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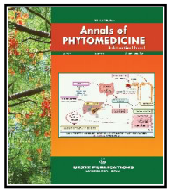

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Evaluation of immune modulation activity, antioxidant activity and phytochemical screening of *Croton bonplandianum* Baill.

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Abstract

Croton bonplandianum Baill. is in the Euphorbiaceae family. Medicinal herbs offer anticancer, antioxidant, anti-inflammatory, and wound healing properties. We have decided to work in this innovative area. This was accomplished by prelude phytochemical analysis, LC-MS analysis, immunological modulation, and antioxidant activity of *C. bonplandianum* ethanolic extract (CBEE). Preliminary phytochemical research indicated the being there of phytochemicals such as sugars, quinines, and glycosides in *C. bonplandianum* leaf extract. The total flavonoids and phenolics content in leaf extracts were measured spectrophotometrically. The total phenolics content and total flavonoids content were resolute near are present 254.58 g/ml and 59.35 g/ml, respectively.

LC-MS analysis was used to analyze the chemical composition of the ethanol of *C. bonplandianum* leaf extract. Using the LC-MS approach, we effectively detected key chemicals such as quercetin, phytol, kaempferol, and trehalose. The protective antioxidant capacity of *C. bonplandianum* leaves was determined using the DPPH test. The concentration of *C. bonplandianum* extract increased significantly, and the inhibitory concentration (IC_{50}) value was found to be 57.05 g/ml.The cytotoxicity of the extract was assessed on the THP-1 cell line (a model for evaluating immunological modulation activities) using the MTT test. The cytotoxicity activity was moderate, with an IC_{50} of 213.5 ± 0.084 µg/ml. Protein profiling was analyzed with ELISA-IL4. The *C. bonplandianum* ethanolic extract significantly decreased IL-4 inflammatory cytokine production compared to the control (64.58 pg/ml or .00006458 µg/ml). Cells treated with the IC_{50}/2 dose of the sample showed a decrease of IL-4 inflammatory cytokine production 0.82 times (53.33 pg/ml or .00005333 µg/ml).

1. Introduction

Herbal medicines, primarily derived from plants, are increasingly recognized as potential therapeutic agents for various human ailments. The bioactive phenolics and polyphenol compounds found in fruits, seeds, and leaves have shown promise in treating malignant cells, regulating cell growth, and exhibiting anti-inflammatory and antioxidant properties. Compared to conventional medicine, herbal medicines generally have fewer reported side effects, making them a potentially safer alternative over time (Saggoo *et al.,* 2010; Prathyusha, 2022; Rao *et al.,* 2023; Yadav *et al.,* 2022). However, translating laboratory findings into clinical trials remains a challenge, necessitating further research to validate their efficacy and safety, especially for conditions like ischemic stroke. In the traditional Indian medical system, specific herbs, plant-based formulations, and decoctions are recommended to enhance immunity and combat illnesses (Thakur, 2022). One such plant with medicinal potential is *C. bonplandianum,* a deciduous shrub that thrives in clay and sandy soils across South America, South India, Bangladesh, and Pakistan (Qaisar *et al.,* 2013). The plant's bark, roots, seeds, and latex possess various therapeutic properties. For instance, the fresh juice relieves headaches, while the latex heals cuts and wounds and exhibits antifungal activity against ringworm fungi (Mohan and Divya, 2017).

C. bonplandianum leaves are rich in antibacterial compounds and beneficial for treating high blood pressure, skin issues, cuts, wounds, and as antiseptics (Somit *et al.,* 2013). Recent studies have also revealed its twig extract's anticancer effects and other bioactive potentials like antioxidant, antihypertensive, repellent, analgesic, anthelminthic, and larvicidal activities (Islam *et al.,* 2011; Singha *et al.,* 2022; Jeeshna *et al.,* 2011). Phytochemical analysis of the leaves identified various compounds, including steroids, tannins, flavonoids, saponins, terpenoids, glycosides, phenolics, anthraquinones, alkaloids, cholesterol, carbohydrates, and proteins. Further studies assessed its antioxidant and immunological modulation activities using DPPH procedure, MTT assay, and protein profiling.

2. Materials and Methods

2.1 Materials

In this study, all solvents utilized were sourced from Merck, India, while chemicals were procured from Himedia, ensuring the highest quality and consistency in our experimental procedures.

2.2 Collection and authentication of plant materials

The plant materials were gathered from Lucknow, Uttar Pradesh, India, in September and authenticated by the taxonomic division at Banaras Hindu University (BHU), Varanasi, India, under voucher specimen number Euphor. 2022/1.

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2.3 Preparation of extract

After collection, the leaves were carefully dried in the shade and sunlight, followed by one week of shade drying to ensure the best extraction conditions. The leaves that had been dried were subsequently pulverized, weighed, and kept in airtight containers to ensure their integrity. Ethanol leaf extract was then made using the Soxhlet extraction process, which involved placing powdered leaves in a muslin bag and extracting them with ethanol. The obtained herbal extract was concentrated, refrigerated, and used in pharmacological study following the approach stated by (Alam *et al*., 2017).

2.4 Physicochemical analysis

Physicochemical parameters include loss on drying, extractive values, ash values, foaming Index, pH of the drug done according to the standard protocol (Hussain *et al*., 2014).

2.5 Phytochemical screening

The *C. bonplandianum* ethanolic extract (CBEE) was submitted to analysis for active phytoconstituents including phenols as well flavonoids, alkaloid compounds, tannins, saponins, and terpenoids, *etc.,* according to the standardized methodology (Tiwari *et al.,* 2011).

2.5.1 Phytochemical quantification

2.5.1.1 Total phenolics content

The extracted solution (1 mg/ml) was combined with 20 µl of Folin-Ciocalteau reagents (diluted 1:10) and 50 µl of 2.5% aqueous Na_2CO_3. After 1 h, the mixes were analyzed on 765 nm with a spectrophotometer. The extract's phenol concentration was measured using the gallic acid solution as the standard and a curve for calibration (Maurya and Singh, 2010; Bamne *et al.,* 2023).

2.5.1.2 Total flavonoids content

The extract was treated with 0.1 ml of 10% aluminium chloride and 0.1 ml of 1 M sodium acetate, and then incubated for 30 min under ambient temperature. The absorbance of the resultant combination was subsequently measured at 510 nm. The amount of flavonoids compound of the extract was assessed assuming quercetin as the reference substance, and the total amount of flavonoids concentration was reported as mg of quercetin equivalent per gramme (QE mg/g) of the sample's dry weight, based on a standard curve.

2.5.2 LC-MS analysis

The plant extract sample weighing 100 mg was dissolved in 50 ml of LC-MS grade methanol in a 100 ml round bottom flask. After vigorous shaking for 10 min, the flask was filled to volume with methanol and subjected to 10 min of bath sonication at ambient temperature. The resulting solution was then filtered through a 0.45 µm syringe filter. After filtration, a suitable dilution was made for insertion directly in the LC-MS system using an auto-injector. Prior to running the instrument, chromatographic conditions were optimized considering the compound's properties and molecular weight range. The molecule was analyzed in the 70-790 m/z range using a mobile phase comprising 0.1 per cent formic acid and methanol at an average flow rate of 0.2 ml/min. The vaporization temperature was kept at 350°C, and the scan was run in APC mode. The chromatographic settings were carefully modified as appropriate, and the tests were run in triplicate (N=3).

2.5.3 Antioxidant activity

The extract's DPPH radical scavenging potential was assessed at doses of 20, 40, and 80 µg/ml. A newly produced methanol solution containing 0.15 mM DPPH was vigorously vortexed into the sample. The resultant mixture was then incubated at the ambient temperature under dark conditions for 20 min before being measured for absorbency at the wavelength of 517 nm using UV-VIS spectroscopy. Butylated hydroxytoluene (BHT) had been employed as a positive control in the experiment (Siddiqui *et al.*, 2020; Qadir *et al.*, 2022).

For the purpose of assessing the degree of DPPH radical scavenging activity, the corresponding percentage of inhibition was computed using the formula:

$$\% \text{ Antioxidant activity} = [(Ac - As)/Ac] \times 100$$

where, Ac is absorbency from the control,

As is the absorbency from the sample

2.5.4 Cytotoxic activity against THP-1 cell line (immune modulation activity)

The THP-1 cell line, generated from human leukemia monocytic cells, is an excellent model for investigating monocyte/macrophage activity, signalling mechanisms, and nutrient/drug transport. This model, widely used in research, allows for the investigation of changes in macrophage and monocyte activity, which is critical for understanding immune system functioning, such as infection phagocytosis, antigen presentation to T cells, cytokine production, and tissue healing and immune response regulation (Chanput *et al*., 2014).

2.5.5 Cell culture

The impact of CBEE on the cytotoxicity of THP-1 cells, a human leukemia monocytic cell line obtained from NCCS Pune, was assessed using the MTT assay. THP-1 cells were seeded at a density of 10,000 cells per well in a 96-well plate and cultured for 24 h in DMEM medium (Dulbecco's Modified Eagle medium-AT149-1L) complemented among 10% FBS and 1% antibiotic solution at 37°C with 5% CO_2. Following incubation, cells were exposed to various doses of CBEE, while untreated cells served as the control group (Morgan, 1998; Tihauan *et al.,* 2020).

2.5.6 Cell viability assay by microscopy

Following a 24 h treatment period, the plate of cells was observed using an inverted culture microscope (Olympus eK2) fitted with an AmScope digital camera (10 MP Aptima CMOS). Microscopic images were captured and any observable changes in cell morphology such as cell rounding or shrinking, cytoplasmic granulation, and vacuolization were noted as indicators of cytotoxicity (Chithra *et al.,* 2020).

2.5.7 MTT assay

Following a 24 h incubation period, MTT Solution at varying concentrations (1, 10, 50, 100, 250, 500, 1000) was introduced to the cell culture and further incubated for an additional 2 h. Subsequently, the culture supernatant was collected, and the cell layer matrix was dissolved in 100 µl of dimethyl sulfoxide (DMSO-SRL-Cat No.-67685) to dissolve the formazan crystals. Absorbance was subsequently deliberate at 540 and 660 nm using an Elisa plate

reader (iMark, Bio-Rad, USA). The optical density of formazan generated in untreated control cells was designated as 100% vitality (Fotakis and Timbrell, 2006; Thomas *et al.,* 2023). The IC_{50} was determined using the freeware Graph Pad Prism-6.

2.5.8 Protein profiling assay by ELISA- IL4

The experiment was performed up accordingly to the ELISA kit instructions (Human IL-4GENLISATMELISA- Cat No. KB1066). 100 µg/ml of IL-4 standard was applied to the plate, followed by 50 µg/ml of diluted detection antibody. Incubate at 37°C for 30 min before sealing the plate. The plate was rinsed four times with wash buffer (1X) and the buffer was blotted by vigorously pressing it upside down on absorbent paper. In every single well, add 100 µl of dilute Streptavidin-HRP solution, cap the plate, and then incubate for 30 min. at 37°C. After washing the medium using wash buffer (1X), 100 µl of a substrate composed of TMB (3, 3', 5, 5'-Tetramethylbenzidine) solution was added and incubated in the dark for a period of 30 min. To halt the reaction, add 100 µl of the stopping solution each and every well and record the value of the absorbance at 450 nm within 30 min (Human IL-4 GENLISATM ELISA-Cat No. KB1066 kit).

3. Results

3.1 Physicochemical analysis

The physicochemical attributes of powdered leaves from *C. bonplandianum*, including the extractive values in petroleum ether, ethanol, and water; total ash value; acid insoluble ash value; water soluble ash value; loss on drying; foaming index; and pH of the drug (1% solution) are presented in Table 1.

Table 1: The physicochemical characters of powdered drug of leaves *C. bonplandianum*

Physicochemical parameters		Values
Extractive values	Petroleum ether	3.51 %w/w
	Ethanol	9.25 %w/w
	Water	5.34 %w/w
Ash values	Total ash value	4.31 %w/w
	Acid insoluble ash value	0.48 %w/w
	Water soluble ash value	3.58 %w/w
	Loss on drying	2.23 %w/w
Foaming index		3.87
pH of the drug (1% solution)		8.18

3.2 Phytochemical screening

Throughout history, plant-derived compounds have been revered for their therapeutic potential. A qualitative investigation of plant ethanol extract demonstrates the existence phenols, terpenoids, steroids, saponins, flavonoids; also tannins presented in Table 2.

3.3 Phytochemical quantification

Quantitative analysis exposed the incidence of phenols (254.58 µg/ml) and flavonoids (59.35 µg/ml) as secondary metabolites presented in Table 3.

Table 2: Phytochemical screening of ethanolic leaf extract of *C. bonplandianum*

Compound's name	Test	Present/ Absent (+/-)
Tannin	Phenazone test	+
Terpenoids	Salwoski test	+
Glycoside	Sodium picrate test	+
Phenolics	Litmus test	+
Flavonoid	Alkaline reagent test	+
Steroid	Liebermann-Burchard test	+
Saponin	Foam test	+
Carbohydrate	Molisch's test	+
Anthraquinone	Bromine test	–
Protein	Biuret test	+

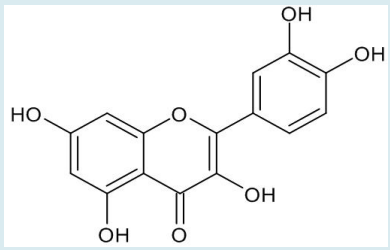
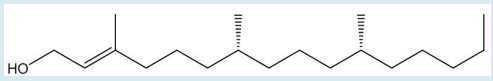
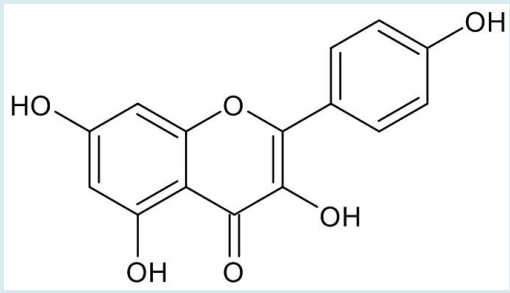
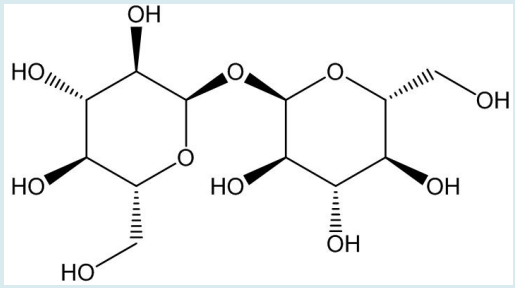
Table 3: Secondary metabolite content in the leaf extract of *C. bonplandianum*

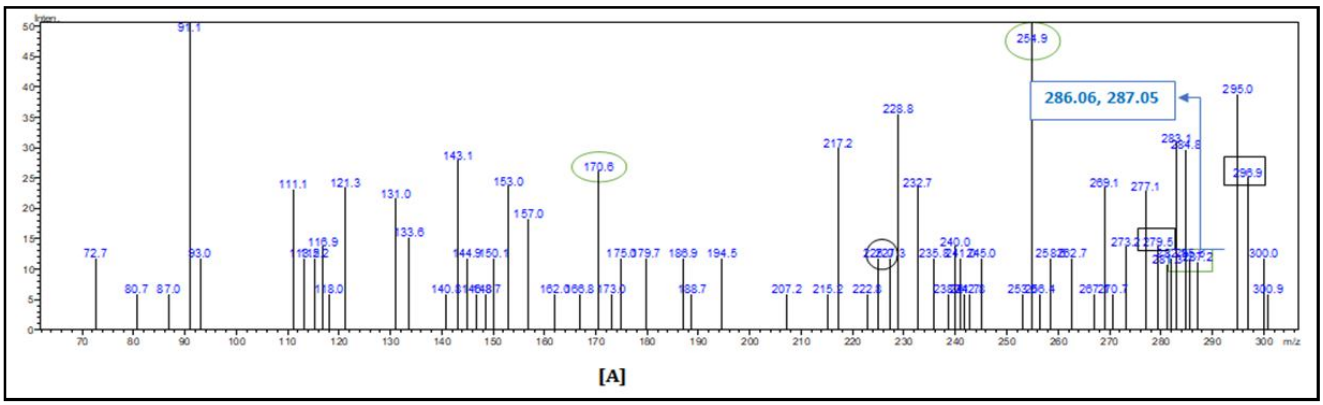
Ethanolic extract	Total phenolic content	Total flavonoid content
C. bonplandianum	254.58 µg/ml	59.35 µg/ml

3.4 LC-MS analysis for phytochemical screening

We have successfully identified the below compounds (Table 4, Figure 1) by LC-MS method and reported above. The identified compounds are shown antiallergic properties. However, our research findings revealed that the proposed hypothesis going in a positive direction and need more investigation to prove this hypothesis.

Table 4: Compounds identified in liquid chromatography-mass spectrophotometry (LC-MS)

Compound identified	Identified exact mass (m/z)	Chemical structure
Quercetin	270.6, 254.9	
Phytol	296.9	
Kaempferol	286.06, 287.05	
Trehalose	343.3, 360.1	



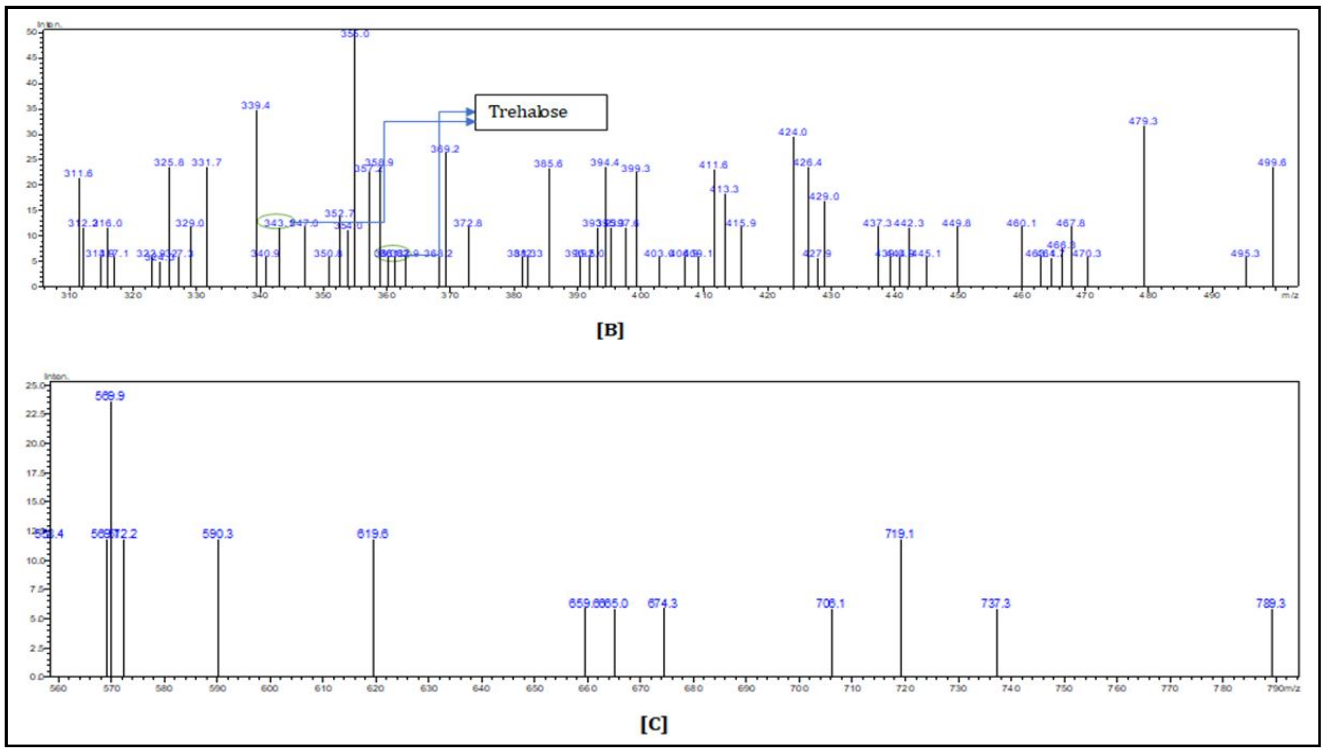


Figure 1: Experimental graphs of mass spectra of *C. bonplandianum* plant extract where {A} m/z mass spectra of with scanning range 70 to 300, {B} 310 to 490, {C} 490 to 790, respectively.

3.5 DPPH assay for antioxidant activity

The DPPH technique was used to compare the antioxidant activity of *C. bonplandianum* extract to the reference medication BHT (Figure 2). The results demonstrated robust free radical scavenging antioxidant activity, with a considerable rise in activity with increasing *C. bonplandianum* extract concentration, with an IC_{50} value of 57.05 g/ml.

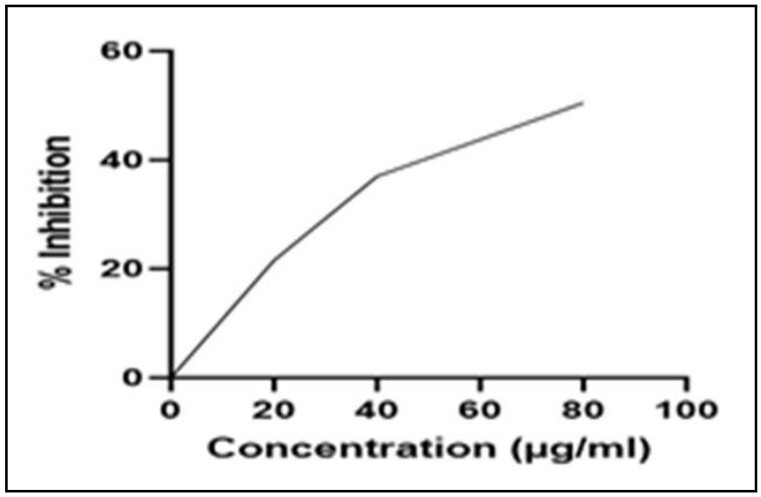


Figure 2: Antioxidant activity of *C. bonplandianum* leaf extract.

3.6 Immune modulation activity

The MTT experiment revealed cytotoxic activity in sample CBEE (IC_{50} = 213.5 ± 0.084 µg/ml) when exposed to varied doses in the THP-1 cell line (Figures 3 and 4).

Table 5: *C. bonplandianum* leaves extract IC_{50}

Sample code	IC_{50} value (µg/ml)
CBEE	213.5 ± 0.084

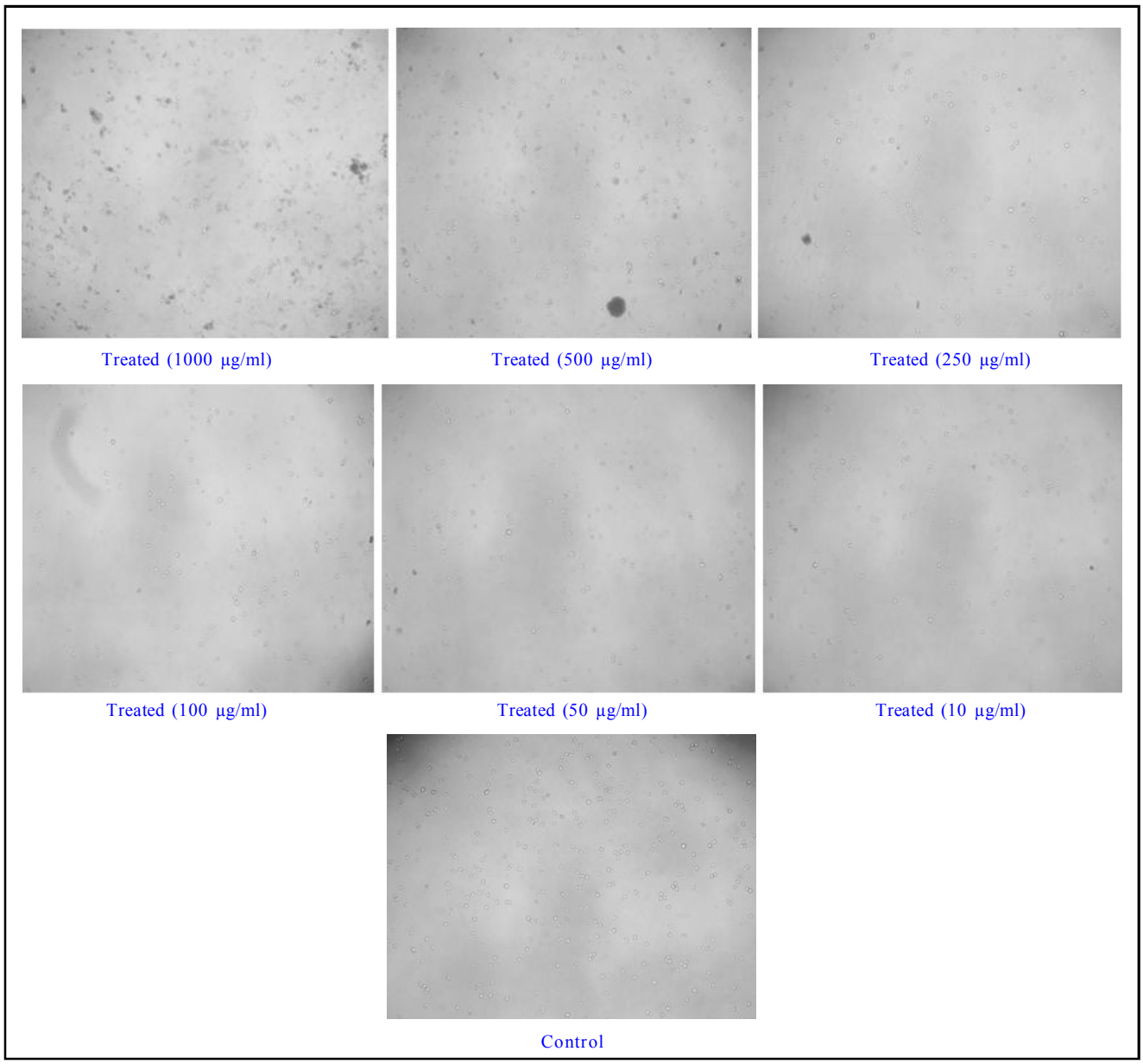


Figure 3: Cytotoxic activity of *C. bonplandianum* leaf extract against THP-1.

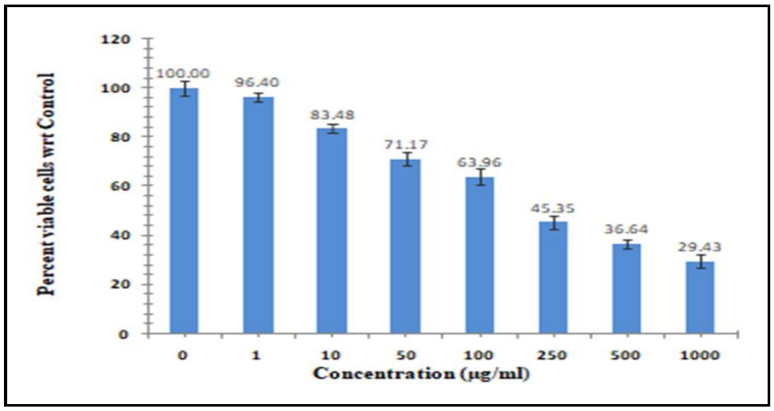


Figure 4: Cytotoxic activity of *C. bonplandianum* leaf extract against THP-1.

3.7 Protein expression analysis (Immuno fluorescence microscopy)

As per the results obtained after analysis, it was observed that the sample CBEE is down regulating the IL-4 inflammatory cytokine production with respect to control (64.58 pg/ml or .00006458 µg/ml).

When cells, treated with $IC_{50}/2$ dose of CBEE sample, IL-4 inflammatory cytokine production was decreased (0.82 times less than control, *i.e.,* 53.33 pg/ml or .00005333 µg/ml).

Table 6: Protein expression analysis of *C. bonplandianum* leaf extract

	Mean (pg/ml Lysate)	SD	SEM
Control	64.5833	14.1421	7.0711
CBEE	53.3333	11.1959	5.5979

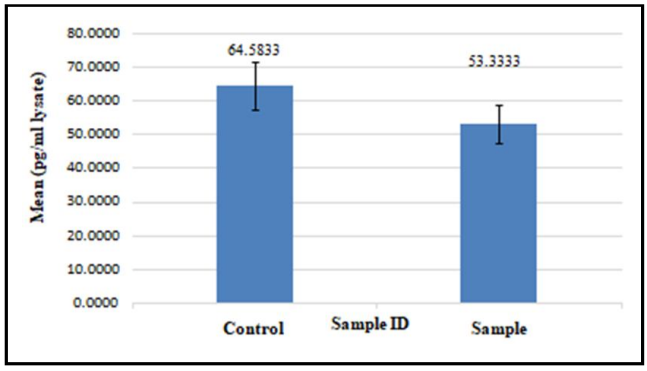


Figure 5: Protein expression analysis of *C. bonplandianum* leaf extract.

4. Discussion

This aspect of the study involved analyzing the chemical composition of the *C. bonplandianum* extract. Phytochemical analysis focuses on identifying and quantifying the various chemical compounds present in plants (Velavan, 2015). In this case, the analysis revealed the presence of specific compounds such as quercetin, phytol, and kaempferol. These compounds are known for their potential health benefits, including antioxidant and anti-inflammatory properties.

Antioxidant activity refers to the ability of a substance to neutralize harmful free radicals in the body, thus reducing oxidative stress and preventing cellular damage (Sen *et al*., 2010). The study assessed the antioxidant capacity of the *C. bonplandianum* extract using a method called DPPH radical scavenging activity assay. This assay measures the ability of the extract to donate electrons to the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical, thereby reducing it to a stable molecule. The significant antioxidant capacity observed suggests that the extract has potential protective effects against oxidative damage.

Immunological modulation refers to the ability of a substance to modulate the immune response in the body. In this study, we assessed the immunomodulatory effects of the *C. bonplandianum* extract using the THP-1 cell line. THP-1 cells are a type of human monocytic cell line commonly used in immunological research (Chanput *et al*., 2014). We observed a decrease in the production of IL-4, which is an inflammatory cytokine involved in allergic and immune responses. This suggests that the extract may have anti-inflammatory properties and could potentially modulate the immune system.

Future research should focus on isolating and characterizing bioactive compounds, investigating their mechanisms of action, and evaluating their therapeutic potential in preclinical and clinical studies.

5. Conclusion

The study investigated *C. bonplandianum* extract's physiochemical analysis, phytochemical screening, and phytochemical quantification in order to standardize it. Physiochemical study showed the amount and type of components in various solvents, whereas phytochemical screening provided insight into composition of *C. bonplandianum* composition and potential applications as a source of phytochemicals such as saponins, flavonoids, alkaloids, and phenolics. Phytochemical analysis revealed significant phenolics and flavonoids levels, indicating antioxidant action. Secondary metabolites detected by LC-MS identified substances having industrial and medical potential. The DPPH experiment revealed dose-dependent antioxidant activity in the sample, which is well correlated with total phenolics and flavonoids content. Based on our findings, we may infer that the ethanolic extract of *C. bonplandianum* has a high concentration of bioactive chemicals.

In asthma and allergies, immune responses are often characterized by an overactive reaction to harmless substances. Macrophages can contribute to this process by promoting inflammation. *C. bonplandianum* causes cytotoxicity against macrophages; it might suppress inflammation, and *C. bonplandianum* ethanolic extract decrease IL-4 inflammatory cytokine production, which could potentially reduce symptoms associated with asthma or allergies.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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