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Evaluation of Immunostimulant Activity and Inhibition of Cytokine Storm Activity of Proprietary Herbal Formulation Virulina®

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Abstract: Immunostimulation is strengthening of immune system through induction of nonspecific immune responses. Synthetic drugs have harmful side effects, enormous cost and are ineffectual in controlling several pathological disorders. Therefore, nowadays herbal immunostimulants have been considered as superior and less toxic opportunity for the management of disorders as well as their complications. The objective of current investigation is formulation of Virulina®-Natural Solutions (VL®-NS) polyherbal formulation and its evaluation for immunostimulant and cytokine storm inhibition activity. The immunostimulant & anti-inflammatory potential of Virulina® were determined by using *in-vitro* phagocytosis through polymorphonuclear analysis indicating the invasion of leukocytes. *In-vitro* cytokine storm inhibitory activity were also performed using mouse macrophage cells (RAW264.7) treated with polyinosinic-polycytidylic acid to test the ability of Virulina® to attenuate the immune responses. The inflammatory responses were evaluated by endotoxin-lipopolysaccharide (LPS) model following the endotoxin challenges of aggravating the inflammatory mediators (cytokines) thereby, the LPS derived from *E. coli* as antigen at a dose of 10 $mgkg^{-1}$, i.p was used for the systemic inflammation. VL®-NS exhibited enhanced phagocytic efficacy at 100 $mgml^{-1}$ in PMN function test. VL®-NS significantly decreases the expression of cytokine such as interleukin-6, tumor necrosis factor-α & vascular endothelial growth factor in RAW264.7 cell culture. The increase in LPS-induced cytokine levels in rat serum was dose dependently & significantly ($p<0.05$) inhibited by VL®-NS treatment. VL®-NS tends to be potent immunostimulant therapy as superior and less toxic opportunity for strengthening of immune system for the management of several inflammatory disorders.

Keywords: Immunostimulant; polyherbal formulation; phagocytosis; polymorphonuclear analysis; cytokine storm inhibitory activity; interleukin-6, tumor necrosis factor-α.

1. Introduction

Immunotherapy is a revolutionizing treatment paradigm for the advanced therapeutic approach of immunodeficiency diseases nowadays. Recently, the current scenario of the research is employed in the designing of immunostimulatory formulation to induce the adjuvant immune response for better tolerance of the immunodeficiency conditions like respiratory tract infections, chronic obstructive pulmonary disease, diabetes, and cancer. The

herbal medicinal plants as a natural source of therapeutic aid in improving the immune system and the various herbal plants have gained a significant attention in the research of polyherbal formulation as adjuvant enhancement of immune system. Therefore, the herbal immunostimulants have been considered as superior and less toxic opportunity for the management of disorders as well as their complications [1-12]. Although the advancement in the field of the synthetic medicines in the management of immunodeficiency disorders but due to the numerous reported harmful side effects and enormous cost related problems with synthetic medicines, the alternative complementary therapy have replaced the synthetic treatment with natural herbal medicines tends to patient compliance with lesser side effects and low medication adherence. The literature supports traditional herbal treatment or folklore medicines used in the treatment of enhancing the immune system including plants like *Emblica officinalis* [13], *Terminalia chebula* [14], *Berberis Aristata* [15], *Solanum xanthocarpum* [16], *Zingiber officinale* [17], *Marshdenia Tenacissima* [18], *Tinospora Cordifolia* [19], *Alhagi Maurorum* [20], *Picrorhiza kurroa* [21], *Azadirachta indica* [22], *Glycrrhiza glabra* [23], *Trachyspermum ammi* [24], *Clerodendrum serratum* [25], *Cedrus deodara* [26], *Andrgraphis paniculata* [27], *Syzigium aromaticum* [28], *Cinnamomum tamala* [29], *Myristica fragrans* [30], *Aconitum heterophyllum* [30], *Curcuma longa* [32], *Santalum album* [33] and *Sida cordifolia* [34], *Desmodium gangeticum* [35]. These plants tend to possess active phytoconstituents having anti-oxidant, anti-inflammatory and immunomodulatory effects. The current research investigated the polyhedral formulation Virulina®-Natural Solutions (VL®-NS) as a combination of several immunostimulant herbs designed by using the standard powder formulation method (Table 1) likely to be effective therapy in improving the immune system. VL®-NS was assessed for phagocytosis through in-vitro polymorphonuclear (PMN) function test. The in-vitro cytokine storm inhibitory activity of VL®-NS was assessed in mouse mononuclear macrophage leukemia cells and in-vivo pharmacological activity was performed in rats through induction of lipopolysaccharide mediated cytokine storm activity. Therefore, the current study provides a new research of (VL®-NS) polyherbal formulation comprising traditional herbal plants listed in table 1 having active phytoconstituents possessing anti-oxidant, anti-inflammatory activity and tends to modifying the immune system for management of several inflammatory disorders.

Table 1. Composition of proprietary herbal formulation Virulina®-Natural Solutions.

Dravya	Scientific name	Part used	Amount (mg)
Triphala (Amla, Harde & Baheda)	*Emblica officinalis, Terminalia chebula, Terminalia bellirica*	Fruit	50
Daruharidra	*Berberis aristata*	Fruit and stem	50
Kantakari	*Solanum xanthocarpum*	Root and fruit	100
Trikatu (Sunthi, Maricha, Pippali)	*Zingiber officinale, Piper nigrum, Piper longum*	Root	100
Moorba	*Marsdenia tenacissima*	Root	50
Guduchi	*Tinospora cordifolia*	Stem	100
Jawasa	*Alhagi maurorum*	Aerial part	50
Kutaki	*Picrorhiza kurroa*	Root and stem	50
Trayamana	*Gentiana kurroo*	Root	50
Netrabala	*Sida cardifolia*	Root	50
Neem Chaal	*Azadirachta indica*	Bark, leaf and seed	50
Mulethi	*Glycyrrhiza Glabra*	Root	50
Kuda chhal	*Holarrhena Antidysenterica.*	Seed and bark	100
Ajwain	*Trachyspermum ammi*	Fruit	50
Bharangi	*Clerodendrum Serratum*	Leaf and root	100
Sahjan beej	*Moringa oleifera*	Seed	50

Kiratatikta	*Swertia chirayita*	Whole plant extract	50
Vacha (Bal)	*Acorus Calamus,*	Root	50
Chitrak	*Plumbago zeylanica.*	Root	50
Devdaru	*Cedrus deodara*	Bark	50
Chavya	*Piper retrofractum*	Root and fruit	50
Patolpatra	*Richosanthes dioica*	Leaf	50
Kalmegh	*Andrographis paniculata*	Root	100
Lavang	*Syzygium aromaticum*	Flower	100
Kamal	*Nelumbo nucifera Gaertn*	Flower and rhizome	100
Kakoli (tuber root)	*Lilium polyphyllum D*	Flower and bulb	50
Tejpatra	*Cinnamomum Tamala*	Leaf and bark	50
Talispatra	*Taxaceae*	Leaf and bark	50
Javitri	*Myristica Fragrans*	Seed	50
Ativisha	*Aconitum heterophyllum*	Root	100
Haldi	*Curcuma longa*	Root and stem	50
Dalchini	*Cinnamomum verum*	Leaf and bark	50
Khas	*Chrysopogon zizanioides*	Root	50
Shwet Chandan	*Santalum album*	Root	50
Khareti	*Sida cordifolia Linn*	Bark and seed	50
Shaliparni	*Desmodium gangeticum*	Root	100
Prishniparni	*Uraria picta*	Root, leaf	100

2. Materials and Methods

2.1. Reagents

Endotoxin-lipopolysaccharide (LPS) derived from *E. coli* were procured from Sigma-Aldrich (St. Louis, USA). The PMN leucocytes were retrieved from plasma sample of protocol of healthy volunteers aged between 18-25 years was approved by Institutional Human Ethics Committee, Chitkara University, Punjab (IHEC/DHR/CU/PB/20/16). Milliplex kit, mouse macrophage cells (RAW264.7), Indomethacin and polyinosinic-polycytidylic acid were procured from Sigma-Aldrich (St. Louis, USA). All the solvents, reagents and chemicals used were of analytical Grade.

2.2. Animals

The study was conducted under the guidelines of CPCSEA and approved by Institutional Animals Ethical Committee, Chitkara College of Pharmacy, Chitkara University, Punjab under registration number 1181/PO/ReBi/S/08/CPCSEA. Inbreeded Wistar albino rats of both sexes (200-250 gm) were accommodated in appropriate facilities and habituated for duration of 7 days prior to the initiation of experiments. The animals (Wistar rat) were freely accessed with feed and distilled water for 24 h. Experimental animals were randomly divided into five groups of six rats each (three male and three female) for cytokine storm activity. The test group animals were treated with orally administration of VL®-NS in 1.0 ml distilled water whereas the Vehicle/Control animals were given distilled water (p.o).

2.3. Preparation of VL®-NS formulation

The polyherbal powder formulation consisting of immunostimulant herbs (VL®-NS) was manufactured using traditional herbs with data from traditional text and pharmacological studies for individual immunomodulatory and anti-inflammatory activity, by the standard powder formulation method (Table 1). The herbal ingredients were powdered separately, passed through 80 # sieve and mixed in ascending order with continuous trituration to get

homogeneous mixture. The herbal blend was further mixed with lactose (diluents) and magnesium stearate (lubricant) for appropriate flow properties for packaging as powders or as capsules as VL®-NS formulation [36].

2.4. Immunostimulant activity of VL®-NS

2.4.1. In-vitro phagocytosis test

Immunomodulatory activity of VL®-NS (5, 10, 20, 50, 100 $mgml^{-1}$ suspension) was evaluated through in-vitro phagocytosis test using PMN function test. Approximately 5 ml peripheral venous blood was collected in sterile heparinized tube from healthy volunteers as per standard procedures in a sterile heparinized tube. Neutrophils have been withdrawn by sedimentation with gradient density of Ficoll Hypaque. The RBC-PMN pellet was precipitated by adding dextran. The supernatants comprising over 90 percent of PMN cells were retrieved and the density of the cells was modified (1×10^6 $cellsml^{-1}$) using MEM. *Candida albicans* (1×10^6 $cellsml^{-1}$) was utilized as test microorganism. The PMN cells were blended with *Candida albicans* and incubated for one hour at 37ºC under 5% CO_2 atmosphere, in the presence VL®-NS (as 5, 10, 20, 50, 100 $mgml^{-1}$ suspension). The control was the identical solution without VL®-NS. Subsequent to incubation, cytosmears were created followed by fixation through methanol, staining by Giemsa and examined via oil-immersion microscopic technique (100×) to establish phagocytic activity. Approximately 100 neutrophils were examined to count the cells containing ingested microorganisms [37-43]. Phagocytosis parameters were calculated using following equations.

$$\% \text{ Phagocytosis} = \frac{\text{PMN cells with phagocytosis activity}}{\text{Total number of PMN cells}} \times 100 \qquad \text{Equation 1}$$

$$\text{Phagocytic index} = \frac{\text{Number of Candida albicans engulfed}}{\text{Total number of neutrophils}} \qquad \text{Equation 2}$$

2.4.2. In-vitro cytokine storm inhibitory activity of VL®-NS

Cytokine production in mouse mononuclear macrophage leukemia cells (RAW264.7) in culture medium [10% fetal bovine serum, 100 Uml^{-1} of penicillin and 100 μgml^{-1} of Streptomycin in Dulbecco's Modified Eagle Medium (DMEM)] was measured through multiplex bead-based cytokine assay. The cells were treated with polyinosinic-polycytidylic acid [poly (I:C)] (control); poly (I:C) along with indomethacin (reference) and poly (I:C) along with different doses of VL®-NS (25/50/100/150/200 μgml^{-1}) (test) and subsequently incubated in 5% CO_2 incubator within a humidified atmosphere at 37ºC. After 24 h treatment, the interleukin-6 (IL-6); tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF)] liberated from treated cells were calculated in supernatants of cell culture via Milliplex kit (Millipore, USA) [44-47].

2.4.3. In-vivo pharmacological activity

2.4.3.1. Treatment schedule for animals

VL®-NS (250 and 500 $mgkg^{-1}$ body weight, p.o. twice daily) was administered to the test group animals for five days and the vehicle was administered to control animals. The

VL®-NS was orally administered on each of two days before immunization i.e., on the day of immunization and on each of two days following immunization (*i.e.* Days –2, -1, 0, +1, +2).

2.4.3.2. Induction of LPS mediated cytokine storm activity

The rats were immunized by injecting endotoxin-lipopolysaccharide (LPS) derived from *E. coli* (10 $mgkg^{-1}$, i.p). The 2nd day after the immunization, blood samples were taken via retro-orbital puncture. After sampling, blood was immediately mixed with anticoagulant, followed by centrifugation to separate serum. After separating serum, samples were frozen until subsequent examination. All animals were breathing spontaneously during the experiments. For determination of interleukin-1 (IL-1), IL-6 and TNF-α, ELISA kits were employed in accordance with the protocols provided by manufacturer Progenbiolab technologies, Delhi [48-51].

2.4.4. Acute toxicity study of VL®-NS

The acute toxicity study of VL®-NS was carried out in rats according to the recommended standards. Wistar rats (n=3) were weighed before commencement of experiment. A single maximum tolerated dose (2000 $mgkg^{-1}$ p.o) of VL®-NS was given to test animals and monitored for mortality as well as changes in behavior over 14 days. Control animals were delivered distilled water [52].

2.4.5. Calculation of human equivalent dose (HED) from animal dose

From in-vivo pharmacological activity test in Wistar rats, effective animal dose of VL®-NS was determined. Subsequently, HED was calculated from animal dose using following formula:

$$\text{Human equivalent dose } \left(\frac{\text{mg}}{\text{kg}}\right) = \text{Animal dose } \left(\frac{\text{mg}}{\text{kg}}\right) \times \left[\frac{\text{Animal Weight (kg)}}{\text{Human Weight (kg)}}\right]^{0.33} \quad \text{Equation 3}$$

2.5. Statistical Analysis

The reported findings are compiled from three separate trials and reflect a mean ± standard deviation (SD). Significant differences ($p < 0.05$) were investigated by analysis of variance (ANOVA) and Dunnett test using GraphPad Prism version 6.01 (GraphPad Software, CA, USA).

3. Results and Discussions

3.1. In-vitro phagocytosis test

Following drug particle or droplet deposition, particles must disperse within the fluids of epithelial cells. Physiologic factors, formulation of drug as well as its physicochemical properties such as dissolution characteristics play an essential role in pulmonary drug dissolution. Slow dissolution as a rate-limiting step is obligatory as it prolongs lung retention, although with a simultaneous increase in the possibility that drug particles may get cleaned by mucociliary clearance.

VL®-NS was evaluated at different concentrations of 5, 10, 20, 50 and 100 $mgml^{-1}$. It exhibited significant action at 100 $mgml^{-1}$ concentrations. The VL®-NS tends to have phagocytosis capacity of ingesting foreign microbes, migration of neutrophils at the first

stage of immune response indicating as key role in influencing the immune system. Neutrophil activation caused an increase in innate cellular immune response. These findings of an in-vitro PMN function test revealed that phagocytosis as well as phagocytic index for VL®-NS augmented considerably (Figure 1). This indicated that VL®-NS enhanced the phagocytic efficacy of the PMN cells at 100 $mgml^{-1}$ through elevated engulfment of *Candida albican* in opposition to control, consequently inducing an unspecific immune system response. Since the VL®-NS demonstrated hopeful immunostimulant behavior through *in-vitro* research, therefore *in-vivo* animal investigation was conducted [37-43].

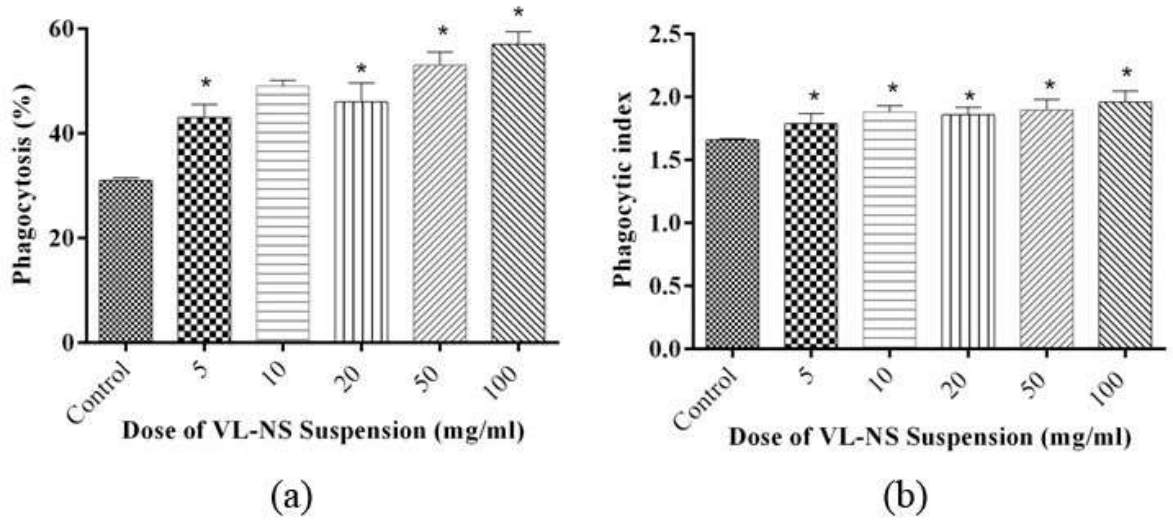

Figure 1. Effect of virulina®-natural solutions (VL®-NS) on phagocytic efficacy of polymorphonuclear leucocytes. Results are presented as mean ± SD, (n=3), *p < 0.05 versus control.

3.2. In-vitro Cytokine storm inhibitory activity of VL®-NS

Cytokines are a potent source of tiny proteins (5-20 kDa) which have been responsible for cell modulation as well as inflammation. The multiplex cytokine method was used to assess cytokine secretion in mouse mononuclear macrophage leukemia cells (RAW264.7) in a culture medium. Following 24 h incubation of cells with poly (I:C)/indomethcacin/VL®-NS, the cytokines discharged from cells were quantified by Milliplex kit. The results revealed that VL®-NS substantially lowered overproduction of cytokines in poly (I: C)-induced RAW 264.7 cells by activation as well as normal T-cell expression (Figure 4) [44-47].

Viral & bacterial pathogens disrupt the immunity function which renders individuals across recurrent airway illnesses [47]. Viral infections cause type-1 interferon reactions through inducing targets for identification of endosomal or cytosolic patterns which are essential inflammatory initiators *i.e.* cytokines [53,54]. This is widely established that viral & bacterial illnesses tend to recurrent pathogenesis of extreme sepsis, which is marked through an intense synthesis of several inflammatory cytokines [55-58]. Sepsis remained a serious challenging issue with high death rates even after antibiotics. Since, VL®-NS herbal immunostimulant decreases cytokines levels; therefore, it could be explored for management of viral and bacterial disorders in several clinical diseases [59-61]. Furthermore, in today's scenario, serious inflammatory reaction in various infections like cytomegalo, influenza,

variola, corona and avian H5N1 influenza viruses is believed to cause rigorous, unregulated cytokine development (cytokine storm). Therefore, it is crucial to concentrate on therapies which regulate the hyper-inflammation stimulated by viruses [62]. The current study revealed that VL®-NS ameliorated inflammatory response by lowering cytokines levels, consequently VL®-NS might be explored to attenuate hyper-inflammation associated with viruses.

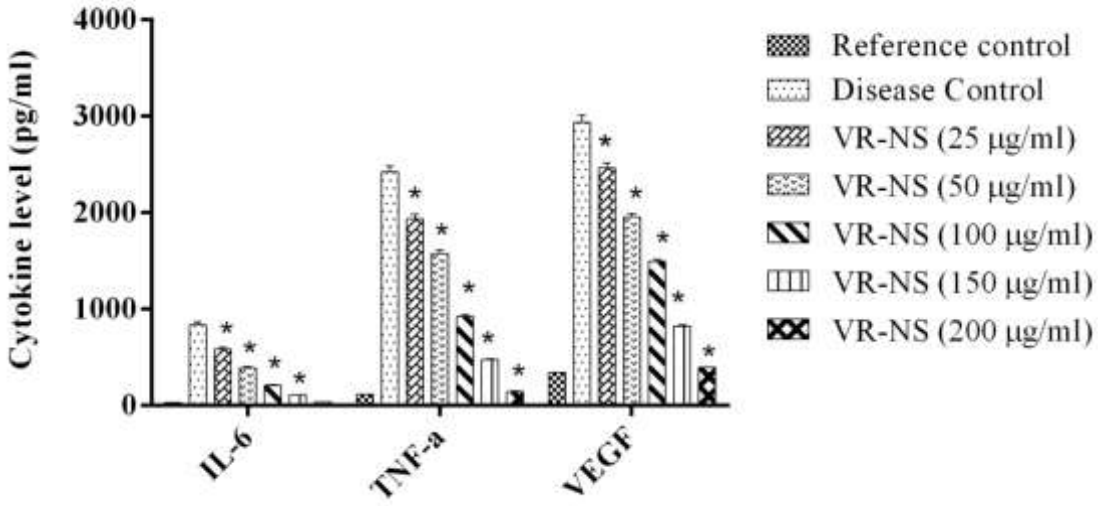

Figure 4. Effect of virulina®-natural solutions (VL®-NS) on cytokines levels in cytokine storm inhibitory activity in RAW264.7 cells. IL-6: Interleukin-6; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor. *p < 0.05 versus control.

3.3. In-vivo Induction of LPS mediated cytokine storm activity

Serum cytokines levels are elevated in inflammatory diseases, bacterial & viral infections. Tumor necrosis factor (TNF)-α and interleukins are important inflammatory mediators in sepsis conditions in human body. In sepsis, these inflammatory responses are exacerbated by a cytokine storm that can lead to morbidity & mortality. A dose-related rise in 48 h immune responses in rats occurred per oral delivery of 200 and 500 mg/kg VL®-NS for 5 days. In Wistar rats, LPS induced tolerance leads to significant production of pro-inflammatory cytokines TNF-α, IL-1 and IL-6, respectively in comparison to vehicle control group. Pre and post treatment of rats with LPS challenge, VL®-NS (200 and 500 $mgkg^{-1}$) significantly ($p<0.05$) dose dependably decrease the level of cytokines like TNF-α, IL-1 and IL-6 as compare to disease control group. Post-treatment with VL®-NS effectively attenuated LPS-induced cytokine secretion. Interleukins reduces the secretion of pro-inflammatory cytokines and also lowers neutrophil infiltration induced by various antigens. This increase in primary immune response to LPS asserted that perhaps the macrophages as well as T-and B-lymphocyte subgroups implicated in antibody production are most responsive. VL®-NS possibly promotes the proliferation of lymphocytes, which would in turn leads to cytokine synthesis which activates certain immune cells like B-cells and several other T-cells. Across specific as well as non-specific immune pathways, VL®-NS has been shown to have valuable immunostimulatory function [48-51].

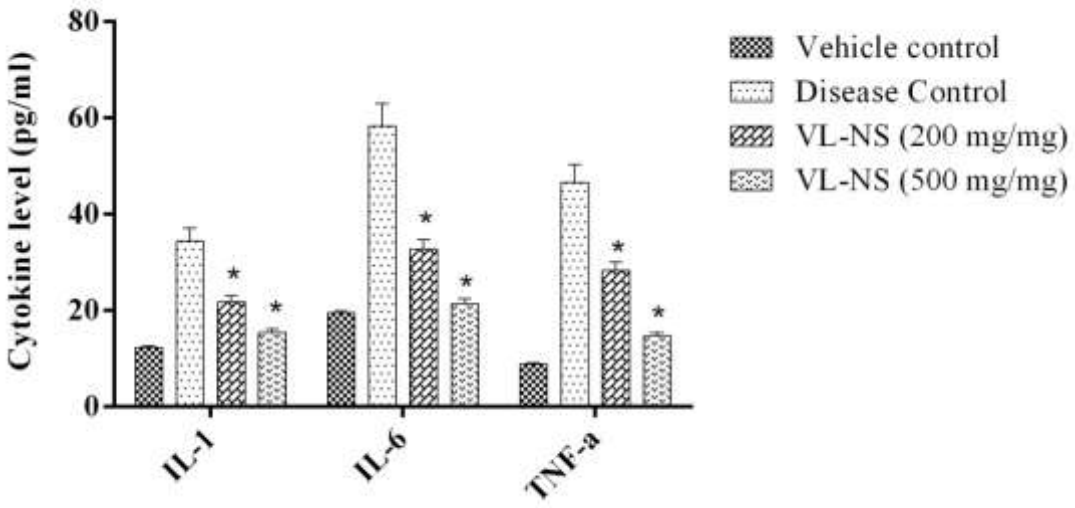

Figure 2. Effect of virulina®-natural solutions (VL®-NS) on lipopolysaccharide mediated cytokine storm activity in Wistar rats. IL-1: interleukin-1; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α. Results are presented as mean ± SEM, (n=6), *p < 0.05 versus control.

3.4. Acute toxicity study of VL®-NS

Outcomes of single dose toxicity study revealed that VL®-NS LD-50 was safe in Wistar rats [52]. Animals have shown no change in body weight when administrated at dose level of 2000 $mgkg^{-1}$, p.o. No morbidity and behavioral changes have been observed in treated animals.

3.5. Calculation of human equivalent dose (HED) from animal dose

The highest effective dose of VL®-NS in Wistar rats during in-vivo pharmacological test was found 500 $mgkg^{-1}$. Therefore, estimated human equivalent dose of VL®-NS calculated through conversion factor was 4800 mg per day.

4. Conclusions

Synthetic drugs have detrimental side effects, huge cost and are inadequate in controlling pathological facades of numerous disorders such as respiratory tract infections, chronic obstructive pulmonary disease, diabetes, and cancerous conditions. Therefore, in present investigation, Virulina®-Natural Solutions (VL®-NS) polyherbal immunostimulant formulation consisting of several herbs was developed by the standard powder formulation method. VL®-NS exhibited enhanced phagocytic efficacy at 100 $mgml^{-1}$ as estimated through polymorphonuclear function test. VL®-NS significantly reduced excess production of cytokines in RAW264.7 macrophage cells as estimated through multiplex cytokine assay. This polyherbal formulation demonstrated decrease in cytokine storm activity in LPS tolerance animals at dose of 200 and 500 $mgkg^{-1}$. In Wistar rats, no death or morbidity was observed in any animal that indicates its safety at the single maximum tolerated dose of VL®-NS. No adverse effect was seen on the animals with respect to behavior, body weight and lethargy. HED of VL®-NS was found 4800 mg per day. Conclusively, VL®-NS immunostimulant have been considered as superior and less toxic opportunity for strengthening of immune system for management of several ailments.

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Conflict of Interest

The authors declare no conflict of interest.

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