDA compared to the positive control group. Rats treated with palmitic acid showed significant increases in GPx and significant decreases in GSH, MDA, and UA compared to the positive control group. Rats injected with palmitic acid and the extract showed noticeable increases in GSH and GPx and noticeable decreases in MDA and UA compared to the positive control group. Accordingly, this study tried to investigate the neuroprotective effect of Ashwagandha extract and the linoleic acid isolated from it against TCE-induced neurodegeneration in rats.

1 Introduction

Neurodegenerative disorders effect on a large number of people worldwide. Recent studies have revealed that a combination of genetic and environmental factors can contribute to an individual's increased risk of developing them. Neurodegenerative diseases effect on multiple sides of human functioning, limiting the ability to perform basic tasks (e.g., speech, movement, stability, and balance) (Rapp et al.,, 2015). This is because of the loss of neuronal function and subsequent loss of brain function. Common neurodegenerative diseases contain Alzheimer's disease (AD), a complex age-related disease characterized by progressive memory loss (Jorfi et al.,, 2023), and Parkinson's disease (PD), a neurodegenerative movement disorder characterized by the progressive loss of dopaminergic neurons and the formation of Lewy bodies in affected brain regions (Teleanu et al.,, 2022). Mechanisms of degeneration include central nervous system damage, demyelination, neurological dysfunction, and oxidative stress resulting from free radical formation by reactive oxygen species (ROS), associated with mitochondrial dysfunction, microglia activation, and cell death (Kumar et al.,, 2022). Despite increasing efforts to develop appropriate treatments for neurodegenerative diseases, a lot of work remains to be done to find an

effective cure (Teleanu et al.,, 2022). Natural products are a potential reason of novel bioactive compounds that may lead to the development of novel treatments for various diseases (Anand et al.,, 2019). For example, Withania Somnifera (L. Dunal), a medicinal plant belonging to Ayurveda, the traditional Indian medical system, is an evergreen woody shrub of the nightshade family and has diverse therapeutic effects, including anti-inflammatory, anti-cancer, antimicrobial, anti-hepatoprotective, and anti-diabetic properties. Its potential positive effect against neurodegenerative diseases is intriguing, as known medical treatments only delay disease of getting worse and provide symptomatic relief and are not free of side effects. It is considered a regenerative agent because it promotes mental and physical health, revitalizes the body in cases of dementia, and preserves longevity. (W. somnifera) is potentially beneficial for several neurological disorders, such as Alzheimer's and Parkinson's disease. It contains biologically active compounds with numerous pharmacological effects. A study has shown that Ashwagandha extract and its active components possess antioxidant properties and rescue neurons from toxins by regulating antioxidant enzymes, beta-amyloid clearance, neurite growth, calcium influx, lipid peroxidation, inflammation, and other debilitating processes associated with Alzheimer's and Parkinson's diseases (Lerose et al.,, 2024). Therefore, this study aimed to investigate the neuroprotective effect of Ashwagandha extract and its isolated linoleic acid fraction against trichloroethylene-induced neurodegeneration in rats.

2 Materials and Methods

2.1 Ashwagandha roots were obtained from local markets in Nineveh Governorate. The roots were washed with distilled water to remove impurities and then left to dry at room temperature. After drying, the roots were ground using an electric grinder until they became a fine powder. They were then stored in airtight glass containers until ready to use.

Preparing the alcoholic extract of Ashwagandha roots

Weigh 20 grams of Ashwagandha root powder and steep it in 100 ml of 70% ethanol. The mixture was stirred for 24 hours at room temperature using a shaker. The mixture was then filtered using sterile filter paper and placed in a refrigerated centrifuge at 2,500 rpm for 10 minutes to separate fine impurities. It was then transferred to a rotary evaporator to evaporate the solvent under reduced pressure, and the extract was dried at a suitable temperature. The crude extract was scraped using a clean tool, then stored in a sterile, tightly sealed glass container, and refrigerated at 2-4°C until used in the experiment. This method was described by (Dhanani et al.,, 2017).

Isolation of Linoleic Acid

Linoleic acid was measured by reverse-phase chromatography (HPLC) using a Shim-pack C18 chromatography column (250 mm × 4.6 mm) with a fluorescent detector at excitation and emission wavelengths of 265 and 315 nm, respectively. The column temperature was set at 50°C. Gradient separation was used with a mobile phase of acetonitrile-water mixture, with the following ratios: A (85-15%) from 0 to 4 min, B (87-13%) from 5 to 8 min, C (96-4%) from 9 to 14 min, at a flow rate of 1.5 ml/min. Pre-injection LA was obtained by reacting 1 ml of sample with 250 µl of 9-fluorenylmethyl chloroformate solution and 25 µl of sodium phosphate buffer (0.05 M, pH 9.3) at 40°C for 10 min. 100 µl of the mixture was injected into an HPLC device, and the quantity was estimated by comparing the results with the standard pure linoleic acid (Majnooni et al.,, 2016).

Experimental Design

Forty-eight laboratory animals were divided into eight groups and administered trichloroethylene (TCE) orally at a dose of 200 mg/kg for 6 weeks to induce neurodegeneration (Ilieva et al.,, 2022).

The laboratory animals were then dosed with Ashwagandha alcoholic extract at a dose of 300 mg/kg for 4 weeks (Balkrishna et al.,, 2022), palmitic acid at a dose of 0.24 mg/kg for 4 weeks (Ogbaja et al., 2021), and linoleic acid at a dose of 150 µg/kg for 4 weeks (Tofighi et al.,, 2021), according to the groups below.

Group 1: The negative control group, fed only water and protein feed throughout the experiment.

Group 2: The positive control group, fed daily with trichloroethylene (TCE) for 6 weeks to induce neurodegeneration.

Group 3: Received TCE for 6 weeks, then, treated with linoleic acid for 4 weeks.

Group 4: Received TCE, then, treated with palmitic acid for 4 weeks.

Group 5: Received TCE for 6 weeks, then, treated with an alcoholic extract of Ashwagandha for 4 weeks.

Group 6: Received TCE for 6 weeks, then, treated with a combination of the alcoholic extract and linoleic acid for 4 weeks.

Group 7: Received TCE, then, treated with a combination of the alcoholic extract and palmitic acid for 4 weeks.

Group 8: Received linoleic acid for 4 weeks, then, neurodegeneration was induced by TCE administration for 6 weeks.

Estimating Glutathione Concentration in Serum

The concentration of glutathione (GSH) in the blood serum of male laboratory rats was determined using Mann's reagent (dithiobis-2-nitrobenzoic acid) (DTNB). The method relies on its reduction by the SH group in glutathione, forming a yellow-colored compound. The concentration depends on the intensity of the color, which absorbs at a wavelength of 412 nm.(Sedlak et al.,, 1968)

Estimating Malondialdehyde Concentration in Serum

The method relies on the reaction of lipid peroxides, particularly MDA, with Thiobarbituric acid in an acidic medium. The absorbance is measured at a wavelength of 532 nm, which affects its concentration in the sample (Muslih et al.,, 2002).

Estimating Glutathione Peroxidase Concentration in Serum

Glutathione peroxidase catalyzes the formation of oxidized glutathione (GSSG) from reduced glutathione (GSH) and reduces toxic hydrogen peroxide. To non-toxic hydroxyl compounds, GPX catalyzes the oxidation of GSH by hydrogen peroxide to produce GSSG. GSH can react with DTNB to form compounds with characteristic absorption peaks at 412 nm using a Spectrophotometer.

Estimation the concentration of Serum Uric Acid

Uric acid concentration in the serum of male rats was determined by the enzyme-based method using a ready-made analysis kit from the Spanish company Spinreact.

3 Results

Table 1: The experimental groups
different letters indicate the significant differences (P<0.05)

The Groups (Mean + Standard Deviation)								Biomarkers and Units
Group 8	Group 7	Group 6	Group 5	Group 4	Group 3	positive control 2	negative control 1	
2.80 ± 0.27 a	2.84 ± 0.24 a b	3.36 ± 0.47 C b	2.94 ± 0.48 b a	2.44 ± 0.16 a	2.84 ± 0.41 b a	2.57 ± 0.54 a	3.741 ± 0. 42 C	**GSH µmol/l**
2.18 ± 0.17 b d C	2.06 ± 0.30 a b d C	1.67 ± 0.53 a	1.74 ± 0.28 a b	2.26 ± 0.20 d C	1.93 ± 0.33 a b C	2.42 ± 0.56 d	1.88 ± 0.23 a b C	**MDA µmol/l**
4.68 ± 0.37 b C	4.82 ± 0.58 b C d	5.20 ± 0.37 C d	5.41 ± 0.41 d	4.49 ± 0.51 b	4.99 ± 0.36 b C d	3.67 ± 0.51 a	6.0 ± 0.58 e	**GPx U/L**
0.95 ± 0.17 a	0.88 ± 0.37 a	1.41 ± 0.28 b	1.07 ± 0.18 a	0.83 ± 0.10 a	0.98 ± 0.16 a	0.9 ± 0.28 a	1.43 ± 0.47 b a	**UA mg/dl**

4 Discussion

Changes in the levels of Serum Glutathione:

Induction of neurodegenerative disorders in positive control male laboratory rats using TCE resulted in a significant decrease (0.54±2.57 µmol/l) at the probability level (P<0.05) in serum glutathione levels when compared to the negative control group (0.42±3.741 µmol/l). This decrease is attributed to the oxidative stress caused by TCE and the excessive consumption of antioxidants, especially glutathione, resulting in the induced neurological dysfunction (Srivastava et al.,, 2024). The effect of linoleic acid showed a significant increase (0.41 ± 2.84 µmol/l) at the probability level (P>0.05) in serum glutathione levels when compared to the positive control (0.54 ± 2.57 µmol/l). This is attributed to the fact that linoleic acid contributed to reducing oxidative stress resulting from free radicals, as well as activating cells and reducing their death, which led to an increase in its

level in the serum (Lee et al.,, 2022). The palmitic acid group also showed a significant decrease (0.16 ± 2.44 µmol/l) at the probability level (P>0.05) in serum glutathione levels when compared to the positive control (0.54 ± 2.57 µmol/l). This is attributed to the fact that palmitic acid is one of the acids. Saturated fats increase reactive oxygen species, which exacerbates oxidative stress, increases cell damage, and reduces the level of antioxidants such as glutathione (Uchiyama et al.,, 2025).

The alcoholic extract of the plant had a positive effect, showing a significant increase (0.48 ± 2.94 µmol/l) at the probability level (P>0.05) in serum glutathione levels when compared to the positive control (0.54 ± 2.57 µmol/l). This is attributed to the anti-oxidative stress role of the compounds present in the ashwagandha extract, as the extract reduces fatty acid damage, regulates stress stimuli, and promotes balance in the body, which raises glutathione levels (Basudkar et al.,, 2024).

The extract group showed Together, linoleic fatty acid significantly increased serum glutathione levels (470±3.36 µmol/l) at a probability level of (P>0.05) compared to the positive control (0.54±2.57 µmol/l). This is attributed to the fact that the extract is rich in withanolides, which combat oxidative stress, contributing to the formation of antioxidants and maintaining tissue integrity from inflammation associated with oxidative stress (Pullaiah et al.,, 2025). The acid has antioxidant properties, and these properties also contributed to reducing oxidative stress and raising serum glutathione levels (Friend, 2012). The results for the extract and palmitic acid together showed a significant increase (0.24 µmol/l ±2.84) at the probability level (P>0.05) in the serum glutathione level when compared with the positive control group (0.54 ±2.57 µmol/l). This is because palmitic acid intake causes stress and inflammation of astrocytes, which may lead to a decrease in glutathione (Sivasubramanian et al.,, 2024). However, the presence of the extract containing active compounds (Pullaiah et al., 2025), including ferulic acid, which was also measured in the extract, improved the ability to inhibit free radicals resulting from toxins or oxidized fats (palmitic), reflecting a positive effect that contributes to raising the level of glutathione. (Zhai et al.,, 2023). Linoleic acid also showed a significant increase (0.27 µmol/l ± 2.80) at the probability level (P>0.05) in serum glutathione levels, when compared to the positive control (0.54 ± 2.57 µmol/l). This increase is due to the antioxidant properties of the acid, which increase the activity of antioxidants, such as GSH, while reducing Malondialdehyde levels. This indicates a reduction in intracellular oxidative stress (Zhang et al.,, 2024).

Changes in Serum Malondialdehyde levels:

Inducing a neurodegenerative disorder in positive control male laboratory rats using TCE resulted in a significant increase (0.56±2.42 µmol/l) at a probability level of (P>0.05) in serum malondialdehyde levels when compared to the negative control (0.23±1.88 µmol/l). This increase is due to treatment with TCE, which generated reactive oxygen species, which attack the lipid membrane of cells. Malondialdehyde is a clear biological indicator of lipid peroxidation (Lin et al.,, 2022), and its increase indicates the presence of oxidative stress (Mohideen et al.,, 2021). Linoleic fatty acid showed a significant decrease (0.33 µmol/l ±1.93) at the probability level (P>0.05) in the serum malondialdehyde level when compared with the positive control group (0.56 µmol/l ±2.42). This is due to the fact that unsaturated fatty acids, such as linoleic, do not only play a role in building membranes, but also participate in regulating the stressful environment, which reduces the malondialdehyde level (He et al., 2020). As for the effect of palmitic acid, it showed a significant decrease (0.33±2.26 µmol/l) at the probability level (P>0.05) in malondialdehyde levels in serum when compared to the positive control (0.56±2.42 µmol/l). Although palmitic acid is classified as a saturated acid associated with oxidative stress, the results of our study are consistent with studies indicating that low concentrations of palmitic acid can protect against oxidative damage in cells (Palomino et al.,, 2022). The effect of the alcoholic root extract showed a significant decrease (0.28±1.74 µmol/l) at the probability level (P>0.05) in the level of malondialdehyde in the serum when compared with the positive control group (0.56±2.42 µmol/l). This decrease is due to the important antioxidant properties of the biological compounds present in the extract, such as (Withanone), which reduces the accumulation of free radicals and inhibits cellular damage to lipids resulting from oxidative stress, which contributes to reducing the level of malondialdehyde (Sprengel et al.,, 2025).

The effect of Ashwagandha root extract and linoleic acid together showed a significant decrease (1.67 µmol/l 0.56±2.42) at the probability level (P>0.05) in the level of malondialdehyde in the serum when compared to the positive control (0.56 µmol/l 0.56±2.42). The reason for this decrease is that Ashwagandha has antioxidant properties according to its active compounds such as flavonoids that reduce free radicals (Sprengel et al.,, 2025). Linoleic acid, which is classified among omega-6 acids, performs functions according to its concentration and the ratio between it and omega-3 acids, since linoleic acid contributes to oxidation processes, which reduces malondialdehyde levels in the serum (Mariamenatu et al.,, 2021).

The effect of the alcoholic extract and palmitic acid showed a significant decrease (2.06 ± 300 µmol/l) at the probability level (P>0.05) in the serum malondialdehyde level when compared with the positive control group (2.42 ± 0.56 µmol/l). This decrease is attributed to the biological activity of active compounds such as quercetin, which was measured in the alcoholic extract of Ashwagandha, which has an

effective role against oxidative stress and enhances antioxidant activity, leading to a reduction in free radical damage (Namdev et al., 2023). The low concentration of palmitic acid can also provide slight protection against oxidative stress (Palomino et al., 2022). Linoleic acid showed a significant decrease (0.17 ± 2.18 µmol/l) at the probability level (P>0.05) in the serum malondialdehyde level when compared with the positive control (0.56 ± 2.42 µmol/l). This is due to the fact that linoleic acid enhances the fluidity of membrane phospholipids, which limits lipid saturation and its susceptibility to oxidation. Linoleic acid contributes to the activation of the Ascorbate Glutathione Cycle (ascorbic acid and glutathione), which is one of the most important defense systems within the cell, which increases the level of ascorbic acid and glutathione, which in turn neutralizes oxidation processes and reduces malondialdehyde formation (Ou et al.,, 2025).

Changes in Serum Glutathione Peroxidase Levels:

Inducing neurodegenerative disorders in positive control male laboratory rats using TCE resulted in a significant decrease (0.51 ± 3.67 U/L) at a probability level of (P>0.05) in serum glutathione peroxidase levels when compared to the negative control group (0.58 ± 6.0 U/L). Exposure to TCE disrupts the balance within liver cells due to an increase in reactive oxygen species, which reduces the synthesis of glutathione peroxidase. The liver is the organ responsible for regulating antioxidant activity. This confirms the cells' failure to cope with the excessive accumulation of free radicals resulting from exposure to TCE (Lou et al.,, 2024).

As for linoleic acid, it showed a significant increase (0.36 U/L ± 4.99) at a probability level of (P>0.05) in glutathione levels. Serum peroxidase levels were significantly higher when compared to the positive control group (U/L 0.51±3.67). This is attributed to the fact that linoleic acid significantly increased glutathione peroxidase levels, reflecting its antioxidant activity. It has important properties that activate Nuclear Factor Releasing Factor (Nrf2), a regulator of many antioxidant enzymes. In cases of oxidative stress, it is transported to the nucleus and stimulates antioxidants, including glutathione peroxide (Zhang et al., 2024).

Treatment with palmitic acid also showed a significant increase (U/L 0.51±4.49) at the probability level (P<0.05) in serum glutathione peroxidase levels when compared to the positive control group (U/L 0.51±3.67). This is attributed to the fact that palmitic acid is often known to cause cytotoxicity at high concentrations. However, the results of our study indicated that exposure to low doses activates cellular defense against oxidation, improves mitochondrial respiratory capacity, and enhances ATP production, which increases GPx levels. This is consistent with the findings of (Mthembu et al.,, 2024).

The effect of Ashwagandha plant extract revealed a significant increase (U/L 0.41 ± 5.41) at the probability level (P<0.05) in serum glutathione peroxidase levels when compared to the positive control group (0.51 U/L ± 3.67). This is attributed to the extract being rich in numerous phenolic and flavonoid compounds, as we found when identifying some of them, such as gallic acid and rutin, which have the ability to inhibit free radicals and enhance cellular defense against oxidative stress (Khojah et al.,, 2020).

The alcoholic extract of Ashwagandha and linoleic acid also noticeably increased (0.37±5.20 U/L) at the probability level (P<0.05) in serum glutathione peroxidase levels when compared to the positive control (U/L 0.51±3.67). Linoleic acid stimulates the cellular defense pathway (Yang et al.,, 2020). The plant roots also contain a group of active alkaloid compounds and withanolides, such as withasomnine, withanolide A, and withanosides, as well as compounds such as β-sitosterol and D-glucoside. These compounds are responsible for their stronger activity against oxidative stress and increase glutathione peroxidase levels (Wiciński et al.,, 2024). The plant extract and palmitic acid combination showed a significant increase (U/L 0.580±4.82) at the probability level (P<0.05) in serum glutathione peroxidase levels when compared to the control. Compared to the positive control group (U/L 0.51 ± 3.67), this is due to the presence of withanolides and Withaferin A in the extract, which are compounds responsible for combating free radicals (Wiciński et al., 2024). Moderate concentrations of saturated palmitic acid may provide cellular protection against oxidative stress. This interaction between the compounds may increase the level of antioxidants such as glutathione peroxidase (Palomino et al., 2022). Finally, linoleic acid has an effect on glutathione peroxidase, showing a significant increase (U/L 0.37 ± 4.68) at the probability level (P<0.05) in serum glutathione peroxidase levels when compared to the positive control group (0.51 U/L ± 3.67). This increase after treatment with linoleic acid is attributed to its ability to activate the antioxidant pathway, which stimulates the production of antioxidant enzymes for oxidation such as GPx (Yang et al., 2020).

Changes in the levels of Serum Uric Acid:

Inducing neurodegenerative disorders in positive control male laboratory rats using TCE resulted in a significant decrease (0.28±0.9 mg/dl) at the probability level (P<0.05) in serum uric acid levels when compared to the negative control (0.47±1.43 mg/dl). Exposure to TCE leads to increasing of the free radical production, in addition to high and severe oxidative stress (Srivastava et al., 2024). Uric acid is an essential natural antioxidant, as it works to remove and combat free radicals, especially reactive oxygen species (ROS). This decrease is explained by the consumption of the acid to combat the oxidative stress caused by TCE (Mahomoodally et al.,, 2022). The groups treated with linoleic acid, Ashwagandha alcohol extract, and the protective linoleic acid group, respectively, showed a non-significant increase (0.16 mg/dl ± 0.98), (0.18 ± 1.07 mg/dl), (0.17 ± 0.95 mg/dl) at the probability level (P>0.05) in uric acid levels in the serum of laboratory rats when compared with the positive control group

(0.28 ± 0.9 mg/dl), in which neurodegeneration was induced. The palmitic acid and alcoholic extract groups together and palmitic acid alone showed a non-significant decrease (mg/dl 0.37±0.88), (mg/dl 0.10±0.83) at the probability level (P>0.05) in the uric acid levels in the blood serum of laboratory rats when compared with the positive control group (mg/dl 0.28±0.9) in which neurodegeneration was induced. Treatment with the alcoholic extract of Ashwagandha and linoleic acid together showed a noticeable increase (0.28 mg/dl ± 1.41) at the probability level (P>0.05) in serum uric acid levels when compared to the positive control (0.28 mg/dl ± 0.9). This is due to the treatment with the alcoholic extract, which resulted in an increase in uric acid levels in the rats' serum. This is due to the ability of the plant compounds to improve kidney function, reduce this induced toxicity, and regulate uric acid secretion (Vedi et al.,, 2014). Linoleic acid also has an indirect function that contributes to raising uric acid levels, as it increases uric acid secretion, acting as an antioxidant against lipid peroxidation resulting from unsaturated acids such as linoleic acid (Guelcin et al.,, 2008).

5 Conclusions

This study showed that Ashwagandha root extract has a neuro-therapeutic effect against neurodegeneration caused by trichloroethylene (TCE) used in laboratory rats. This is due to the existing of bioactive substances. This therapeutic effect appears through combating oxidative stress and improving its biomarkers. The best results were obtained when using the alcoholic extract and the isolated linoleic acid from these together, which indicates the possibility of considering this combination as a natural therapeutic agent for neurodegenerative disorders with further clinical testing.

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