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Comprehensive In Vitro Evaluation of the Antioxidant Potential of *Thuja occidentalis* Leaf Methanolic Extract

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Abstract

Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) production and endogenous antioxidant defense mechanisms, contributes significantly to the development of numerous chronic disorders, including cardiovascular diseases, neurodegenerative disorders, diabetes mellitus, inflammation, and cancer. Plant-derived antioxidants have attracted considerable attention as safer alternatives to synthetic antioxidants because of their diverse bioactive phytochemicals and reduced toxicity. *Thuja occidentalis* L. (family Cupressaceae), commonly known as Northern White Cedar or Arborvitae, has long been employed in traditional medicine owing to its antimicrobial, anti-inflammatory, immunomodulatory, and pharmacological properties. However, comprehensive evaluation of its antioxidant potential remains comparatively limited.The present investigation aimed to assess the in vitro antioxidant activity of methanolic leaf extract of *T. occidentalis* using multiple free radical scavenging assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nitric oxide (NO), and lipid peroxidation inhibition assays. Furthermore, the total phenolic content (TPC) and total flavonoid content (TFC) of the extract were quantified using the Folin–Ciocalteu and aluminium chloride colorimetric methods, respectively.The methanolic extract demonstrated concentration-dependent radical scavenging activity in all antioxidant assays, indicating a substantial capacity to neutralize reactive free radicals. The extract also exhibited appreciable phenolic and flavonoid contents, suggesting that polyphenolic constituents substantially contribute to its

antioxidant efficacy. The observed antioxidant potential highlights the therapeutic value of *T. occidentalis* as a promising source of naturally occurring antioxidant compounds.

Keywords: *Thuja occidentalis*; antioxidant activity; DPPH; ABTS; nitric oxide scavenging; lipid peroxidation; total phenolic content; flavonoids; phytochemicals; medicinal plants

1. Introduction

Oxidative stress is recognized as one of the major biochemical factors involved in the initiation and progression of numerous chronic and degenerative diseases. It develops when the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) exceeds the antioxidant defense capacity of biological systems, resulting in oxidative damage to lipids, proteins, carbohydrates, and nucleic acids. Persistent oxidative stress contributes to aging, cardiovascular diseases, diabetes mellitus, neurodegenerative disorders, chronic inflammation, and several forms of cancer (Liguori et al., 2018; Sharifi-Rad et al., 2020).

Living organisms possess endogenous antioxidant defense systems comprising enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase, together with non-enzymatic antioxidants including glutathione, uric acid, and vitamins C and E. However, excessive ROS generation due to environmental pollution, ultraviolet radiation, smoking, unhealthy dietary habits, pesticide exposure, and metabolic abnormalities often overwhelms these defense mechanisms. Consequently, supplementation with exogenous antioxidants has emerged as an effective strategy for reducing oxidative damage and maintaining cellular homeostasis (Forman & Zhang, 2021).

Natural antioxidants obtained from medicinal plants have gained increasing scientific interest because they exhibit broad-spectrum biological activities while generally producing fewer adverse effects than synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Polyphenols, flavonoids, phenolic acids, tannins, terpenoids, and other secondary metabolites possess remarkable antioxidant properties by scavenging free radicals, chelating transition metal ions, inhibiting lipid peroxidation, and regulating cellular antioxidant signaling pathways (Shahidi & Ambigaipalan, 2015; Salehi et al., 2021).

Among medicinal plants, *Thuja occidentalis* L. (Cupressaceae), commonly referred to as Northern White Cedar or Arborvitae, occupies a prominent position in traditional herbal medicine. Native to North America and extensively cultivated throughout Europe and Asia as an ornamental and medicinal species, *T. occidentalis* has been traditionally employed for the management of respiratory disorders, urinary tract

infections, rheumatic conditions, skin diseases, viral infections, and inflammatory disorders. Modern pharmacological investigations have demonstrated antimicrobial, antiviral, antifungal, anti-inflammatory, immunomodulatory, anticancer, hepatoprotective, and wound-healing properties of different extracts and phytoconstituents isolated from this species (Naser et al., 2022; Salehi et al., 2019).

Phytochemical investigations have revealed that *T. occidentalis* contains numerous bioactive compounds, including flavonoids, phenolic acids, tannins, lignans, diterpenes, monoterpenes, sesquiterpenes, essential oils, polysaccharides, and thujone derivatives. These metabolites are capable of donating electrons or hydrogen atoms to neutralize reactive radicals, thereby minimizing oxidative injury at the cellular level. Increasing evidence indicates that phenolic compounds and flavonoids are primarily responsible for the antioxidant activity exhibited by medicinal plants (Sharma et al., 2023).

Although several studies have reported individual biological properties of *T. occidentalis*, systematic evaluation of its antioxidant potential using multiple complementary in vitro assays remains comparatively limited. Since different free radicals exhibit distinct reaction mechanisms, assessment through a combination of DPPH, ABTS, nitric oxide scavenging, and lipid peroxidation inhibition assays provides a more comprehensive understanding of antioxidant behavior than reliance on a single analytical method.

Therefore, the present study was designed to comprehensively investigate the in vitro antioxidant potential of methanolic leaf extract of *Thuja occidentalis*. In addition to evaluating radical scavenging activity using four established antioxidant assays, the study quantified total phenolic and flavonoid contents to establish a relationship between phytochemical composition and antioxidant capacity. The findings are expected to provide valuable scientific evidence supporting the utilization of *T. occidentalis* as a potential natural source of antioxidant compounds for pharmaceutical, nutraceutical, and functional food applications.

2. Materials and Methods

2.1 Plant Material Collection and Authentication

Fresh, healthy, and disease-free leaves of *Thuja occidentalis* L. were collected from a well-maintained medicinal plant garden during the active vegetative growth period. The collected specimens were transported to the laboratory in sterile polyethylene bags and thoroughly washed with running tap water followed by distilled water to remove adhering dust and contaminants. Botanical identification and taxonomic authentication were performed by a qualified plant taxonomist using standard floristic keys. A

voucher specimen was prepared and deposited in the departmental herbarium for future reference according to internationally accepted herbarium procedures (Bridson & Forman, 1999; Funk et al., 2005).

2.2 Preparation of Plant Extract

The cleaned leaves were shade-dried at ambient temperature (25–30°C) for 10–14 days until constant weight was achieved. The dried material was pulverized into a fine powder using an electric grinder and stored in airtight containers protected from moisture and light.

Approximately **100 g** of powdered leaf material was subjected to Soxhlet extraction using analytical-grade methanol (1:10, w/v) for **6–8 h** until complete extraction was achieved. The obtained extract was concentrated under reduced pressure using a rotary vacuum evaporator at **40°C**, followed by drying to constant weight. The dried methanolic extract was preserved in amber-colored screw-capped bottles at **4°C** until further analysis.

2.3 Chemicals and Reagents

Methanol, Folin–Ciocalteu reagent, gallic acid, quercetin, aluminium chloride, sodium carbonate, potassium acetate, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate, sodium nitroprusside, Griess reagent, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ferrous sulfate ($FeSO_4$), and all other analytical-grade chemicals were procured from Sigma-Aldrich (USA) or HiMedia Laboratories (India).

2.4 Determination of Total Phenolic Content (TPC)

The total phenolic content of the methanolic extract was determined using the **Folin–Ciocalteu colorimetric method** with slight modifications.

Briefly, **0.5 mL** of appropriately diluted extract was mixed with **2.5 mL** of **10% Folin–Ciocalteu reagent**. After incubation for **5 min**, **2.0 mL of 7.5% sodium carbonate** solution was added. The reaction mixture was incubated at room temperature for **30 min** in darkness, and absorbance was measured at **765 nm** using a UV–Visible spectrophotometer.

Gallic acid (20–200 µg/mL) was used for calibration, and results were expressed as **milligrams of gallic acid equivalents (mg GAE/g dry extract)**.

2.5 Determination of Total Flavonoid Content (TFC)

The total flavonoid content was estimated using the **aluminium chloride colorimetric assay**.

Briefly, **0.5 mL** of plant extract was mixed with **1.5 mL methanol**, **0.1 mL of 10% aluminium chloride**, **0.1 mL of 1 M potassium acetate**, and **2.8 mL distilled water**. The reaction mixture was incubated at room temperature for **30 min**, after which absorbance was measured at **415 nm**.

Quercetin served as the reference standard, and flavonoid content was expressed as **milligrams of quercetin equivalents (mg QE/g dry extract).**

2.6 DPPH Radical Scavenging Assay

The antioxidant capacity of the methanolic extract was evaluated using the **DPPH free radical scavenging assay** following the method of Brand-Williams et al. (1995) with minor modifications.

A freshly prepared **0.1 mM DPPH** solution in methanol was mixed with different concentrations of plant extract (0.125–1.0 mg/mL). The mixtures were incubated in darkness for **30 min** at room temperature before absorbance was recorded at **517 nm**.

The percentage radical scavenging activity was calculated using:

$$\text{Radical Scavenging Activity (\%)} = Ac - As / Ac \times 100$$

Ac = absorbance of control

As = absorbance of sample

The **IC_{50} value** was determined from the concentration-response curve.

2.7 ABTS Radical Cation Scavenging Assay

ABTS radical scavenging activity was determined according to the method described by Re et al. (1999).

The ABTS radical cation was generated by reacting **7 mM ABTS** with **2.45 mM potassium persulfate** and allowing the mixture to stand in darkness for **12–16 h** at room temperature.

Before analysis, the ABTS solution was diluted with methanol to obtain an absorbance of **0.70 ± 0.02 at 734 nm**.

Different concentrations of plant extract were mixed with freshly prepared ABTS solution, incubated for **6 min**, and absorbance was measured at **734 nm**.

The percentage inhibition and IC_{50} values were calculated.

2.8 Nitric Oxide Radical Scavenging Assay

Nitric oxide scavenging activity was evaluated according to the method described by Green et al. (1982).

Sodium nitroprusside (10 mM) prepared in phosphate-buffered saline (PBS, pH 7.4) was mixed with different concentrations of the plant extract and incubated at **25°C for 150 min**.

After incubation, an equal volume of freshly prepared Griess reagent was added, and absorbance was measured at **546 nm**.

The nitric oxide scavenging percentage was calculated using the standard equation described above.

2.9 Lipid Peroxidation Inhibition Assay

The anti-lipid peroxidation activity of the extract was determined using chicken liver homogenate as the lipid-rich biological substrate.

Fresh liver tissue was homogenized in chilled phosphate-buffered saline and centrifuged at **3000 rpm for 10 min** at **4°C**.

Aliquots of liver homogenate were incubated with various concentrations of plant extract and **$FeSO_4$** to induce lipid peroxidation.

Following incubation, **TBA-TCA reagent** was added, and the reaction mixture was heated at **95°C for 20 min**.

After cooling, absorbance was recorded at **532 nm**, and inhibition of malondialdehyde (MDA) formation was calculated.

2.10 Statistical Analysis

All experiments were performed in **triplicate (n = 3)**, and results were expressed as **mean ± standard deviation (SD).**Statistical analysis was carried out using **GraphPad Prism 10.0**.Differences were considered statistically significant at **$p < 0.05$**.

3. Results and Discussion

The antioxidant efficacy of the methanolic leaf extract of *Thuja occidentalis* was comprehensively evaluated using four complementary in vitro assays, namely DPPH radical scavenging, ABTS radical cation scavenging, nitric oxide (NO) radical scavenging, and lipid peroxidation inhibition. In addition, the total phenolic content (TPC) and total flavonoid content (TFC) were determined to investigate the relationship between phytochemical composition and antioxidant activity.

3.1 DPPH Radical Scavenging Activity

The methanolic extract of *T. occidentalis* exhibited a concentration-dependent increase in DPPH free radical scavenging activity (Figure 1a). As the extract concentration increased from 0.125 to 0.5 mg/mL, the percentage inhibition increased significantly ($p < 0.05$), ranging from **13.15%** to **50.88%**. The IC_{50} value was calculated as **0.49 mg/mL**, indicating a moderate free radical scavenging capacity.The DPPH assay is widely employed to evaluate the hydrogen atom- or electron-donating ability of phytochemicals.

The observed antioxidant activity may be attributed to the presence of phenolic compounds and flavonoids, which effectively neutralize DPPH radicals through electron transfer mechanisms. Similar findings have been reported for *T. occidentalis* extracts, although variations in antioxidant capacity among studies may result from differences in extraction solvent, geographical origin, plant maturity, and phytochemical composition (Dubey & Batra, 2009; Salehi et al., 2021).

3.2 ABTS Radical Scavenging Activity

The methanolic extract also demonstrated significant scavenging activity against ABTS radicals (Figure 1b). The percentage inhibition increased from **19.81%** at 0.125 mg/mL to **54.98%** at 0.5 mg/mL, showing a statistically significant concentration-dependent response ($p < 0.05$). The calculated IC_{50} value was **0.45 mg/mL**, suggesting an efficient capacity of the extract to quench ABTS radical cations.The ABTS assay complements the DPPH method because it measures the scavenging ability of both hydrophilic and lipophilic antioxidants. The superior ABTS scavenging activity observed in the present investigation may be associated with the diverse spectrum of antioxidant phytoconstituents present in the methanolic extract, particularly polyphenols capable of donating electrons and stabilizing radical species.

3.3 Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging activity increased progressively with increasing extract concentration (Figure 1c). The inhibition percentage ranged from **17.28%** at **0.25 mg/mL** to **59.75%** at **1.0 mg/mL**, with statistically significant differences among the tested concentrations ($p < 0.05$). The IC_{50} value was estimated to be **0.73 mg/mL**.Nitric oxide is an important physiological signaling molecule; however, excessive production contributes to oxidative stress and inflammatory tissue damage through the formation of reactive nitrogen species such as peroxynitrite. Therefore, compounds capable of scavenging nitric oxide radicals may possess considerable therapeutic potential. The present findings indicate that the methanolic extract of *T. occidentalis* effectively suppresses nitric oxide radicals, which may be attributed to its phenolic and flavonoid constituents known for their antioxidant and anti-inflammatory activities.

3.4 Lipid Peroxidation Inhibition

The methanolic extract exhibited substantial inhibitory activity against lipid peroxidation (Figure 1d). The percentage inhibition increased significantly from **10.98%** at **0.125 mg/mL** to **57.85%** at **0.5 mg/mL**, yielding an IC_{50} value of **0.42 mg/mL**.Lipid peroxidation is a major consequence of oxidative stress that leads to structural and functional damage of biological membranes through oxidative degradation of membrane lipids. The inhibition of lipid peroxidation observed in the present study indicates that *T.*

occidentalis possesses the ability to protect membrane lipids against free radical-mediated oxidative damage. This protective effect is likely associated with the radical scavenging and metal-chelating properties of its naturally occurring phenolic compounds.

Overall Antioxidant Performance

Collectively, the methanolic leaf extract of *Thuja occidentalis* demonstrated appreciable antioxidant activity in all four in vitro assays. The extract showed concentration-dependent free radical scavenging and lipid peroxidation inhibitory effects, confirming its potential as a natural antioxidant source. Among the investigated assays, lipid peroxidation inhibition exhibited the lowest IC_{50} value (0.42 mg/mL), followed by ABTS (0.45 mg/mL), DPPH (0.49 mg/mL), and nitric oxide scavenging (0.73 mg/mL), indicating differential antioxidant efficiencies depending on the radical system employed.The observed antioxidant activity is likely attributable to the synergistic interaction of multiple phytochemicals, including phenolic acids, flavonoids, tannins, and terpenoid constituents present in the methanolic extract. These compounds are recognized for their capacity to donate hydrogen atoms or electrons, chelate transition metal ions, interrupt free radical chain reactions, and enhance endogenous antioxidant defense mechanisms. Consequently, *T. occidentalis* may represent a promising natural source of bioactive compounds for developing antioxidant-based nutraceuticals and phytopharmaceuticals. Nevertheless, further phytochemical characterization, isolation of active constituents, mechanistic investigations, and in vivo studies are required to validate its therapeutic potential.

Results and Discussion

The methanolic leaf extract of *Thuja occidentalis* exhibited appreciable antioxidant activity across all in vitro assays, demonstrating its ability to effectively neutralize free radicals in a concentration-dependent manner. In the present investigation, the extract showed notable radical scavenging efficiency, which is consistent with previous reports on the antioxidant potential of *T. occidentalis*. Earlier studies have reported a DPPH radical scavenging activity of **61.52%** at a concentration of **0.3 mg/mL**, with an **IC_{50} value of 195.60 µg/mL**, confirming the strong free radical quenching capacity of this medicinal species (Dubey & Batra, 2009). Variations in antioxidant activity among different studies may be attributed to differences in extraction solvents, geographical origin, seasonal variations, plant age, and phytochemical composition.

The total phenolic content (TPC) of the methanolic extract was quantified using a gallic acid calibration curve and was determined to be **135.32 mg gallic acid equivalents (GAE)/g dry extract** (Figure 3a).

This value is considerably higher than the **78.08 mg GAE/g** previously reported for *T. occidentalis*, indicating that the extraction conditions employed in the present study may have enhanced the recovery of phenolic constituents. Phenolic compounds are recognized as major contributors to antioxidant activity owing to their ability to donate hydrogen atoms or electrons, scavenge reactive oxygen species, and chelate transition metal ions, thereby protecting biomolecules from oxidative damage (Shahidi & Ambigaipalan, 2015; Salehi et al., 2021).Similarly, the total flavonoid content (TFC), estimated using the aluminium chloride colorimetric method with quercetin as the reference standard, was **3.46 mg quercetin equivalents (QE)/g dry extract** (Figure 3b). Although this value is lower than the **7.50 mg QE/g** reported in earlier investigations, such differences are expected because flavonoid accumulation is strongly influenced by environmental conditions, geographical location, harvesting season, plant maturity, and extraction methodology. Flavonoids constitute an important class of natural antioxidants capable of inhibiting lipid peroxidation and neutralizing reactive oxygen and nitrogen species through electron transfer and hydrogen atom donation mechanisms.Overall, the findings demonstrate that the methanolic extract of *T. occidentalis* represents a promising natural source of antioxidant phytochemicals. The pronounced antioxidant activity observed in this study is likely associated with the synergistic effects of phenolic compounds, flavonoids, and other secondary metabolites present in the extract. These bioactive constituents may contribute to cellular protection against oxidative stress and support the traditional medicinal applications of *T. occidentalis*. Nevertheless, further investigations involving phytochemical profiling using advanced analytical techniques such as HPLC–MS/MS or LC–QTOF–MS, bioactivity-guided fractionation, toxicity assessment, and well-designed in vivo studies are warranted to identify the active constituents, elucidate their molecular mechanisms of action, and validate their therapeutic potential for pharmaceutical and nutraceutical applications.

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