Original Research Article

Unraveling the Medicinal Properties of *Tinospora cordifolia* in Chicken Lymphocytes: Antioxidant and Immunomodulatory Perspectives

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Abstract

Medicinal plants have been integral to traditional medicine for centuries, offering natural and cost-effective solutions for various ailments. The bioactive compounds, such as alkaloids, flavonoids, phenolics, terpenoids, and glycosides, exhibit diverse biological activities, that include antioxidant, anti-inflammatory, antimicrobial and anticancer properties. These compounds play a vital role in neutralizing free radicals, reducing oxidative stress and maintaining cellular health. *Tinospora cordifolia* (Giloy), a climber from the Menispermaceae family, is revered in Ayurveda as "Amrita" or the "root of immortality." Known for its immunomodulatory and therapeutic properties, it has been traditionally used to treat conditions like fever, inflammation, respiratory disorders, and metabolic diseases. This study evaluates the antioxidant and immunomodulatory effects of a 50% hydromethanolic extract of *Tinospora cordifolia* stems (TCE) using chicken lymphocytes. The antioxidant activity of TCE was assessed through various *in vitro* assays and the Maximum Non-Cytotoxic Dose (MNCD) was determined using the MTT assay. Immunomodulatory effects were evaluated via the Lymphocyte Proliferation Assay (LPA) and the antioxidant status of TCE-treated lymphocytes was analysed using cell-based assays. Results showed that TCE exhibited significant antioxidant activity and enhanced B and T cell proliferation in mitogen-stimulated lymphocytes. These findings highlight the therapeutic potential of *Tinospora cordifolia*, which can be attributed to its rich phytochemical composition and robust biological activities. The study validates its traditional uses and supports its application in developing nutraceuticals and functional foods. Future research should focus on identifying bioactive compounds, elucidating underlying mechanisms and conducting clinical studies to optimize its utilization in modern healthcare systems.

Keywords: *Tinospora cordifolia*; Immunomodulatory; Antioxidants; Lymphocytes; Chicken

1. Introduction

Medicinal plants have served as natural remedies since prehistoric times due to their therapeutic chemical components [1]. India's extensive biodiversity and ancient medical traditions (Ayurveda, Siddha, Unani, Amchi, and local practices) provide comprehensive knowledge for using plants in healthcare [2]. The natural process of oxidation generates energy necessary for biological cycles but also leads to the formation of free radicals or reactive oxygen species (ROS). When free radicals like hydroxyl radicals and hydrogen peroxide are produced excessively and uncontrollably, they can harm the body and contribute to oxidative stress [3]. Reliance on phytomedicines for their antioxidant and medicinal properties is currently at an unprecedented level. These natural products are increasingly preferred over synthetic antioxidants or chemicals due to safety concerns. Plants' major constituents - polyphenols and flavonoids - possess antioxidant and medicinal properties, working to inhibit or suppress free radical redox reactions in biological systems. It notes that numerous researchers have documented the importance of herbal medicine in treating various diseases, with herbal treatments remaining widely practiced due to their accessibility and cost-effectiveness [4].

The poultry industry is undergoing significant growth, contributing extensively to the global economy. Poultry products, such as meat and eggs, serve as vital sources of protein, ensuring food security, driving economic growth, and supporting the livelihoods of farmers [5]. Various climatic factors influence animal farming systems, with environmental stress gaining particular attention in poultry farming due to growing public awareness and abundant scientific research [6]. Changes in environmental variables such as sunlight, temperature, humidity and metabolism can disrupt the physiological balance of poultry, increasing their susceptibility to environmental pathogens [7]. To meet the rising global demand for food, enhancing poultry growth rates and preventing infectious diseases are critical. However, the extensive use of antibiotics in poultry feed has raised concerns about the emergence of antibiotic-resistant pathogens, which negatively affect poultry performance and pose potential risks to human health. As a result, alternative solutions to antibiotic growth promoters are being explored. These include herbal plants, probiotics, fruit by-products, organic acids, nanomaterials, and combinations of phytogenic feed additives. Natural herbal plants offer significant health benefits compared to synthetic drugs, while probiotics act as feed additives that improve digestion, strengthen immunity, and extend the freshness and shelf-life of food products. Similarly, organic acids function as natural preservatives and hygiene enhancers, inhibiting microbial growth and ensuring the quality and longevity of poultry products [8,9].

Tinospora cordifolia (commonly known as Giloy) is a medicinal plant used in both Ayurvedic and Unani systems. Belonging to the family Menispermaceae, it is also referred to as Guduchi, Heart-leaved moonseed, Amrita, Gurach, and Tinospora [10]. This plant grows in tropical regions, particularly in India, Sri Lanka, Bangladesh, and China [11,12]. *Tinospora cordifolia* is a glabrous, succulent, and woody climbing shrub that flourishes in tropical areas, often reaching considerable heights as it ascends the trunks of large trees [12]. The plant is characterized as a large deciduous, extensively spreading climbing shrub with several coiled branches and distinct morphology. Its filiform, fleshy stem is rich in minerals like copper, calcium, phosphorus, iron, zinc, and manganese, making it valuable for treating metabolic disorders. The plant features white to gray bark, and its stem powder, known as "Guduchi-satva," has distinctive characteristics including a creamish to dark brown colour and bitter taste [13,14]. Various compounds have been identified in *Tinospora cordifolia*, including alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoids, phenolics,

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aliphatic compounds, and polysaccharides [15]. Traditionally used in Ayurvedic medicine, this plant is valued for its therapeutic applications in treating conditions such as jaundice, rheumatism, urinary disorders, skin diseases, diabetes, anaemia, inflammation, allergies, and for its radioprotective and anti-periodic properties [1]. *Tinospora cordifolia* demonstrates various medicinal effects, including anti-periodic, anti-spasmodic, anti-inflammatory, immunomodulatory, anti-tumor, anti-allergic, and antipyretic activities, with strong antioxidant effects found in extracts of its stem, bark, roots, and leaves [16]. Tripathi *et al.*[17] noted that the root of *Giloy* serves as an effective laxative and supports bowel health. The plant's starch is used domestically to address chronic fever, relieve burning sensations, and improve energy and appetite levels. Additionally, its roots and stems are sometimes utilized as part of treatments for snake and scorpion bites [18]. The immunomodulatory benefits of three *Tinospora* species in the form of guduchi-satwa—a recognized dosage form—have been established [19]. This study, therefore, aims to investigate the antioxidant and immunomodulatory potential of a hydromethanolic extract of *Tinospora cordifolia* stems in chicken splenocytes culture system.

2. Materials and Methods

2.1. Collection of Plant sample and Extract Preparation

The stems of *Tinospora cordifolia* were sourced from the Medicinal Plants Research and Development Centre (MRDC) located at G. B. Pant University of Agriculture and Technology in Pantnagar, Uttarakhand. Dr. D. S. Rawat, Assistant Professor, Department of Biological Sciences, College of Basic Sciences and Humanities, authenticated the plant material (Voucher specimen No. 1635). Following collection, the stems were processed to create a 50% hydromethanolic extract (TCE) using the methodology described by Deb *et al.*[20].

2.2. Phytochemical Analysis of TCE

Presence of phytoconstituents in TCE was determined via different quantitative analyses.

2.2.1. Total Phenolic Content

Total phenolic content was measured by following the Folin-Ciocalteu protocol established by Ben Mansour *et al.*[21]. The procedure involved mixing sample extract (100 µl), Folin reagent (250 µl), and 20% sodium carbonate solution (500 µl), then diluting to 5 ml with water. After a 30-minute incubation period at room temperature, absorbance readings were taken at 765 nm using a spectrophotometer. Results were expressed as gallic acid equivalents (GAE), calculated using a gallic acid standard curve.

2.2.2. Total Flavonoid Content

The measurement of total flavonoid content was done using a colorimetric method established by Ben Mansour *et al.*[21]. The analysis involved a sequential process: first combining 1 mL of diluted sample with 4 mL water and 300 µL of 5% sodium nitrite solution. After a 5-minute incubation, 300 µL of 10% aluminum chloride was introduced. Following 6-minute incubation, 2 mL of 1M sodium hydroxide was added, and the solution was immediately brought to 10 mL with water. Absorbance measurements were taken at 510 nm, and the results were expressed as rutin equivalents, calculated using a rutin standard calibration curve.

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2.3. *In Vitro* Antioxidant Assays

2.3.1. Total Antioxidant Capacity

The antioxidant properties of the *Tinospora cordifolia* extract (TCE) were assessed using the methodology described by Sharma and Singh [22]. The process involved mixing 0.1 ml of the sample solution with 1 ml of a reagent mixture comprising 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The prepared solutions were sealed in tubes and heated in a boiling water bath at 95ºC for 90 minutes. After cooling to room temperature, the absorbance of each aqueous solution was measured spectrophotometrically at 695 nm. The results were quantified in terms of ascorbic acid equivalents, expressed as milligrams of ascorbic acid per gram of dried extract.

2.3.2. Ferrous Reducing Antioxidant Power Assay (FRAP)

The FRAP was performed using a method of Meena *et al.*[23]. From the stock concentrations, 10 µL was taken from each concentration and 1.5 mL FRAP reagent (10:1:1 of 300 mM Sodium Acetate buffer: 20 mM FeCl3: freshly prepared TPTZ in 40 mM HCl), and finally volume was rendered up to 5 mL by adding distilled water. After that, the reaction mixture was allowed to incubate for 30 min at room temperature in a dark room. At 593 nm, absorbance was measured. TCE concentration was given in the form of ascorbic acid equivalents, expressed as milligrams of ascorbic acid per gram of dried extract.

2.3.3. Metal Ion Chelation Assay

Metal chelating activity was measured by the method described by Adusei *et al.*[24]. 0.1 mM FeSO4 and 0.25 mM of ferrozine, forming an Fe^{2+}-ferrozine complex, were subsequently added into 0.2 mL of the extract. After incubation at room temperature for 10 min, absorbance of the mixture was recorded at 562 nm. EDTA was used as a positive control.

2.3.4. Reducing Power Capacity

The reducing power of TCE was analyzed using Nićiforović *et al.*'s [25] method. TCE and standard in different concentrations were combined with sodium phosphate (pH 6.6, 2.5 ml) and 1% potassium ferricyanide (2.5 ml), then incubated at 50°C. After adding 2.5 ml of 10% trichloroacetic acid, the mixture was centrifuged, and the top layer was mixed with 1 ml of 0.1% of ferric chloride. Absorbance was measured at 700 nm, with higher absorbance indicating greater reducing power.

2.3.5. DPPH Radical Scavenging Assay

The antioxidant activity of the extract was assessed using a modified DPPH method from Do *et al.*[26]. Varying concentrations of the extract (2.5 mL) were combined with a methanolic DPPH solution (2.5 mL, 0.3 mM) and incubated in the dark for 20 minutes at room temperature. The reduction in absorbance was then recorded at 517 nm using a UV-VIS spectrophotometer.

2.3.6. NO Scavenging Assay

The NO scavenging activity of TCE was evaluated following the method of Jagetia *et al.*[27]. Different concentrations of the extract were combined with 5 mM sodium nitroprusside in phosphate-buffered saline and incubated at 25°C for 150 minutes. The resulting samples were then reacted with Griess reagent, and absorbance was measured at 546 nm, using potassium nitrite standard solutions processed similarly with Griess reagent for reference.

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2.3.7. Superoxide Anion Radical Scavenging Assay

This activity was measured by the reduction of NBT according to a method reported by Iqbal *et al.*[28]. 1 mL of reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM), and various concentrations of plants extracts. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated.

2.3.8. Hydroxyl Radical scavenging Assay

Following Rathee *et al.*[29], this assay measured chromogen production to assess antioxidant activity. A reaction mixture with 2- deoxyribose (2.8 mM), Fe (III) chloride (20 lM), EDTA (100 lM) (EDTA and Fe (III) chloride were mixed prior to the addition of 2-deoxyribose) and H_2O_2 (200 lM) without or with different concentrations of TCE in 10 mM potassium phosphate buffer (pH 7.4) was initiated by adding ascorbic acid (final concentration 300 µM) and incubated at 37°C for an hour. Afterward, TBA in 50 mM NaOH (1 ml, 1% w/v) and TCA (1 ml, 2.8% w/v aqueous solution) were added, the mixture was heated, and chromogen formation was measured at 532 nm.

2.4. Collection of Spleens

The study utilized spleens obtained from healthy broilers aged 5-7 weeks of age. These spleens were collected at a local slaughterhouse and immediately preserved in sterile Dulbecco's phosphate buffer saline (DPBS). Following standard protocols and maintaining sterile conditions, the spleen tissues were immediately processed to extract lymphocytes.

2.5. Lymphocytes Extraction from Chicken Spleens

Lymphocytes were isolated from chicken spleens following Ambwani *et al.*[30] protocol. Spleens were minced in RPMI-1640 medium, and density gradient centrifugation was used to separate the lymphocytes. The cell suspension was layered on lymphocyte separation media (density 1.077 g/ml) and centrifuged at 400 x g for 30 minutes. Lymphocytes were then collected, washed twice with DPBS at 250 x g for 5 min at room temperature, and resuspended in RPMI-1640. A trypan blue exclusion test confirmed over 95% cell viability.

2.6. Estimation of Maximum Non-Cytotoxic Dose (MNCD)

To determine the maximum non-cytotoxic dose (MNCD) of TCE, chicken lymphocytes (1×10^6 cells/ml) were placed in a 96-well plate and exposed to various TCE concentrations (15–1000 µg/ml) in triplicate. Cells were incubated for 68 hours at 40°C with 5% CO_2, after which cytotoxicity was assessed. Following incubation, the media from each well was carefully removed. Then 20 µl of MTT solution (5 mg/ml stock) was added, and the plate was further incubated in the dark for 4 hours. Formazan crystals formed were dissolved in DMSO, and absorbance at 570 nm was measured using an ELISA Reader. Cell survival rate was expressed as a percentage by comparing treated and control cells.

2.7. Lymphocytes Proliferation Assay

The lymphocyte proliferation assay (LPA) was conducted according to the protocol by Ambwani *et al.*[30]. Lymphocytes were stimulated with mitogens—Concanavalin A (ConA), Phytohaemagglutinin M (PHA-M), and *Escherichia coli*(serotype 0111:B4) derived lipopolysaccharide (LPS)—each at 5 µg/ml concentration in RPMI-1640 medium. 200 µl lymphocyte suspension (1×10^6 cells/ml) in 96-well plate were exposed to the MNCD of TCE alongside different mitogens in triplicate.

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2.8. Assessment of Antioxidant Potential of TCE Exposed Cells

The lymphocytes treated with MNCD of TCE alongside untreated controls and ascorbic acid as a positive control were incubated for 68 hours in a CO_2 incubator. After incubation, the cells were collected and lysed to create cell lysates. These lysates were stored at -80°C for later analysis of their antioxidant properties.

2.8.1. Membrane Lipid Peroxidation (LPO)

Malondialdehyde (MDA) serves as a valuable indicator of lipid peroxidation in biomedical studies due to its ability to readily react with thiobarbituric acid (TBA) leading to formation of a distinctive MDA-TBA_2 complex that can be measured spectrophotometrically at 532 nm, producing a characteristic red-pink coloration. The results were expressed in nanomoles of MDA per mg of protein[31].

2.8.2. Nitric Oxide Assay

NO (nitric oxide) is converted into nitrite, a stable intermediate that accumulates in the cell medium. Nitrite levels were measured as an indicator of NO production using the Griess method. The procedure involved combining 100 µl of medium of different treatments with an equal amount of Griess reagent in a 96-well plate. The absorbance was then measured at 520 nm using an ELISA reader, and nitrite concentrations were calculated using a sodium nitrite ($NaNO_2$) standard curve [32].

2.8.3. Reduced Glutathione (GSH)

Reduced glutathione (GSH) levels in cell lysates were determined through Ellman's method, where DTNB (5,5′-dithiobis-2-nitrobenzoic acid) reacts with sulfhydryl groups to form a yellow product. The intensity of this yellow color was measured spectrophotometrically at 412 nm, and the results were expressed as millimoles of GSH per milliliter [33].

2.8.4. Superoxide Dismustase (SOD)

The superoxide dismutase (SOD) activity was measured by evaluating its ability to inhibit the reduction of MTT (a tetrazolium dye) by superoxide, which was generated through pyrogallol autooxidation. The reaction was stopped using DMSO, which also helped dissolve the formazan product, and absorbance was measured at 570 nm. One SOD unit was defined as the amount of protein (in mg) needed to achieve 50% inhibition of MTT reduction [34].

2.8.5. Catalase

The catalase (CAT) activity in cell lysate was determined by measuring its ability to break down hydrogen peroxide (H_2O_2) into water and oxygen. The CAT enzyme's activity was quantified by monitoring the reduction in absorbance at 240 nm as H_2O_2 was decomposed over time, with the rate of change in absorbance (ΔA240) serving as a measure of catalase activity[35].

2.9. Statistical Analysis

Results were expressed as means ± standard deviation. Statistical significance between control and treated groups was evaluated using one-way ANOVA, where differences were considered significant when p-values were < 0.05. Statistical analysis was performed using Origin Software.

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3. Results

3.1. Percent Yield of TCE

The percent yield of hydromethanolic extract of *Tinospora cordifolia* stems was obtained to be 18.54% as presented in Table 1, Figure 1.

Table 1: Percent Yield of TCE

S. No.	Plant Material	Weight of Dried plant stems (gm)	Weight of hydromethanolic extract obtained (gm)	Percent Yield (%)
1.	*Tinospora cordifolia*	100	18.54	18.54

(A)

(B)

(C)

Figure 1: *Tinospora cordifolia* (A) Stem, (B) Dried stem powder, (C) Extract of *Tinospora cordifolia* stem (TCE)

3.2. Phytochemical Analyses of TCE

The total phenolic and flavonoid content of BAE was found to be 111.371±1.697 mg Gallic acid equivalent (GAE) /g extract and 83.038 ± 1.39 mg Rutin equivalent (RE) /g extract, respectively (Table 2).

Table 2: Phytochemical Analyses of TCE

S. No.	Quantitative Analyses	TCE
1.	Total Phenolic Content	111.371 ± 1.697 mg GAE/ g dry extract
2.	Total Flavonoid Content	83.038 ± 1.39 mg RE/ g dry extract

3.3. *In Vitro* Antioxidant Assays

3.3.1. Total Antioxidant Capacity

The total antioxidant capacity was evaluated using the phosphomolybdenum method, which assesses the sample's ability to reduce Mo (VI) to Mo (V), resulting in the formation of

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a green phosphate/Mo (V) complex. The results are detailed in Table 3. The total antioxidant capacity of TCE was measured at 192.75±1.52 mg ascorbic acid equivalent (AAE)/g of dry extract (Table 3).

3.3.2. Ferrous Reducing Antioxidant Power Assay (FRAP)

The ferrous ion chelating ability of TCE was assessed by measuring the reduction in absorbance of the Fe (II)-ferrozine complex at 562 nm. The FRAP value of TCE was found to be 107.407±1.25 mg ascorbic acid equivalent (AAE)/g of dry extract (Table 3).

Table 3: Antioxidant Potential of TCE

S. No.	***In vitro* Antioxidant Assays**	**Antioxidant Potential of MAE**
1.	Total antioxidant Capacity (TAC)	192.75±1.52 mg AAE/ g dry extract
2.	Ferrous Reducing Antioxidant Power (FRAP)	107.407±1.25 mg AAE/ g dry extract

3.3.3. Metal Ion Chelation Assay

The chelating effect of TCE was evaluated by assessing its ability to inhibit the formation of the ferrozine-Fe^{2+} complex, indicated by a reduction in colour intensity. The study demonstrated a direct correlation between TCE concentration and its metal ion chelating ability; as the concentration increased, the chelating activity also increased proportionally, as illustrated in Figure 2 (A) and Table 4.

3.3.4. Reducing Power Capacity

The antioxidant activity of TCE was assessed by its capacity to reduce the Fe^{3+}/ferricyanide complex to Fe^{2+}, which is indicated by the formation of Perl's Prussian blue color measured at a wavelength of 700 nm. The results indicated that TCE exhibited a dose-dependent reducing power, as reflected by decreasing absorbance values with increasing concentrations (Figure 2 (B), Table 4).

3.3.5. DPPH Radical Scavenging Assay

This assay measures the ability of antioxidants with hydrogen-donating capacity to reduce the alcoholic DPPH solution. The results revealed that the ability of TCE to scavenge DPPH radicals increased with higher concentrations, as illustrated in Figure 2 (C) and Table 4.

3.3.6. NO Scavenging Assay

When sodium nitroprusside solution in PBS was incubated, it generated nitrite in a linear, time-dependent manner. The introduction of TCE led to a decrease in nitrite production, with higher concentrations of TCE resulting in a more significant reduction. The findings are illustrated in Figure 2 (D) and Table 4.

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3.3.7. Superoxide Anion Radical Scavenging Assay

The capacity of TCE to neutralize superoxide radicals, generated by a PMS/NADH system, was evaluated by measuring light absorption at 562 nm. TCE's effectiveness in neutralizing superoxide radicals significantly increased with higher concentrations (Figure 2 (E), Table 4).

3.3.8. Hydroxyl Radical scavenging Assay

The capacity of TCE to neutralize hydroxyl radicals was assessed by measuring its effectiveness in preventing the oxidation of 2-deoxy-D-ribose by OH radicals, with the oxidation products quantified via their reaction with TBA (thiobarbituric acid). TCE was found to effectively inhibit the degradation of 2-deoxyribose, with its protective effect increasing at higher concentrations (Figure 2 (F), Table 4).

Table 4: Inhibitory Concentration (IC_{50}) value obtained in different *in vitro* Antioxidant Assays

S. No.	*In vitro* Antioxidant Assays	IC_{50} (µg/ml)	
		Standard	**TCE**
1.	Metal Ion Chelation	178.675	223.805
2.	DPPH Radical Scavenging	16.98	64.826
3.	NO Scavenging	57.425	70.252
4.	Superoxide Anion Radical	59.845	85.89
5.	Hydroxyl radical	56.422	66.927

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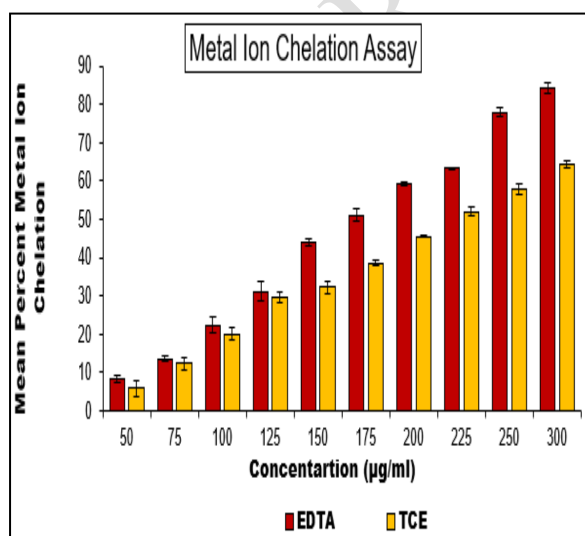

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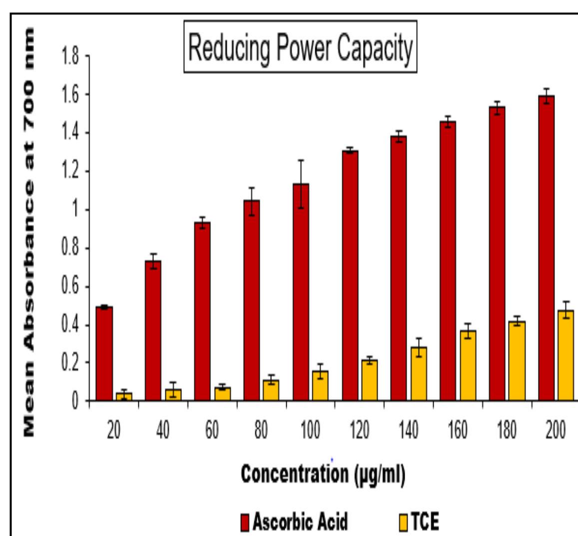

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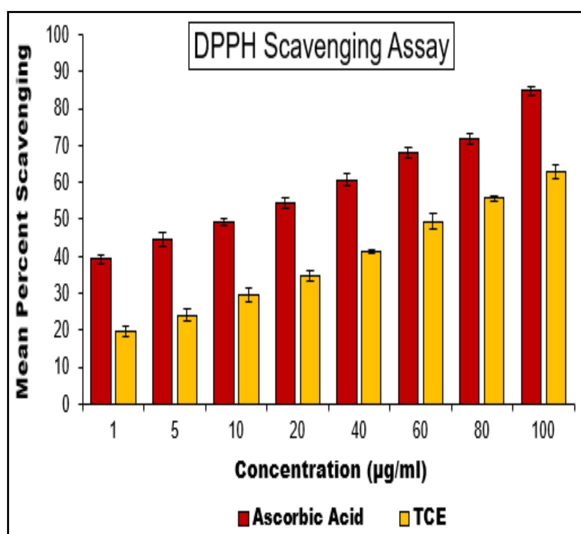

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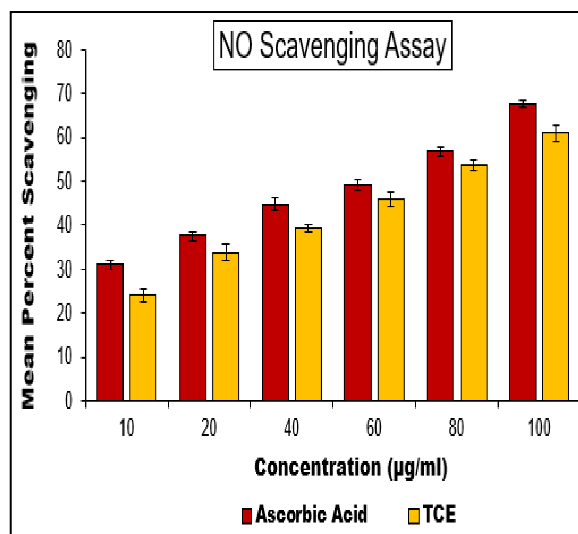

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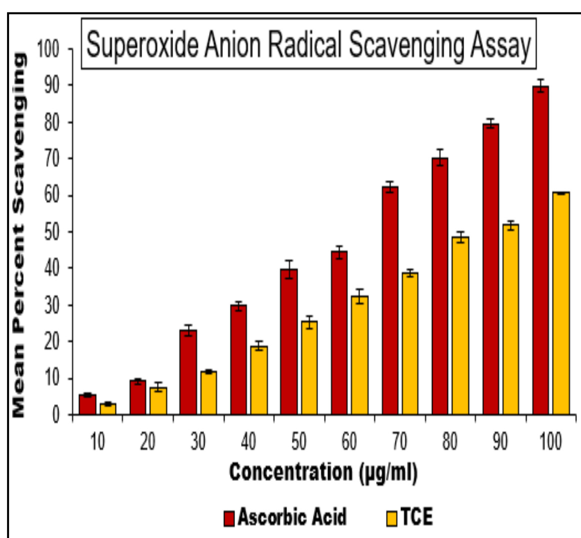

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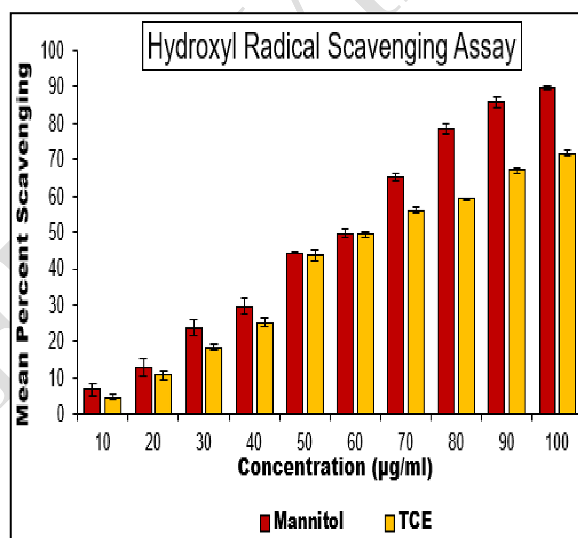


(F)

Figure 2: *In Vitro* Antioxidant Assays. (A) Metal Ion Chelation Assay, (B) Reducing Power Capacity, (C) DPPH Radical Scavenging Assay, (D) NO Scavenging Assay, (E) Superoxide Anion Radical Scavenging Assay and (F) Hydroxyl Radical Scavenging Assay.

3.4. Estimation of Maximum Non-Cytotoxic Dose (MNCD)

The MNCD of the TCE extract was determined to be 300 µg/mL via MTT cytotoxicity assay, as chicken lymphocyte viability demonstrated significant reduction at concentrations exceeding this threshold, as shown in Figure 3.

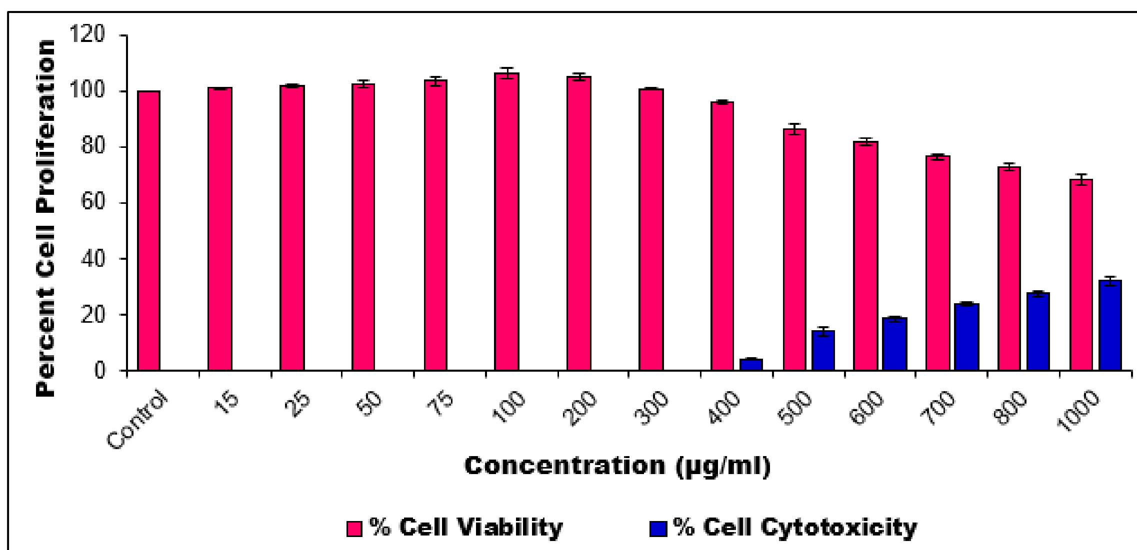


Figure 3: Percent Cell Proliferation of Chicken Lymphocytes when Exposed to Different Concentrations of TCE.

3.5. Lymphocytes Proliferation Assay

Exposure to the MNCD of TCE led to a significant increase in B and T cell proliferation in mitogen-stimulated lymphocytes (LPS, PHA, and ConA) compared to the respective controls. The results are presented in Figure 4.

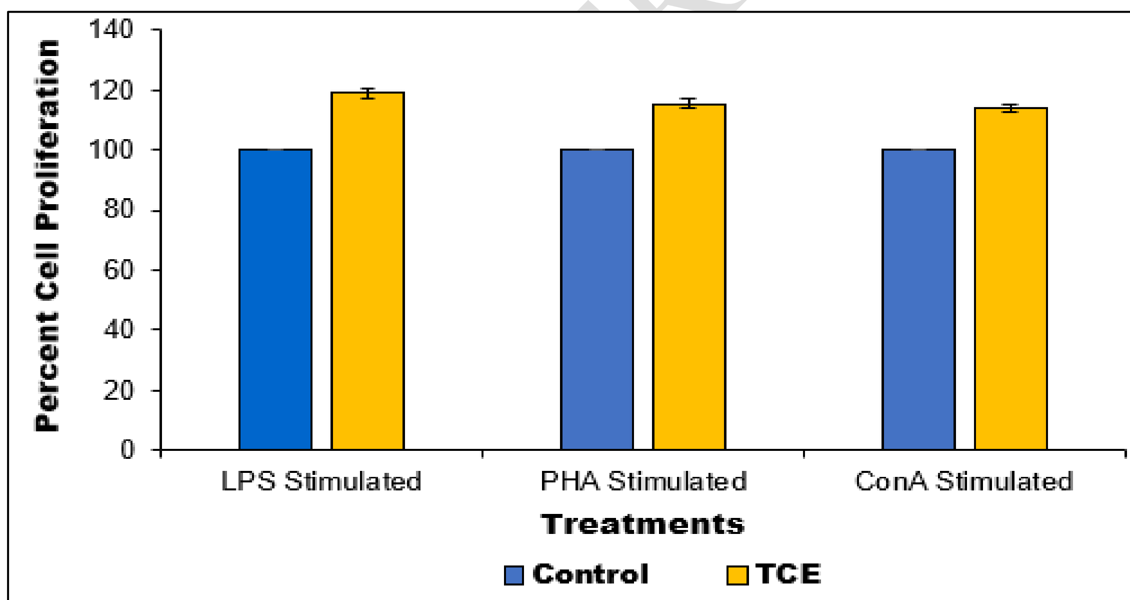
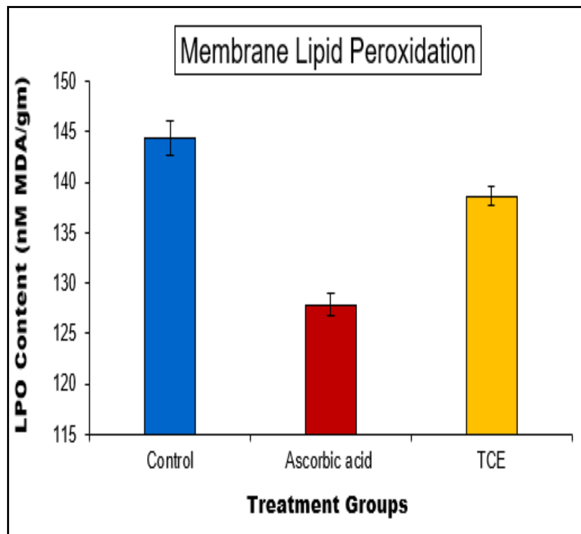


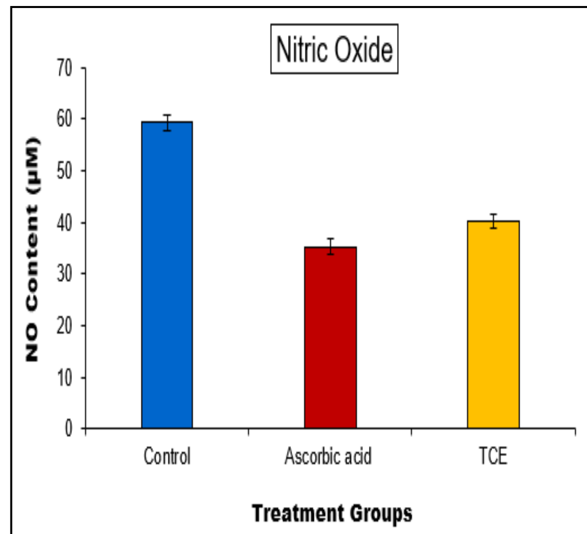
Figure 4: Lymphocyte Proliferation Assay.

3.6. Assessment of Antioxidant Potential of TCE Exposed Cells

When chicken lymphocytes were treated with the MNCD of TCE, their antioxidant profile improved significantly. Analysis revealed reduced levels of lipid peroxidation and nitric oxide, while simultaneously showing increased levels of reduced glutathione and enhanced activity of superoxide dismutase and catalase enzymes, as illustrated in Figure 5 (A-E).



(A)



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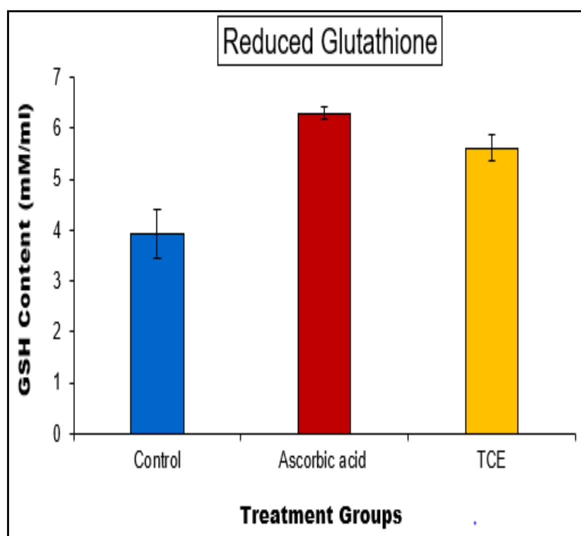

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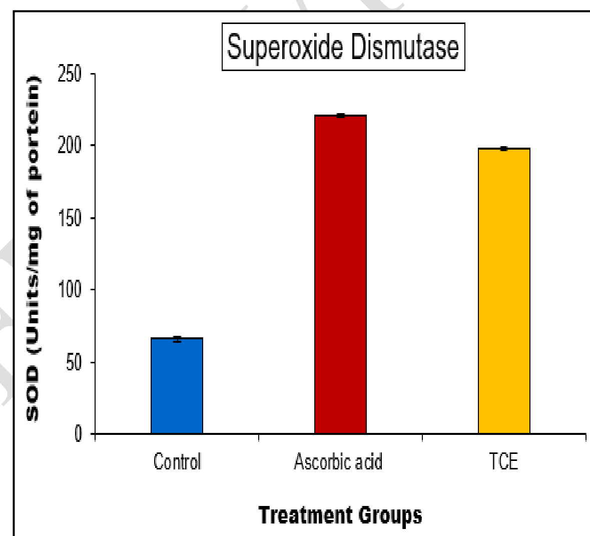

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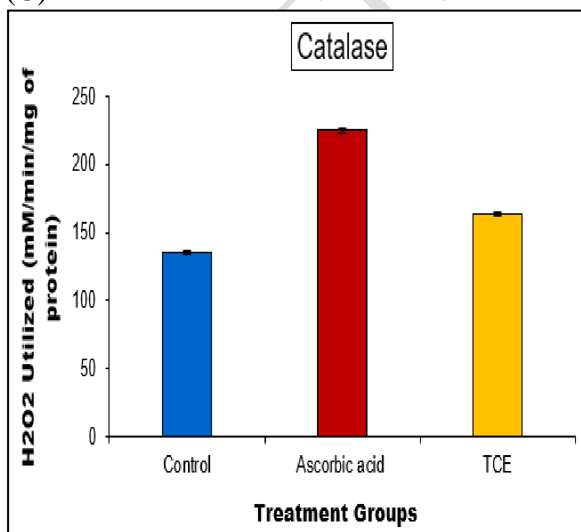

(E)

Figure 5: Antioxidant Potential of TCE Exposed Cells. (A) Membrane Lipid Peroxidation, (B) Nitric Oxide Assay, (C) Reduced Glutathione, (D) Superoxide Dismutase and (E) Catalase.

4. Discussion

The present investigation provides comprehensive insights into the antioxidant and immunomodulatory potential of *Tinospora cordifolia* stem extract (TCE), supporting its traditional therapeutic applications. The evaluation of overall health impacts of plant extracts is crucial prior to their potential use as immune boosters. *Tinospora cordifolia* has gained attention as an immune booster consumed in various forms including tablets, crude extracts, or decoctions [36]. Our study revealed that the hydromethanolic extract of *Tinospora cordifolia* stems yielded 18.54%, containing substantial bioactive compounds with total phenolic content at 111.371±1.697 mg GAE/g extract and flavonoid content at 83.038±1.39 mg RE/g extract. The extensive phytochemical characterization has identified over two hundred different phytochemicals, with diterpenoids representing the most abundant chemical class [37]. These findings differ from those reported by Khan *et al.* [3], who found higher phenolic content (195.45±18.88 mg/g GAE) and lower flavonoid content (21.97±98 mg QE/100g) in methanol extract. Importantly, the phytochemical variations and efficacy of the medicinal values of *Tinospora cordifolia* are dependent on geographical locations and seasons [15].

Oxidative stress is considered a main cause for the development of various diseases. Food materials promote antioxidative defences in the human body to combat non-desirable effects of reactive oxygen species. Plants are able to biosynthesize a wide range of non-enzymatic antioxidative molecules capable of attenuating reactive oxygen species-induced oxidative destruction [38, 39]. In this study, it was observed that TCE exhibited remarkable antioxidant capacity through multiple mechanisms. The TCE demonstrated concentration-dependent activities in various *in vitro* antioxidant assays. These findings correlate with previous studies such as in a study by Polu *et al.* [40], it was reported that the n-butanolic extract of *T. cordifolia* stem (200 mg/mL) demonstrated significant antioxidant activity in DPPH, ABTS, nitric oxide scavenging, and iron chelating assays, supporting earlier findings about its free radical scavenging properties [41]. The methanolic extract of *T. cordifolia* stem proved effective in scavenging superoxide anion radical and inhibited deoxyribose degradation induced by hydroxyl radical, scavenging them directly rather than via chelating iron ion [3]. The anti-oxidant activity evaluation of aqueous, methanolic, and alcoholic extracts using seven in vitro anti-oxidant methods (DPPH, OH, FRAP, TAC, NO, $O2^{-}$, and metal chelation) showed free radical scavenging activity through the inhibition of oxidative molecules [42, 43]. Phytochemical components, especially polyphenols such as flavonoids, phenylpropanoids, and phenolic acids, are responsible for the free radical scavenging and antioxidant activities. Plant polyphenols act as reducing agents and antioxidants through the hydrogen-donating property of their hydroxyl groups [39, 44].

The immunomodulatory aspects of our findings align with previous research showing that modulation in the immune system through stimulation or suppression mechanisms helps in maintaining disease-free lifestyles [45]. The immunomodulatory potential of TCE was evidenced through its effects on chicken lymphocytes. The extract showed a maximum non-cytotoxic dose (MNCD) of 300 µg/mL, above which significant reduction in cell viability was observed. At MNCD, TCE significantly enhanced both B and T cell proliferation in mitogen-stimulated lymphocytes (LPS, PHA, and ConA). Ethanolic extract of *Tinospora cordifolia* stem increased spleen lymphocyte proliferation compared with control and cyclophosphamide treatment [46]. This proliferation indicated increased B and T cell numbers, leading to cytokine and growth factor release and antibody secretion. Flow

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cytometric analysis revealed substantial increases in T cell surface markers (CD3+, CD4+, and CD8+), NK1.1+, and B cell surface markers (CD19+), with Guduchi ImP protein (10 μg/mL) demonstrating significant mitogenic activity (~3-fold higher than control) against murine splenocytes [47]. The presence of immunomodulatory protein (Guduchi ImP protein) suggests its potential use in Ayurvedic preparations for immunomodulatory actions [48]. Natural compounds from *Tinospora cordifolia* have been reported to upgrade phagocytic activity of macrophages, amplify nitric acid production through splenocyte stimulation, and create reactive oxygen species in human neutrophil cells [49]. The plant and its constituent α-D-glucan stimulate NK cells, B cells, and T cells with simultaneous production of immune-stimulatory cytokines [50]. The polysaccharide compound G1-4A enhances proliferation and differentiation of immune cells with expression of the anti-apoptotic gene [51]. Studies have shown that aqueous extract primarily acts on macrophages, stimulating production of effector molecules like nitric oxide and cytokine mediators while augmenting antigen-specific recall response, indicating enhanced antigen-presenting ability of macrophages [52].

The cellular antioxidant profile showed significant improvements upon TCE treatment. The extract reduced lipid peroxidation and nitric oxide levels while simultaneously increasing reduced glutathione levels and enhancing superoxide dismutase and catalase enzyme activities. These findings correspond with Gupta and Sharma [53] who reported similar increases in superoxide dismutase, reduced glutathione, glutathione-S-transferase, glutathione peroxidase, and glutathione reductase. Significantly, *T. cordifolia* reduces lipid peroxidation process regulation, decreasing reactive free radical species in diabetic rat models while up-regulating antioxidant enzymes like catalase and glutathione [54]. Ahera and Wahi [46] observed that ethanolic extract from the stem of *Tinospora cordifolia* led to increased levels of liver mitochondrial enzymes, including GSH, CAT, and SOD, while reducing LPO levels in the liver compared to the vehicle, SRBC, and cyclophosphamide-treated groups.Our comprehensive findings demonstrate that *T. cordifolia* stem contains substantial amounts of phenolic and flavonoid compounds responsible for its marked antioxidant activity.

5. Conclusion

The present investigation conclusively demonstrates the significant antioxidant and immunomodulatory potential of *Tinospora cordifolia* stem extract (TCE). Our findings reveal substantial quantities of phenolic and flavonoid compounds, contributing to its marked antioxidant activity through multiple mechanisms, including strong total antioxidant capacity and significant ferrous reducing power. The extract exhibited significant immunomodulatory properties, enhancing both B and T lymphocyte proliferation while improving cellular antioxidant profiles. These comprehensive results validate the traditional therapeutic applications of *T. cordifolia* and establish its potential for nutraceutical and functional food development.

Future research should focus on detailed characterization of bioactive compounds, elucidation of molecular mechanisms, and clinical validation through controlled trials. Priority areas include optimization of extraction methods for commercial production, development of standardized formulations, and establishment of quality control parameters. Additional studies on potential synergistic effects with other therapeutic agents, comprehensive safety profiling, and investigation of novel delivery systems would facilitate the development of evidence-based natural products. The integration of traditional knowledge with modern scientific approaches, alongside sustainable production methods, would ensure optimal utilization of this valuable medicinal plant in modern healthcare applications.

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Ethical Approval

The due approval of this study was procured from Institutional Animal Ethics Committee, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India (IAEC/CBSH/MBGE/313).

Disclaimer (Artificial Intelligence)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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