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-1β, MIP-1α, and IFN-γ were measured in cell supernatants using ELISA. Results: MTT data showed TEBEH formulation was found safe up to 10.53 µg/mL. At nontoxic concentrations (0.0001052–10.53 µg/mL), TEBEH significantly (P ≤ 0.001) inhibited the expressions of TNF-α, IL-1β, and MIP-1α 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-1β, MIP-1α and IFN-γ. The results showed that the expression of TNF-α, IL-1β, and MIP-1α 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.

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.[1,2] The narrow therapeutic range and serious adverse effects of immunosuppressive drugs have proved almost insurmountable obstacles.[3,4] 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

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

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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 biologically active and can modify neutrophil functions.[15] Magnesium reduces the production of an inflammatory cytokine through activation of NF-κB 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.

The required quantity of each individual ingredient was mixed together with the concentration of 37.64 mg/mL. The composition of each ingredients selected is as follows: zinc chloride (1.04 mg/mL), sodium selenate (1.195 µg/mL), magnesium gluconate (170.6 mg/mL), and ashwagandha root extract powder (≥ 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.

Cytokines assay
The effect of herbomineral-based formulation TEBEH on the production of TNF-α, IL-1β, MIP-1α, and IFN-γ were measured by ELISA method using culture supernatants using a Biotech reader (SIAFRT/Synergy HT multimode reader). For the estimation of TNF-α, IL-1β, MIP-1α, and IFN-γ in LPS (0.5 µg/mL) induced splenocyte cultures were grown for 48 h at 37°C in a humidified atmosphere containing 5% CO₂ for the specified period.

Cell culture and test item (TEBEH) treatment
The splenocyte (0.2 × 10⁶ cells per well) cells were grown in RPMI-1640 medium. The various concentrations of TEBEH were used from 0.00001053–10.53 µg/mL in splenocytes cell culture. The respective VC kept in the assay was DMSO with LPS.

MATERIALS AND METHODS

Chemicals
MTT, LPS, 1-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-1α, and IL-1β 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 Alfa Aesar, USA.

Composition and preparation of herbomineral product, TEBEH
The required quantity of each individual ingredient was mixed together 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 biologically active and can modify neutrophil functions.[15] Magnesium reduces the production of an inflammatory cytokine through activation of NF-κB 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.

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 µg/mL.

Cytokines assay
The effect of herbomineral-based formulation TEBEH on the production of TNF-α, IL-1β, MIP-1α, and IFN-γ were measured by ELISA method using culture supernatants using a Biotech reader (SIAFRT/Synergy HT multimode reader). For the estimation of TNF-α, IL-1β, MIP-1α, and IFN-γ in LPS (0.5 µg/mL) induced splenocytes were exposed to TEBEH at selected nontoxic concentrations (0.00001053–10.53 µg/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 0.0001053, 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].

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].
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 µg/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 rational 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 µg/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-1β expression in immunologic and inflammatory functions during infections is well established.[31] Results exhibited a significant inhibition of the expression of IL-1β 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-1α plays an important role in mediating the acute inflammatory response in trauma hemorrhage and reported that MIP-1α reduction could be beneficial in minimizing the inflammatory responses in several diseases.[32] The data revealed that MIP-1α 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 µg/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-kB) activation in human myelomonoblastic leukemia cells.[40] Zinc has the ability to induce or inhibit the activation of NF-kB[41] and magnesium plays a critical regulatory role in NF-kB activation.[42] Overall, the herbomineral-based formulation, TEBEH, remarkably downregulates the expression of the pro-inflammatory cytokines like TNF-α, MIP-1α, and IL-1β 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-1β) and...
chemokine (MIP-1α) expression as compared with the VC group in mouse splenocyte cells. In brief, the expression of MIP-1α 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-1α, and IL-1β 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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Conflicts of interest
There are no conflicts of interest.

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