Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 2s, https://carcinogenesis.com/

In-Vitro Evaluation Of A Herbomineral Capsule Formulation For Its Immunomodulatory Activity Using Cell Line

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ABSTRACT

Background: Herbomineral formulations, combining medicinal herbs and minerals, have long been used in traditional systems like Ayurveda for immune enhancement and overall health. These formulations are believed to exhibit immunomodulatory, anti-inflammatory, and antioxidant effects through synergistic interactions of bioactive components. With growing interest in natural Immunotherapeutics, scientific validation of such formulations is essential. In vitro cell line models provide a reliable platform for evaluating immunomodulatory activity by assessing parameters like immune cell viability, immune cell activation, and other relevant biomarkers

Objective: This study aimed to evaluate the immunomodulatory potential of a standardized herbomineral capsule formulation in vitro using human PBMCs by assessing cell viability, ADA activity, IgG production, and related biomarkers.

Material and methods: Human peripheral blood mononuclear cells (PBMCs) were cultured and treated with different concentrations of the herbomineral formulation (Herboxid). Cell viability was assessed using the XTT assay, while ADA activity and IgG levels were used to evaluate immunomodulatory effects. LPS and PHA were used to stimulate an inflammatory response, and synergistic effects were analysed

Results:The Herboxid herbomineral capsule demonstrated no cytotoxicity across tested concentrations (12.5–100 µg/mL), with PBMC viability exceeding control levels (highest at 50 µg/mL: 126.59%). ADA activity increased significantly in a dose-dependent manner, peaking at ~10–11 U/L/min at 100 µg/mL ($P < 0.05$), compared to ~2 U/L/min in controls. IgG levels also rose progressively with concentration, with the highest activity at 100 µg/mL. Morphological changes in PBMCs suggested immune cell activation. Co-treatment with LPS and PHA further amplified ADA activity and viability, showing a synergistic immune response, particularly at higher doses (LPS 31 µg/µL + PHA 2 µg/µL), confirming the immunostimulatory potential of the formulation.

Conclusion:This study demonstrates that the Herboxid capsule is non-toxic to PBMCs and enhances immune responses by increasing ADA activity, a key marker of T-cell function. These findings suggest Herboxid's potential as an effective immunomodulatory agent for therapeutic use.

How to Cite: Anuma A. Zine , Pulkit Marwah , Sandeep Dongre, Minakshi Rajgire , Pushpahas Ballal, Chetan Marwah, Sachin Lohe , Sanjay Wate, (2025) In-Vitro Evaluation Of A Herbomineral Capsule Formulation For Its Immunomodulatory Activity Using Cell Line, *Journal of Carcinogenesis*, *Vol.24, No.2s*, 309-318

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1. INTRODUCTION

The immune system plays a crucial role in defending the body against pathogens, maintaining homeostasis, and eliminating abnormal cells. In recent years, there has been growing interest in the development of immunomodulatory agents derived from natural sources due to their potential efficacy, safety, and lower toxicity profiles compared to synthetic drugs [1]. Among these, herbomineral formulations—combinations of medicinal plants and mineral compounds—have been traditionally used in various systems of medicine, particularly Ayurveda, for enhancing immune responses and promoting general health [2].

Herbomineral formulations are known to exert a wide range of pharmacological activities, including anti-inflammatory, antioxidant, and immunomodulatory effects. These effects are attributed to the synergistic interactions between bioactive phytochemicals and minerals present in the formulation. However, scientific validation of these formulations is essential to substantiate traditional claims and explore their potential for modern therapeutic applications [3].

In vitro cell line models offer a valuable platform for initial screening and mechanistic studies of immunomodulatory activity. Such models allow for the evaluation of key immunological parameters such as cytokine production and lymphocyte proliferation in a controlled environment [4].

The present study aims to investigate the immunomodulatory potential of a standardized herbomineral capsule formulation using established in vitro cell line assays. By assessing its effects on immune cell viability, cytokine expression, and other relevant biomarkers, this research seeks to provide preliminary evidence for its mechanism of action and therapeutic relevance. This study not only contributes to the scientific validation of traditional remedies but also supports the development of alternative immunotherapeutic strategies.

2. MATERIALS AND METHODS

2.1 Procurement of plant material and bhasma

The herbal extracts used in this research were generously provided by the *Herbs India company, Hingna, Nagpur, India*. A total of ten different herbal extracts were supplied, including *Ashwagandha ghan, Amalaki ghan, Guduchi ghan, Tulsipatra ghan, Bhuiamla ghan, Sunthi churna, Marich churna, Pippali churna*, *Loha bhasma*, and *Abhrak bhasma*. These extracts were used as-received, without any modification or alteration, and were essential for the experimental phase of this study.

2.2 Formulation of Herbomineral Capsule

All the herbal extracts and minerals, including Ashwagandha ghan, Amalaki ghan, Guduchi ghan, Bhuiamla ghan, Tulsipatra ghan, Sunthi churna, Marich churna, Pippali churna, Loha bhasma, and Abhrak bhasma, are weighed as per the formula. The weighed quantity of herbal extracts and minerals is mixed thoroughly for uniformity. Capsules are filled by using a hand-operated capsule filling machine.

Development of formulation:

Sr. No.	Material	Quantity
1	*Ashwagandha ghan*	10 g
2	*Amalaki ghan*	10 g
3	*Guduchi ghan*	8 g
4	*Tulsipatra ghan*	8 g
5	*Bhuiamla ghan*	8 g
6	*Sunthi churna*	1 g
7	*Marich churna*	1 g
8	*Pippali churna*	1 g
9	*Loha bhasma*	1 g
10	*Abhrak bhasma*	1.5 g

2.3 In vitro evaluation for immunomodulatory activity

2.3.1 Cell culture

PBMC Cell line was maintained in Dulbecco's Modified Eagle Medium and 10% fetal bovine serum (FCS) and 1% antibiotic-antimycotic solution (Himedia, India), containing 10,000 units of penicillin, 10 mg streptomycin, and 25 mg amphotericin B/ml of culture medium. The cells were routinely incubated in 50 ml, poly-L-lysine-coated tissue culture flasks in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

2.3.2 Preparation of stock solution of herbal drug

The drug was in powder form. 50 mg of powder was added to 5 ml of Dulbecco's Modified Eagle Medium and allowed to dissolve for 24 hours, and then the next day it was filtered through a 0.25-micron syringe filter. The filtrate, which was collected, was used as a stock of 10 mg/ml for further experimental purposes.

2.3.3 LPS and PHA

In this project, LPS (Lipopolysaccharide) and PHA (Phytohemagglutinin) were used to stimulate the inflammatory response, and then the herbal drug was added to evaluate its anti-inflammatory activity.

2.3.4 Measurement of cell viability: Trypan blue exclusion assay

Trypan blue staining was a straightforward method to assess cell membrane integrity and, consequently, cell viability. Isolated and washed cells were evaluated for viability and cell count using a hemocytometer. The goal was to determine the density of viable cells in the culture.

In this assay, viable (living) cells did not take up the dye, while non-viable (dead) cells did, due to compromised cell membranes. Non-viable cells lacked the metabolic capacity to expel the intruding dye.

Procedure:

To prepare the samples, 50 µl of washed cell suspension was mixed with 400 µl of PBS and 50 µl of trypan blue solution in a microcentrifuge tube. The mixture was gently homogenized and loaded into a haemocytometer by carefully touching the edge of the coverslip with the pipette tip to enable capillary filling, avoiding over- or underfilling.

Viable cells (colorless and refractile) and non-viable cells (blue-stained) were counted under a light microscope across all four quadrants of the haemocytometer.

To calculate cell concentration per ml, the following formula was used:

Average number of cells in one large square x dilution factor x 10^4

2.3.5 Measurement of cytotoxicity

Procedure:

Cytotoxicity of the compound Herboxid on peripheral blood mononuclear cells (PBMCs) was assessed using the XTT assay. PBMCs were seeded in a 48-well plate at a density of 2×10^4 cells per 15 µl per well. Herboxid was added at five different concentrations to the experimental wells, while control wells received no drug. The plate was incubated at 37 °C for 48 hours.

Following incubation, 10 µl of XTT reagent was added to each well, and the plate was further incubated at 37 °C for 4 hours. The XTT assay measures mitochondrial enzyme activity in metabolically active (viable) cells, which reduces the XTT reagent to a soluble, colored formazan product. Absorbance was measured at 450 nm using a microplate reader. Results were reported as optical density (OD) values, indicating relative cell viability compared to the untreated control.

2.3.6 Adenosine Deaminase (ADA) Assay

Procedure:

Intracellular adenosine deaminase (ADA) activity was assessed after 72 hours of peripheral blood lymphocyte (PBL) culture. Cells were harvested from 96-well plates by centrifugation, washed twice with ice-cold PBS, and resuspended in 0.1 ml of PBS. Cell lysis was performed by six freeze-thaw cycles (−80 °C and room temperature), followed by centrifugation at 10,000 g for 5 minutes at 4 °C. The supernatants were collected for enzymatic analysis.

ADA activity was measured using a colorimetric assay at 37 °C, based on the method of Giusti and Galanti (1984). The assay relies on the Bertholet reaction, in which ammonia released from adenosine forms a blue indophenol complex, quantified spectrophotometrically at 640 nm. Results were expressed as units/liter/min. (7)

The ADA Activity was calculated as follows:

O.D. of Sample X 25 / O.D. of Standard

2.3.7 Statistical Analysis

All the numerical data were expressed as Mean ± SD. Student t-test was used to compare experimental groups using MedCalc statistical software version 10. A P-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 In vitro evaluation of HM capsule

Table 1 and Figure 1 show that Herboxid, at all tested concentrations (12.5–100 µg/ml), did not reduce PBMC viability compared to the control (O.D. 1.43), with p-values > 0.05. This indicates no significant toxicity.

Table 1: Evaluation of cell viability of Hm drug on PBMC Cells

	Readings for Herboxid Drug				
	Control	12.5 µg/ml	25µg/ml	50µg/ml	100µg/ml
	1.43	2.499	1.29	1.886	1.812
	1.522	2.991	1.495	1.734	1.8
		2.566	1.88	1.625	1.526
Mean	**1.476**	**1.748333333**	**1.555**	**1.748333333**	**1.712666667**
% viability	100	118.4507678	105.3523035	118.4507678	116.034327
SD	0.065053824	0.26682641	0.299541316	0.131089028	0.161769383
Difference		0.27233333	0.079	0.27233333	0.23666666
Standard error		0.194201355	0.216745237	0.103480265	0.123290981
95% CI		-0.56324766 to 1.10791432	-0.853579487 to 1.011579487	-0.172906316 to 0.717572976	-0.293811614 to 0.767144934
Test statistic t		1.402	0.364	2.632	1.92
DF		2	2	2	2
Significance level		P = 0.2959	P = 0.7504	P = 0.1191	P = 0.1949

Table 1: Result of the evaluation of Herboxid drug on PBMC Cells

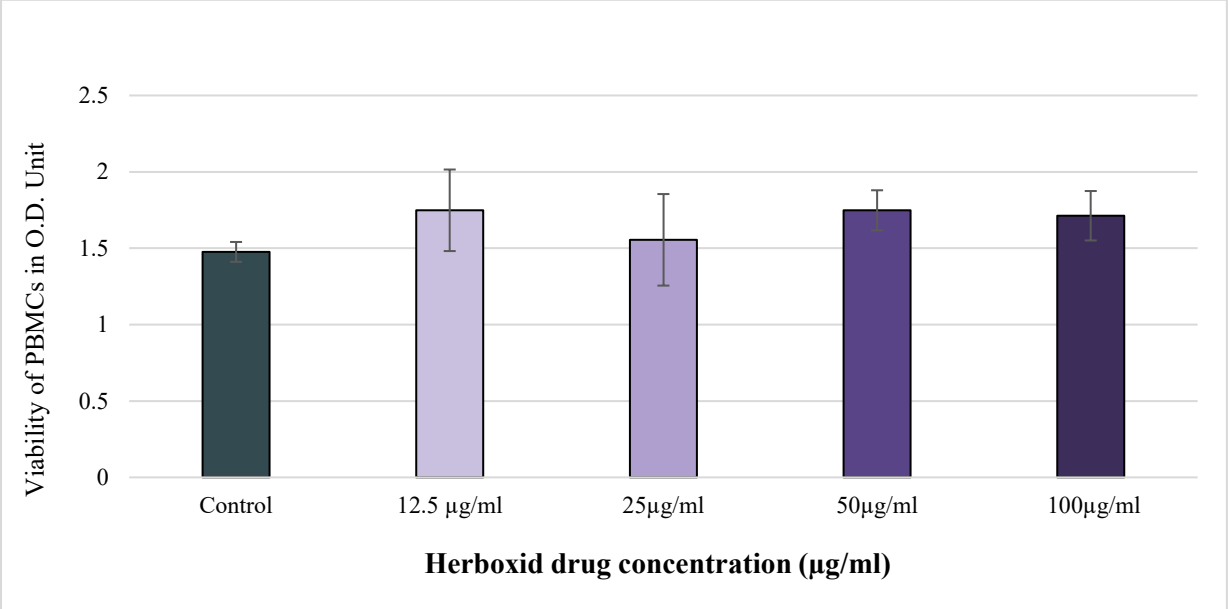


Fig 1: This graph shows the viability (in O.D. Unit) of peripheral blood mononuclear cells (PBMC) with & without Herboxid treatment. P>0.05 vs. control for all concentrations.

3.2 Result of ADA Assay and Morphology of the Cells:

Table 2 and Figure 2 show that Herboxid significantly increased ADA activity in PBMCs in a dose-dependent manner ($P < 0.05$ at 100 µg/ml), suggesting immunomodulatory effects. Morphological changes, including increased cell size, shape variation, granularity, and aggregation, further support Herboxid's role in promoting immune cell activation and proliferation.

ADA Activity					
Mean	4.3333333	5.33333333	6	7.3333333	8.6666667
SD	0.5773503	0.57735027	1	1.1547005	0.5773503
Difference		1	1.66666667	3	4.3333333
Standard error		0.57735026	0.816496578	0.9128709	0.5773503
95% CI		-1.484137672 to 3.484137672	-1.846434559 to 5.179767899	-0.92776656 to 6.92776656	1.849195658 to 6.817471002
Test statistic t		1.732	2.041	3.286	7.506
DF		2	2	2	2
Significance level		P = 0.2254	P = 0.1780	P = 0.0814	P = 0.0173

Table 2: Result of evaluation of Herboxid on ADA Activity

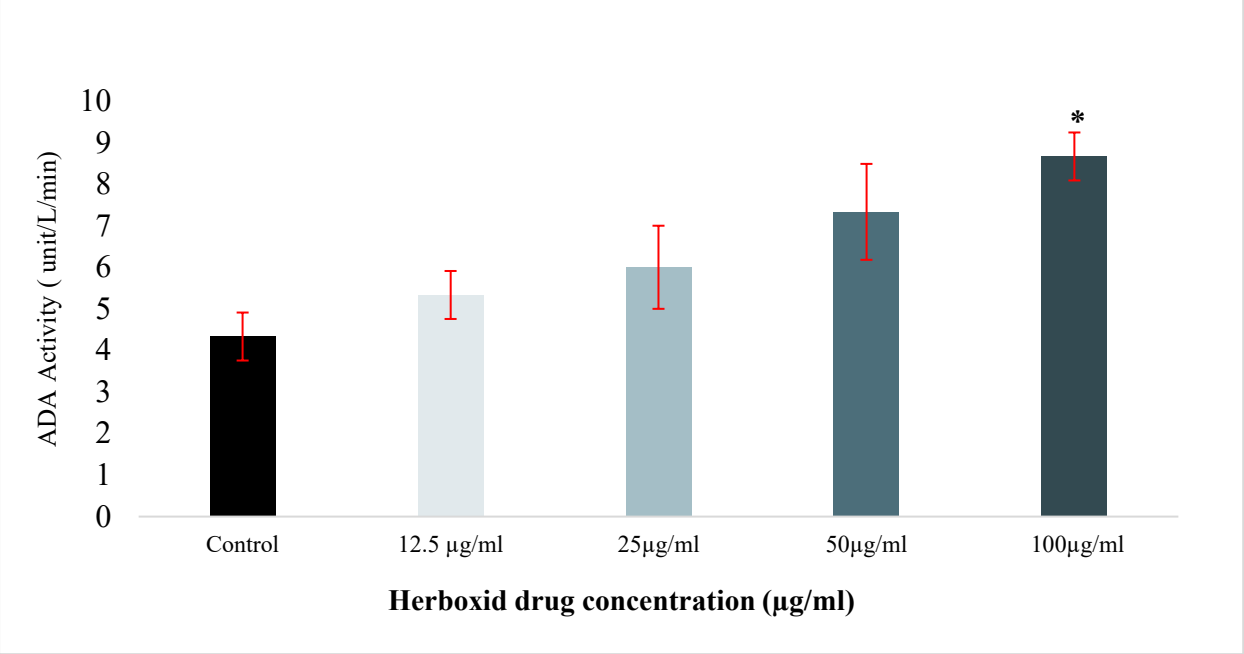


Figure 2. represents the graphs of ADA Assay, after 48 hours of Herboxid treatment. in four different concentrations (like 12.5ug/ml, 250ug/ml, 50ug/ml and 100ug/ml) on PBMC Cells.

P>0.05 vs. control for all the concentrations, except in 100ug/ml Concentration

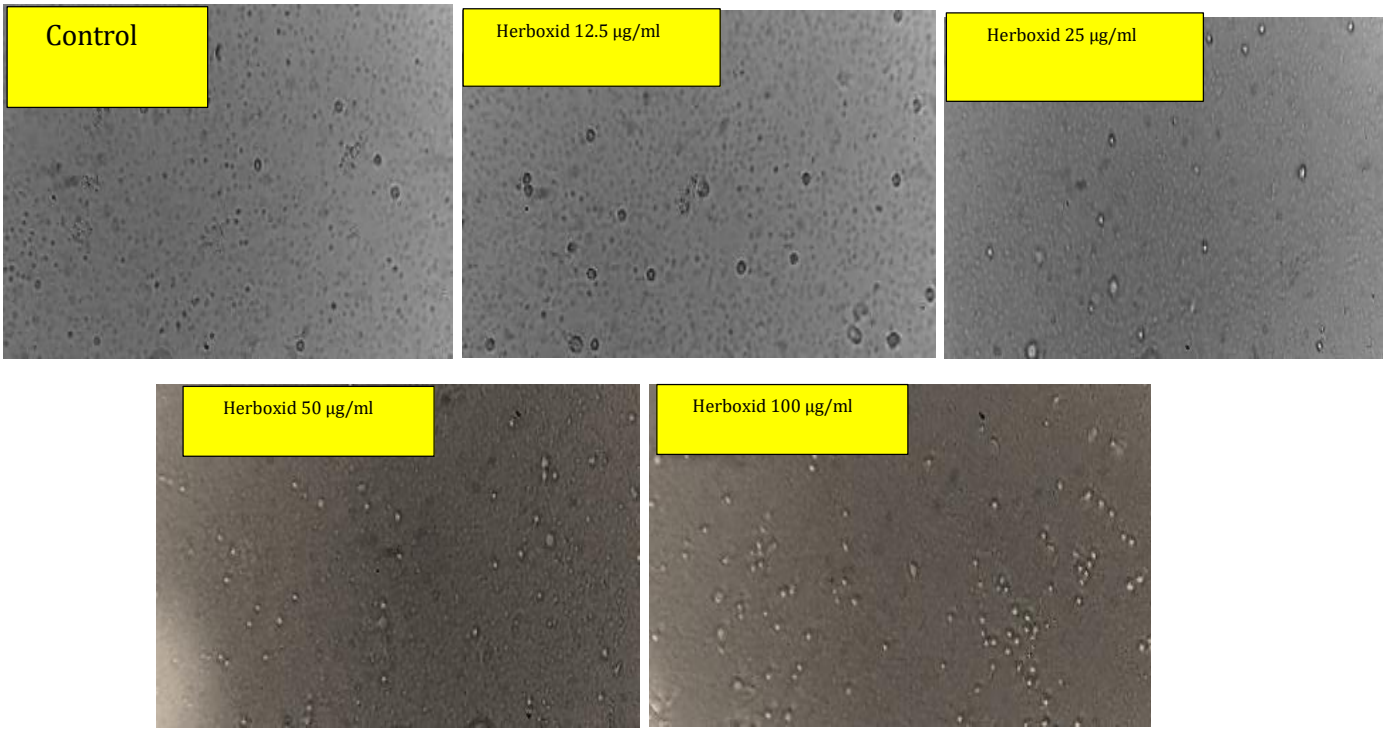


Figure 3 represents morphological changes after treatment with PBMCs

3.5.2 Results of LPS and PHA-induced inflammatory response on PBMC Cells

Figure 4 shows the inflammatory response of PBMCs treated with different LPS and PHA combinations. The combination include\ed LPS 1 µg/µl + PHA 1 µg/µl, LPS 31 µg/µl + PHA 2 µg/µl, LPS 2 µg/µl + PHA 1 µg/µl and LPS 2 µg/µl + PHA 2 µg/µl

A dose-dependent increase in response was observed, with the highest activation seen at LPS 31 µg/µl + PHA 2 µg/µl ($P < 0.05$). Lower concentrations, such as LPS 1 µg/µl + PHA 1 µg/µl, produced a moderate effect, suggesting partial immune cell activation. These results demonstrate how varying concentrations influence PBMC inflammatory responses.

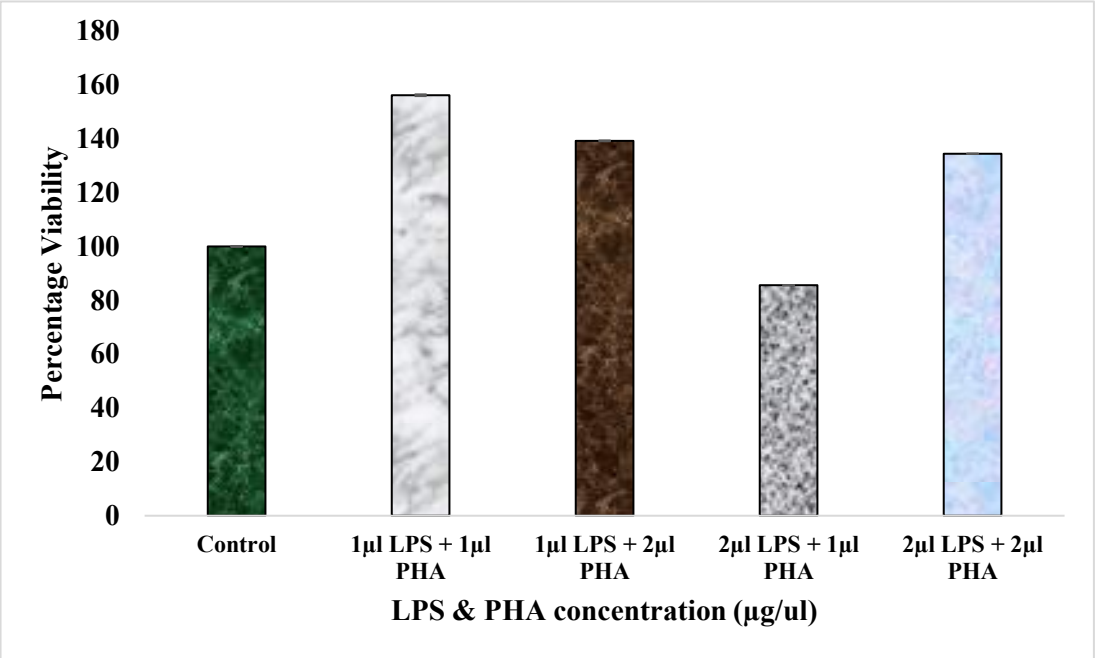


Figure 4: Represents a graph of effect of LPS and PHA on PBMC Cells, when added in Four different combinations with concentrations like LPS 1µg/µl and PHA 1µg/µl, LPS 31µg/µl and PHA 2µg/µl, LPS 2µg/µl and PHA 1µg/µl, and LPS 2µg/µl and PHA 2µg/µl.

Note: $P < 0.05$ vs. control

3.5.3 ADA Activity of LPS and PHA-Induced Inflammatory Response on PBMC Cells

Figure 5 depicts the alterations in Adenosine Deaminase (ADA) activity in PBMC cells upon stimulation with various concentrations of Lipopolysaccharide (LPS) and Phytohemagglutinin (PHA). The graph highlights distinct patterns of enzymatic activity corresponding to the immune activation induced by LPS and PHA.

The data show a dose-dependent increase in ADA activity, with higher LPS and PHA concentrations causing a marked rise, indicating stronger immune activation, while lower doses produced moderate effects.

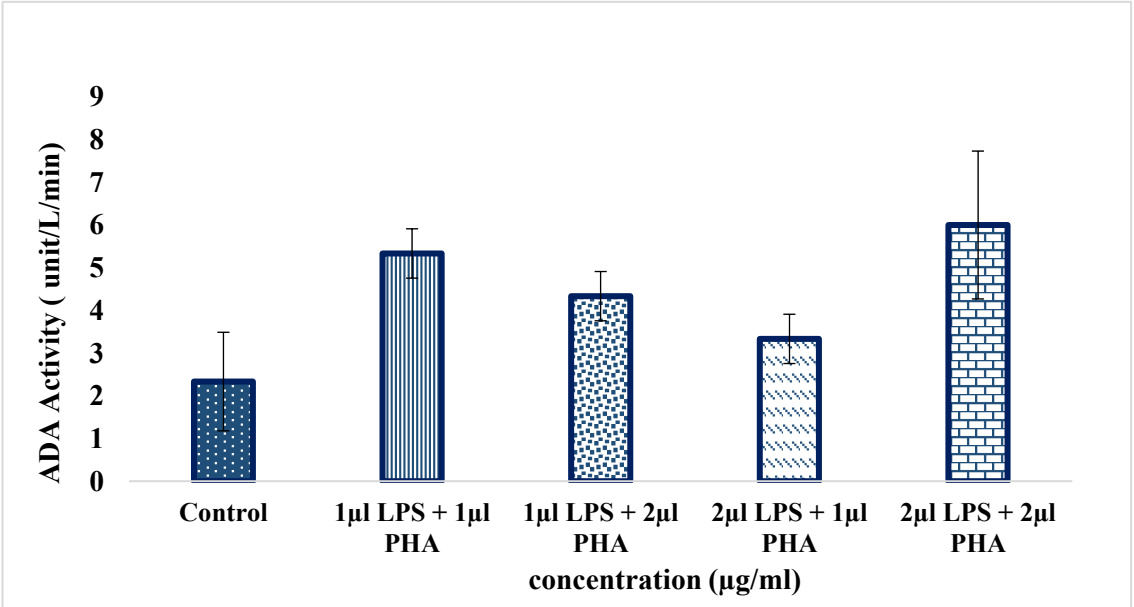


Figure 5: The graph illustrates the changes in ADA activity at various concentrations of LPS (Lipopolysaccharide) and PHA (Phytohemagglutinin) stimulation. Data points represent the enzymatic activity levels in PBMCs, with LPS and PHA showing distinct effects on ADA activity in response to immune activation. Error bars indicate the standard deviation of triplicate experiments.

Note: P < 0.05 vs. control

3.5.4 Results of Cytotoxicity Assay:

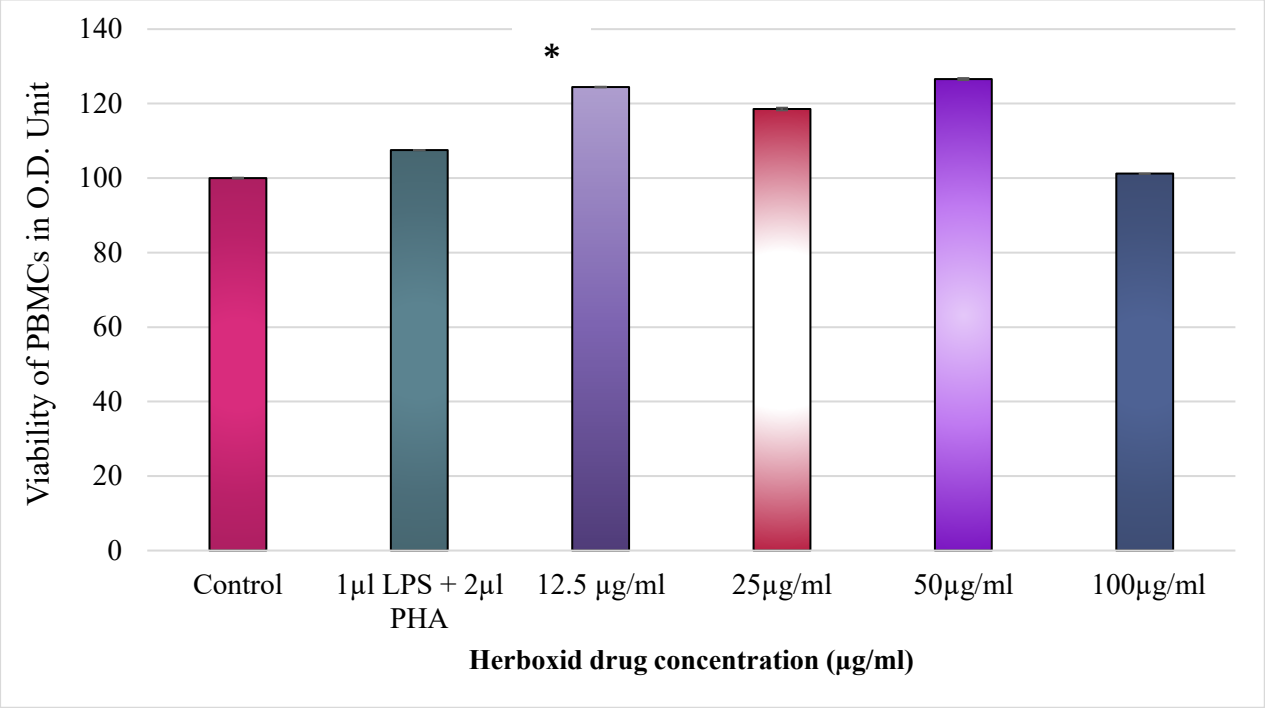


Figure 6 shows a dose-dependent effect of Herboxid on cell viability. Viability peaked at 50 µg/mL (126.59%) and 12.5 µg/mL (124.45%), indicating beneficial effects. The positive control (1 µL LPS + 2 µL PHA) also increased viability (107.52%), confirming the setup. At 100 µg/mL, viability dropped to 101.23%, near control levels, suggesting possible cytotoxicity or saturation at high doses.

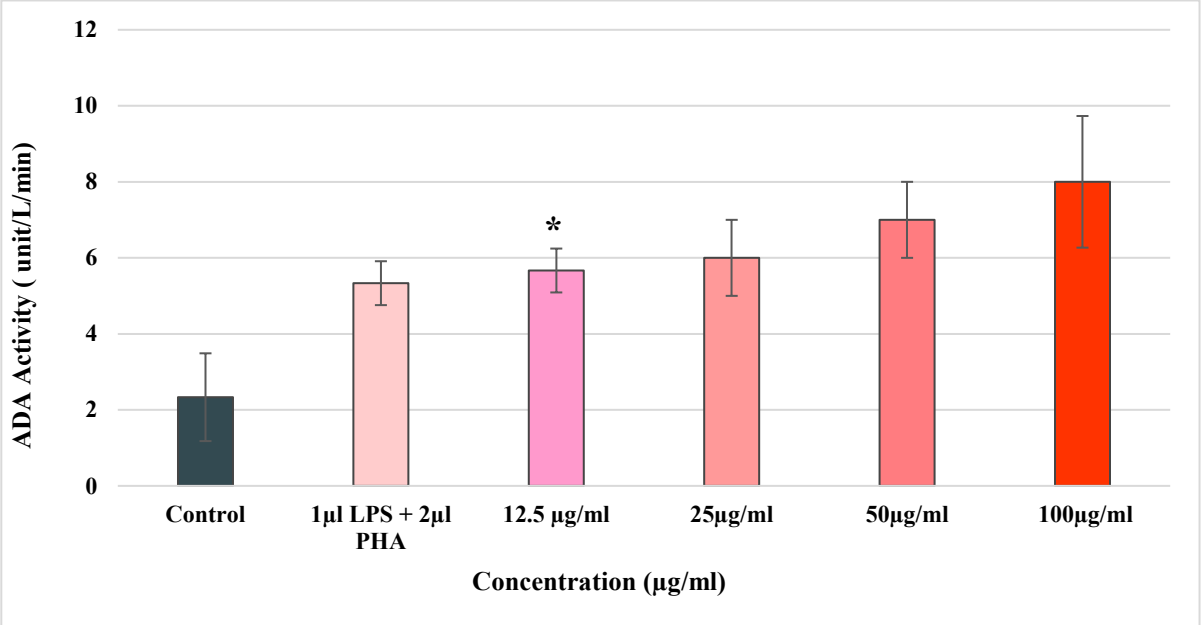
Figure 6: Dose-dependent effect of Herboxid on cell viability in PBMC cells. The data show the percentage viability at various concentrations, with the highest viability observed at 50 µg/mL (126.59%) and 12.5 µg/mL (124.45%). The positive control (1 µL LPS + 2 µL PHA) showed increased viability (107.52%). At 100 µg/mL, viability decreased to 101.23%, suggesting potential cytotoxicity or saturation at higher doses. Error bars represent standard deviation.

Note: P < 0.05 Vs Control

P < 0.05 Vs 1 µL LPS + 2 µL PHA

3.5.5 Dose-Dependent Enhancement of ADA Activity by Herboxid and Immunomodulatory Insights with LPS and PHA Controls

ADA activity showed a clear concentration-dependent increase with Herboxid treatment. The control group had the lowest activity (~2 U/L/min), while the positive control (1 µL LPS + 2 µL PHA) reached ~5 U/L/min, confirming the validity of the experimental setup (P < 0.05). Herboxid treatment significantly elevated ADA levels, starting at ~6 U/L/min at 12.5 µg/mL and peaking at ~10–11 U/L/min at 100 µg/mL. This indicates a strong stimulatory effect on immune activation. Standard deviation bars were minimal, suggesting consistent and reliable data across all concentrations



Note: # P < 0.05 Vs Control

*** P < 0.05 Vs 1 µL LPS + 2 µL PHA**

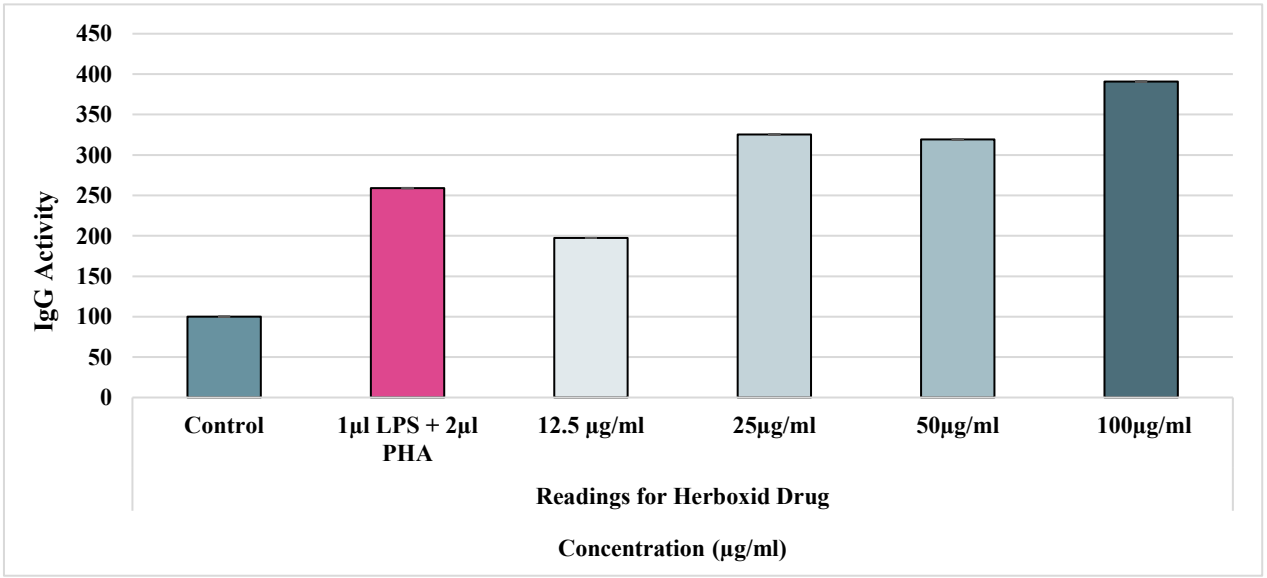
Fig. 7 The results of the ADA (Adenosine Deaminase) assay demonstrate a dose-dependent increase in enzyme activity across the tested concentrations of Herboxid

3.5.6 Effect of Herboxid on LPS and PHA-Induced IgG Activity Production:

The graph shows a dose-dependent increase in IgG activity with Herboxid treatment. The control group had the lowest levels, while the positive control (LPS + PHA) showed a significant increase ($P < 0.05$). Herboxid progressively elevated IgG activity across all tested concentrations, with the highest response at 100 µg/ml, suggesting potential immunomodulatory effects.

Fig 8: The graph illustrates the impact of Herboxid at varying concentrations on IgG activity.

Note: P < 0.05 vs. control P < 0.05 Vs 1 µL LPS + 2 µL PHAs



4. DISCUSSION

This study demonstrates that the herbomineral formulation Herboxid exhibits significant immunomodulatory activity in vitro using PBMCs as the model system. The formulation showed no cytotoxicity at the tested concentrations (12.5–100 µg/mL) and even enhanced cell viability, with a peak at 50 µg/mL. This suggests that the formulation is safe and may

promote lymphocyte proliferation, aligning with the known adaptogenic properties of *Withania somnifera* and *Tinospora cordifolia* used in the formulation.

Herboxid treatment produced a dose-dependent increase in adenosine deaminase (ADA) activity, which is a critical marker of T-lymphocyte activation and cell-mediated immunity. The highest ADA activity observed at 100 µg/mL indicates strong immunostimulatory potential. Additionally, enhanced IgG production and morphological changes in PBMCs, including increased cell size and aggregation, support the activation of both cellular and humoral immune responses.

The synergistic response observed with LPS and PHA co-treatment further confirms the formulation's ability to augment innate and adaptive immune activation. These effects are likely the result of the combined action of phytochemicals and mineral components, where bioactive compounds stimulate immune pathways while mineral bhasma contribute to enzymatic functions.

These findings are in line with the traditional Ayurvedic view that herbomineral preparations may help support immunity. However, as an in vitro study, it is limited in replicating the complexity of the in vivo immune system. Further studies, including animal models and clinical trials, are required to validate efficacy, optimize dosage, and confirm safety in human applications.

Overall, Herboxid demonstrates promising immunomodulatory properties, supporting its potential role as a natural immunotherapeutic or supportive agent in conditions of immune suppression.

5. CONCLUSION:

In this study, the immunomodulatory activity of the Herboxid capsule was evaluated in Peripheral Blood Mononuclear Cells (PBMC). The cytotoxic effects of this herbal formulation were successfully assessed, which allowed the determination of an appropriate therapeutic dose. Additionally, the impact of Herboxid on adenosine deaminase (ADA) activity, a specific marker for T-cell function, was investigated. As a result, valuable insights into the potential therapeutic benefits of Herboxid in modulating immune responses were obtained, emphasizing its relevance in immunotherapy

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