

Effect of a Novel Ashwagandha-based Herbomineral Formulation on Pro-inflammatory Cytokines Expression in Mouse Splenocyte Cells: A Potential Immunomodulator

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ABSTRACT

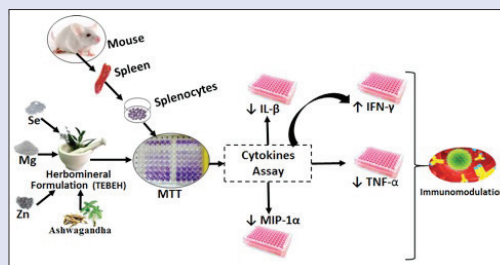
Background: Herbomineral formulations are momentous in an audience of worldwide by virtue of their holistic approach to life. These formulations are widely used as complementary therapies in immunocompromised patients including cancer. Still, there is the need of cost-effective and safe herbomineral-based formulation that can modulate immune response by the regulation of cytokines cascades. **Objective:** Current study, we investigated immunomodulatory effect of TEBEH in LPS-induced cytokines expression levels in mouse splenocytes *in vitro*. **Materials and Methods:** The most effective and safe concentrations of TEBEH were chosen by determining the cell viability of splenocytes using MTT assay. The pro-inflammatory cytokines such as TNF- α , IL-1 β , MIP-1 α , and IFN- γ were measured in cell supernatants using ELISA. **Results:** MTT data showed TEBEH formulation was found safe up to 10.53 $\mu\text{g/mL}$. At nontoxic concentrations (0.0001053–10.53 $\mu\text{g/mL}$), TEBEH significantly ($P \leq 0.001$) inhibited the expressions of TNF- α , IL-1 β , and MIP-1 α in mouse splenocytes as compared with vehicle control. **Conclusion:** In summary, TEBEH may indeed promote an anti-inflammatory environment by suppression of pro-inflammatory cytokines. These observations indicated that TEBEH has potential effects in downregulating the immune system and might be developed as a useful anti-inflammatory product for various inflammatory disorders.

Key words: Inflammation, immunomodulation, splenocytes, pro-inflammatory cytokines, ELISA

SUMMARY

- The present study was undertaken to evaluate an immunomodulatory effect of the herbomineral formulation in LPS-induced mouse splenocytes with the measurement of cytokines expression such as TNF- α , IL-1 β , MIP-1 α and IFN- γ . The results showed that the expression of TNF- α , IL-1 β , and MIP-1 α was

significantly down-regulated while, IFN- γ was significantly up-regulated in mouse splenocytes. It is hypothesized that modulation of the proinflammatory cytokines might occur via NF- κB pathway. Therefore, the herbomineral test formulation might act as an effective anti-inflammatory and immunomodulatory product, and this can be used as a complementary and alternative treatment for the prevention of various types of inflammatory and auto-immune disorders.



Abbreviations used: LPS: Lipopolysaccharide, IL: Interleukin; NF- κB : Nuclear factor kappa-B, TNF- α : Tumor necrosis factor alpha, MIP-1 α : Macrophage inflammatory protein-1 α , IFN- γ : Interferon, MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium, ELISA: Enzyme linked immune sorbent assay, ANOVA: Analysis of variance,

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INTRODUCTION

Cytokines are the key factors in acute and chronic inflammation. Inflammation is characterized by an interplay between pro- and anti-inflammatory cytokines. The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response.^[1-3] The narrow therapeutic range and serious adverse effects of immunosuppressive drugs have proved almost insurmountable obstacles.^[2,3] As a consequence, the finding of lead compound with markedly lower toxicity and higher immunosuppressive activity is of great interest. In recent years, medicinal plants that have been practiced for thousands of years in clinic provide a vast source of pharmaceutical material for the development of effective drugs and offer some unique advantages with low toxicity profiles.^[4,5] Many herbal extracts either *per se* or in combinations with medicinal plants or with minerals may have activities on cytokines.^[6] Herbal medicinal preparations can favorably regulate the whole immune system.^[7] Therefore, anti-inflammatory phytomedicines may be beneficial for the management of chronic

inflammatory disorders due to overactivated immunity.^[8] Vast scientific studies are going on toward dietary phytochemicals that have played a significant role in drug discovery and development, especially in the case of antiproliferation, cytotoxic, and immunomodulatory effects.^[9] The plant-based immunomodulators are gaining special interest, since their possible use in modern medicine was suggested.^[10] TEBEH is a novel proprietary herbomineral formulation consisting of four

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ingredients; an herbal extract (ashwagandha root extract) along with a mixture of minerals (zinc chloride, magnesium gluconate hydrate, and sodium selenate). Several reports have demonstrated its potent antibacterial, immunomodulatory, and antitumor activity due to the presence of withanolides as major active ingredient.^[11,12] Zinc potentiates the immunomodulatory effect by increasing the levels of leukocyte count and the phagocytic index.^[13] Sodium selenate had exhibited the level of the phagocytic index and restored the level of IL-1, IL-6, and TNF- α near to normal.^[14] The selenium-containing compounds are biologically active and can modify neutrophil functions.^[15] Magnesium reduces the production of an inflammatory cytokine through activation of *NF- κ B* pathways, which is a novel innate immunomodulatory mechanism.^[16] The different type of immune cells such as dendritic cells, macrophages, and the spleen can play an important role to stop the acute/chronic inflammation and retrieve a steady-state strategy through the secretion of immunomodulating cytokines.^[17,18] These immune cells have been reported to be useful as cellular models for *in vitro* studies. Therefore, authors used splenocytes to assess the effect of the herbomineral formulation TEBEH on *in vitro* cell culture. In market, several herbal extracts and synthetic immunomodulatory agents are available for the management of various patients suffering from autoimmune diseases, allergies, organ transplantations, and systemic immunocompromise. However, so far, there is no report published about the immunomodulatory activity of *Withania somnifera* extract (synonym Indian ginseng) based herbomineral formulation, TEBEH. Therefore, the aim of this study was to illustrate the immunomodulatory activity of TEBEH in lipopolysaccharide (LPS)-induced mouse splenocytes with the estimation of defined pro-inflammatory cytokines expression level.

MATERIALS AND METHODS

Chemicals

MTT, LPS, L-glutamine, RPMI-1640, penicillin, HEPES, streptomycin, 2-mercaptoethanol, and rapamycin were purchased from Sigma Chemical Co., St. Louis, MO. ELISA kits for all cytokines such as TNF- α , MIP-1 α , and IL-1 β were purchased from R&D Systems, USA. FBS was procured from GIBCO, USA. *W. somnifera* (commonly known as ashwagandha) root extract powder was procured from Sanat Products Ltd., India. The root extract was identified and authenticated by Dabur Research Foundation (DRF), Ghaziabad, India, with voucher number (DOM_EXC/E63). Zinc chloride and magnesium(II) gluconate hydrate were procured from TCI, Japan. Sodium selenate was procured from Alfa Aesar, USA.

Experimental design

The experiment was designed into seven groups. Group 1 contained the splenocyte cells without LPS, denoted as normal control (NC). Group 2 was served as stimulant group that included cells, DMSO along with LPS defined as vehicle control (VC), i.e., negative control. Group 3 was defined as positive control, rapamycin (1 nM). The test item group that included splenocyte cells with LPS along with TEBEH at various concentrations of 0.00001053–10.53 μ g/mL.

Composition and preparation of herbomineral product, TEBEH

The required quantity of each individual ingredient was mixed together with the concentration of 371.64 mg/mL TEBEH. The composition of each ingredients selected is as follows: zinc chloride (1.04 mg/mL), sodium selenate (1.195 μ g/mL), magnesium gluconate (170.6 mg/mL), and ashwagandha root extract powder (\geq 5% of total withanolides, 200 mg/mL). The above formulation was vortexed to achieve a homogenous

solution, which was considered as 100% stock solution. The above stock solution was further diluted in serum-free medium (SFM) to obtain a range of concentrations (in % v/v) for subsequent treatment.

Animal care and housing

C57BL/6 male mouse (8 weeks old) was purchased from Ms. Vivo Biotech Ltd., Hyderabad, India. Rodent laboratory chow diet and drinking tap water were provided *ad libitum* and maintained under controlled conditions a temperature of $22 \pm 3^\circ\text{C}$, humidity of 30–70% and a 12-h light/12-h dark cycle. The approval of the institutional animal ethics committee (IAEC) was obtained before carrying out the animal experiment.

Mouse splenocyte cultures

C57BL/6 male mouse was sacrificed, and the spleen was removed and ground by passing through a sterile plastic strainer under aseptic conditions. After that, the cells were centrifuged twice at 1000g for 5 min. Cells were lysed by lysis buffer (0.15 M NH_4Cl , 0.01 M NaHCO_3 , and 0.1 mM EDTA, pH 7.4) and then the cell pellets were washed twice with RPMI-1640 medium. Further, the cells were resuspended in a sterile RPMI-1640 medium (RPMI-1640 medium plus 10% FBS, 2 mM glutamine, streptomycin and 100 IU/mL penicillin, 15 mM HEPES, and 50 mM 2-mercaptoethanol). The cell counts were performed with the help of hemocytometer and cell viability was determined using trypan-blue dye exclusion technique. The cells were cultured in 96-well tissue culture plates with 0.2×10^6 cells per well. They were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for the specified period.

Cell culture and test item (TEBEH) treatment

The splenocyte (0.2×10^6 cells per well) cells were grown in 96-well culture plates using RPMI-1640 medium supplemented with 10% FBS, 100 μ g/mL of streptomycin, and 100 IU/mL of penicillin. The LPS (0.5 μ g/mL) induced splenocyte cells cultures were grown for 48 h at 37°C in a humidified CO_2 incubator (5% CO_2). The effect of cytotoxicity was determined by exposing cells to different concentrations of TEBEH in a RPMI-1640 medium. The various concentrations of TEBEH were used from 0.00001053–10.53 μ g/mL in splenocytes cell culture. The respective VC kept in the assay was DMSO with LPS.

MTT assay

Cytotoxicity was determined by exposing cells to different concentrations of TEBEH in a RPMI. The respective VC kept in the assay was DMSO with LPS. The number of viable cells was estimated based on the conversion of MTT to formazan dye using a mitochondrial enzyme. The effect of TEBEH on cell viability of splenocytes was determined with the help of the following equation:

$$\% \text{ Cell viability} = 100 - \% \text{ cytotoxicity}$$

where % Cytotoxicity = $\{(\text{O.D. of control cells} - \text{O.D. of cells treated with TEBEH}) / \text{OD of control cells}\} \times 100$.

Cytokines assay

The effect of herbomineral-based formulation TEBEH on the production of TNF- α , IL-1 β , MIP-1 α , and IFN- γ were measured by ELISA method using culture supernatants using a Biotek reader (SIAFRT/Synergy HT multimode reader). For the estimation of TNF- α , IL-1 β , MIP-1 α , and IFN- γ in LPS (0.5 μ g/mL) induced splenocytes were exposed to TEBEH at selected nontoxic concentrations (0.00001053–10.53 μ g/mL). After 48h of incubation, supernatants were analyzed for the secreted levels of cytokines using ELISA as per manufacturer's instructions.^[17-19]

STATISTICAL ANALYSIS

Data analysis was performed with Sigma Plot Statistical Software (Version 11.0). Differences between means (in triplicates) were assessed for statistical differences using one-way analysis of variance (ANOVA) and Student's *t*-test. *P* less than 0.05 was statistically significant. The results are shown as mean ± standard error of mean (SEM).

RESULTS

Assessment of *in vitro* immune cells viability by MTT assay

The concentration that resulted in more than 150% viability was selected for subsequent cytokines estimation. The normal splenocyte cells and VC groups showed 100% cell viability. The rapamycin showed 136.52% cell viability at 1 nM. The percentage cell viability was increased in all the tested concentrations (0.00001053–10.05 µg/mL) with respect to the VC group, which might be due to proliferation in cell culture [Figure 1].

Expression of TNF-α in mouse splenocytes

The results of TEBEH demonstrated a significant suppression of TNF-α levels by 12.80, 13.69, 22.22% (*P* ≤ 0.001), 24.31% (*P* ≤ 0.001), and 30.46% (*P* ≤ 0.001) at the tested concentrations, i.e., at 0.001053, 0.01053, 0.1053, 1.053, and 10.53 µg/mL, respectively, as compared with the VC. The test item at high concentration (10.53 µg/mL) with respect to rest of the tested concentrations showed better response by suppressing the level of TNF-α [Figure 2].

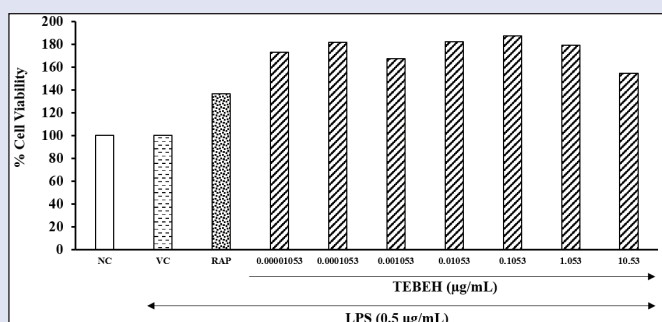


Figure 1: Measurement of cytotoxicity by MTT assay in splenocyte cells. Values are represented as mean ± SEM (triplicates). NC: Normal control; RAP: Rapamycin (1 nM); VC: Vehicle control

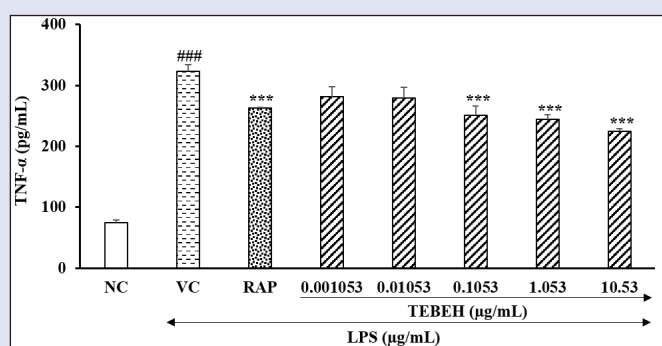


Figure 2: Effect of TEBEH on TNF-α secretion in LPS-mediated splenocyte cells was measured 48 h after exposure. ****P* ≤ 0.001 vs. VC and ###*P* ≤ 0.001 vs. NC (using one-way ANOVA)

Expression of IL-1β in mouse splenocytes

The level of IL-1β in the normal control (NC) group was 19.76 ± 1.13 pg/mL and significantly increased by 79.10% in the VC group (35.37 ± 3.94 pg/mL) after induction with LPS. The test item TEBEH showed a significant (*P* ≤ 0.05) inhibition of IL-1β secretion at the two highest tested concentrations, i.e., at 0.01053 and 0.1053 µg/mL by 34.24 and 37.23%, respectively, as compared with the VC group [Figure 3].

Modulation of MIP-1α expression in mouse splenocytes

The test item TEBEH showed significant (*P* ≤ 0.001) inhibition of MIP-1α secretion by 24.27, 41.30, 45.72, and 18.11% at the tested concentrations, i.e., at 0.00001053, 0.0001053, 0.001053, and 0.01053 µg/mL, respectively, as compared with the VC group [Figure 4].

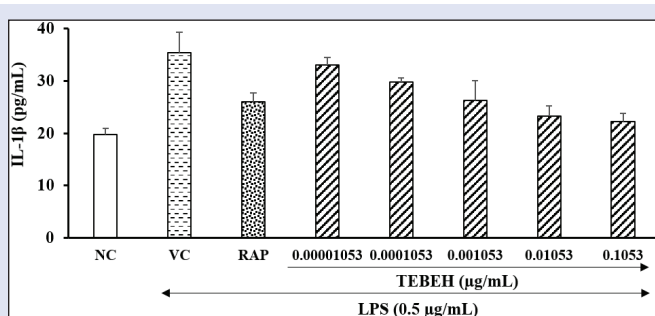


Figure 3: Inhibition of LPS-mediated production of IL-1β by TEBEH. **P* ≤ 0.01 vs. VC (using one-way ANOVA)

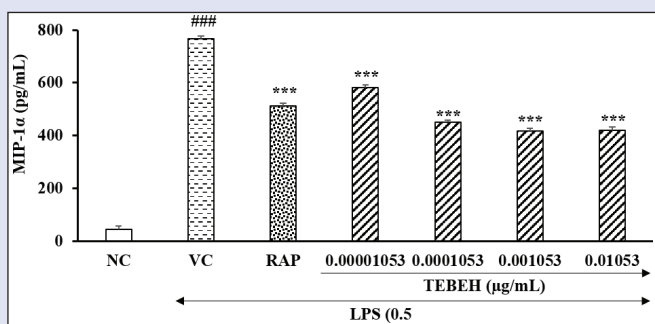


Figure 4: Effect of TEBEH on the expression of MIP-1α in LPS-mediated splenocytes. ###*P* ≤ 0.001 vs. NC and ****P* ≤ 0.001 vs. VC (using one-way ANOVA)

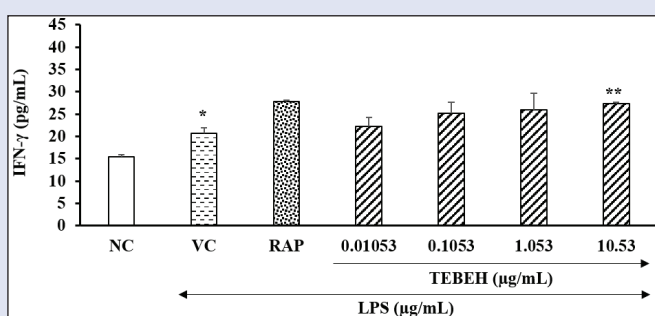


Figure 5: Dose-dependent inhibition of LPS-mediated production of IFN-γ by TEBEH. ***P* = 0.01 vs. VC and **P* ≤ 0.05 vs. NC (using paired *t*-test)

Expression of IFN- γ in mouse splenocytes

The test product TEBEH demonstrated an elevation of IFN- γ as compared with the LPS-stimulated VC group. The result showed the expression of IFN- γ was significantly increased by 21.58, 25.79, and 32.22% ($P \leq 0.01$) at 0.1053, 1.053, and 10.53 $\mu\text{g/mL}$, respectively, as compared to the VC [Figure 5].

DISCUSSION

Cytokines play a key role in immunomodulation. A hallmark of immunity is the production of a multifaceted array of inflammatory cytokines. Disease progression or regression could be possible by estimation the up- and/or downregulation of cytokine signaling cascades in response to pathologic or therapeutic interventions.^[20,21] Immunomodulators can modify the activity of immune function through the impulsive modulation of cytokines.^[22,23] Infection and tissue injury results in alterations in host metabolic and immune homeostasis. These changes result in the secretion of endogenous mediators from a complex cascade of mononuclear phagocyte process. Among these, the most vital host proteins are called cytokines.^[24]

The use of herbomineral products to maintain or improve health has gradually increased across the globe over the last couple of years. Moreover, formulating new products that have the ability to improve the overall health by reducing inflammation is essential because of the potential for long-term effectiveness, decreased toxicities, and lower costs. Therefore, mouse splenocytes was selected as test system to study immune responses and to investigate the anti-inflammatory effects of a newly developed proprietary herbomineral product TEBEH. The TEBEH is a novel proprietary herbomineral formulation containing a mixture of herbal extract like ashwagandha and three minerals, viz., zinc chloride, magnesium gluconate hydrate, and sodium selenate.

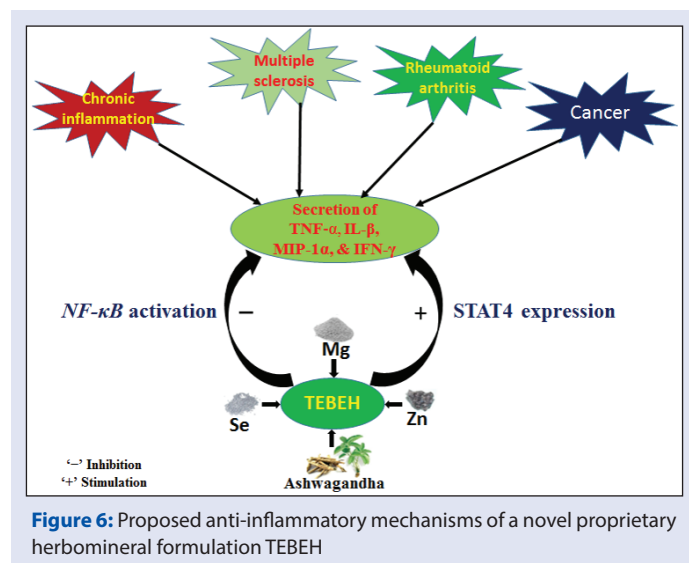
The rationale for selection of each constituents in the TEBEH based on the immunomodulatory activity through same central signaling pathways of the selected component *per se* with specific salt to get the desired solubility of the formulation. The possible anti-inflammatory mechanisms of TEBEH are shown in Figure 6. The metabolic activity is evaluated by measuring the activity of a mitochondrial enzyme succinate dehydrogenase using the MTT test. This test is widely used in the *in vitro* evaluation of the toxicity of any test item.^[25] On the basis of the cell viability using MTT assay, we showed that TEBEH was found to be safe at all the tested concentrations with increased percentage viability ranges from 154.57 to 187.17%. Hence, all the tested concentrations were

selected for the estimation of cytokines. The maximum cell viability was reported as 187.17% at 0.1053 $\mu\text{g/mL}$ [Figure 1]. It can be concluded that the test item showed an increased cell viability at the specified concentrations with respect to the both normal and VC groups. The pro-inflammatory cytokines TNF- α play a central role in inflammation,^[26] immune modulation,^[27] and lymphocyte activation.^[28] In most of the immune-mediated disorders, TNF- α was anticipated as the major factor that controls many disease pathologies.^[29] The role of TNF- α and its alteration was significantly reported to improve insulin resistance, lipid profiles, and so on, in chronic inflammatory diseases patients.^[30] Our results showed a significant downregulation of the expression of TNF- α at the concentration ranging from 0.1053 to 10.53 μL . It is evident that the herbomineral formulation TEBEH could be used against inflammatory disorders by regulating the expression of TNF- α . The importance of IL-1 β expression in immunologic and inflammatory functions during infections is well established.^[31] Results exhibited a significant inhibition of the expression of IL-1 β at the concentrations ranging from 0.01053 to 10.53 μL . The inhibitory effect of TEBEH might play an important role in mediating auto-inflammatory diseases as a complementary and alternate medicine. The results suggest that at higher concentration TEBEH showed better immunosuppressive activity with respect to lower tested concentrations. MIP-1 α plays an important role in mediating the acute inflammatory response in trauma hemorrhage and reported that MIP-1 α reduction could be beneficial in minimizing the inflammatory responses in several diseases.^[32] The data revealed that MIP-1 α level was significantly suppressed at all the tested concentrations after exposure of the novel herbomineral formulation TEBEH in splenocyte cells.

Numerous literatures suggest that IFN- γ expression play a key role in the regulation of visceral adipose tissue inflammatory response,^[33] regulate glucose homeostasis,^[34] and inhibit the inflammatory response of macrophage cells^[35] and many other important inflammatory disorders. This study showed that the level of IFN- γ was significantly increased at higher concentration (10.53 $\mu\text{g/mL}$). Blanchard *et al.* reported that bacterial LPS had induced the expression of pro-inflammatory cytokine IFN- α/β in fresh splenocytes culture by stimulation of the B lymphocytes and macrophages. Because the induction of IFN- γ by bacterial LPS may play an important role in resistance/recovery mechanisms against bacterial infections,^[36] it also emphasized that IFN- γ has the bactericidal and cytotoxic potential of macrophages. From this experiment in the VC group, the level of IFN- γ expression was remarkably increased; which might be due to influence of bacterial LPS. Besides, increased plasma level of IFN- γ has been evident in acute HIV infection that determines the inflammatory cytokine responses.^[37] Increasing levels of IFN- γ can be defined as crucial macrophage activator and also upregulate macrophage antimicrobial activity.^[38] Thus, IFN- γ possibly is upregulated due to upregulation of STAT4 expression through Th1 CD4 T-cell differentiation.^[39] Therefore, it can be concluded that the tested herbomineral formulation TEBEH would be able to increase the production of IFN- γ , which might be used as a supplement in the inflammatory disorders. For instance, ashwagandha was shown to inhibit TNF- α -induced nuclear factor-kappa B (*NF- κ B*) activation in human myelomonoblastic leukemia cells,^[40,41] zinc has the ability to induce or inhibit the activation of *NF- κ B*,^[42] and magnesium plays a critical regulatory role in *NF- κ B* activation.^[16] Overall, the herbomineral-based formulation, TEBEH, remarkably downregulates the expression of the pro-inflammatory cytokines like TNF- α , MIP-1 α , and IL-1 β and upregulates IFN- γ in splenocyte cells.

CONCLUSION

The study results summarize that TEBEH showed better and significant inhibition of pro-inflammatory cytokines (TNF- α and IL-1 β) and



chemokine (MIP-1 α) expression as compared with the VC group in mouse splenocyte cells. In brief, the expression of MIP-1 α was suppressed at all the tested concentrations. Additionally, the level of IFN- γ was significantly upregulated in the highest concentration. Overall, data indicated a significant reduction of TNF- α , MIP-1 α , and IL-1 β and elevation of IFN- γ the pro-inflammatory mediators upon exposure with the TEBEH on splenocyte cells. In conclusion, the herbomineral-based formulation TEBEH might act as an effective anti-inflammatory product and can be used as a complementary and alternative treatment and prevention of various types of inflammatory disorders.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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