

IM-133N - A Useful Herbal Combination for Eradicating Disease-triggering Pathogens in Mice via Immunotherapeutic Mechanisms

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Key Words

carbon clearance test, *Escherichia coli*-induced sepsis, immunomodulator, IM-133N, LC-MS/MS

Abstract

Objectives: The present study was undertaken to evaluate the immunomodulatory (IM) activity of IM-133N, a herbal combination in various immunotherapeutic experimental models.

Methods: The IM activity of IM-133N was evaluated against three experimental models namely, effect of IM-133N against *Escherichia coli* (*E. coli*)-induced abdominal sepsis in mice, and carbon clearance test was performed in Wistar albino rats to evaluate the phagocytic potential of IM-133N, in addition IM-133N was evaluated for its immunoglobulin enhancing potential in rats, where the immunoglobulin levels were measured by zinc sulphate turbidity (ZST) test. Further, IM-133N was subjected for detailed liquid chromatography-mass spectrometry (LC-MS)/MS analysis to identify the probable active constituents present in it.

Results: The findings of the present study has demonstrated very promising IM property of IM-133N in all

the experimental models. Briefly, pretreatment with IM-133N at 125, 250, 500 and 1,000 mg/kg, p.o. doses had protected the mice against *E. coli*-induced abdominal sepsis and mortality, further the effect of IM-133N was found to be significant and dose-dependent. In support of this, in another study administration of IM-133N showed a significant and dose-dependent increase in serum immunoglobulin levels, estimated by ZST test. In line with the above findings, in the carbon clearance test the low doses (125 and 250 mg/kg, p.o.) of IM-133N increased the rate of carbon clearance, whereas the higher doses (500 and 1,000 mg/kg, p.o.) did not sustain the response, and saturation effect was considered as one of the possible reason for futility of higher doses for IM-133N. In addition, A detailed LC-MS/MS analysis of IM-133N showed 17 bioactive phytochemical constituents: namely, apigenin, chaulmoogric acid, mesquitol, quercetin, symphoxanthone, salireposide, β -sitosterol, nonaiecosanol, β -amyryn, betulic acid, oleanolic acid, symplososide, symponoside, symploveroside, symplocomoside, symconoside A and locoracemoside B.

Conclusion: These findings suggest that IM-133N possesses significant IM activity and, hence, could be useful for eradicating opportunistic disease-triggering pathogens via immunotherapeutic mechanisms. The findings also suggest IM-133N may also useful in other immunity disorders.

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1. Introduction

The use of medicinal plants for treating the human diseases has been proficient and long-established [1]. Also, plant based medicines are well-known to augment the body's natural resistance to infections; thus, one of the therapeutic strategies in Ayurvedic medicine is to provide protection against diseases by stimulating the immune response [2]. The term immunomodulation denotes a strengthening or a weakening of cellular and humoral immunity and non-specific defense factors [3]. Extreme manifestations of the immunomodulating actions of biologically-active substances are immunosuppression and immunostimulation. Immunostimulation comprises a prophylactic or therapeutic concept which aims at the stimulation of the nonspecific immune system [4], which primarily implies the non-antigen dependent stimulation of the function and efficiency of granulocytes, macrophages, the complement system and natural killer cells [5].

At present, immunomodulators from various sources are being widely used in the treatment of pathological conditions associated with either enhanced or diminished immune response, such as asthma, cancer, autoimmune disorders and so on [6]. The scope of plant based medicines as immunomodulators is increasing because of their wide safety margin compared to synthetic agents (e.g., corticosteroids), biological response mediators (interleukins, interferons) and antibody reagents [7]. A number of Indian medicinal plants and various 'rasayanas' have been claimed to possess immunomodulatory (IM) activity [8]. In efforts to study the IM activities of Indian medicinal plants and potent plant product combinations, a number of studies were carried out with an aim to identify combinations that could be evaluated clinically and used in conditions where the host's immunity is impaired [9, 10].

On the basis of preliminary results obtained from different experimental models, a herbal combination, IM-133N, was developed. The purpose of the present study was to evaluate the IM activity of IM-133N with respect to both humoral and cell mediated immunity. The herbal combination IM-133N was manufactured by The Himalaya Drug Company, Bangalore, India. It contains mixture of spray-dried aqueous extracts of *Prosopis glandulosa* and *Symplocos racemosa* which were separately prepared and mixed in a ratio of 3 : 2 respectively.

2. Materials and Methods

All the liquid chromatography-mass spectrometry (LC-MS/MS) grade solvents used in this study were procured from Merck Ltd. (Mumbai, India). All other chemicals and reagents were of analytical grade and were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). The LC-MS/MS instrument used in our study comprised a high-performance liquid chromatograph (HPLC) coupled with an API 2000 mass spectrometer (Applied Biosystem/MDS SCIEX, Canada) coupled with an electron-spray ionization source with an electron multiplier detector and a chromatographic system. Batch acquisition and data pro-

cessing were controlled by using Analyst® Software Version 1.5 (Applied Biosystems/MDS Analytical Technologies, CA, USA).

The parameters of the mass spectrometer were optimized by using a 1-mg mL⁻¹ sample solution of IM-133N prepared in Double distilled mineral (DM) water. The sample was directly and continuously infused (flow injection analysis) into the electron spray-MS (ES-MS) by using a syringe pump. The intensity of the response was checked in both the positive and the negative ionization modes. A response with good intensity was obtained in the negative mode. Other parameters, such as the de-clustering potential (DP: - 80 V), ion spray voltage (IS: - 4,500 V), pressures of the nebulizing gas (GS1 - 30 pascals per square inch (psi) and GS2 - 40 psi) and the curtain gas (CUR = 25 psi), focusing potential (FP = - 400 V), entrance potential (EP = - 2 V), source temperature (TEM = 0°C) and EP - 10 V, were optimized with respect to the ionization-intensity response.

Male Swiss albino mice (20 - 25 g) and Wistar albino rats (200 - 220 g) were obtained from the Central Animal House, AI - Ameen College of Pharmacy, Bangalore, and housed three animals per cage with paddy husk as bedding material. Animals were housed at a temperature of 25 ± 2°C and a relative humidity of 40% - 60%. A 12-hour light and dark cycle was followed. The animals had free access to feed (Amrut Feeds Ltd., Bombay, India) and ultraviolet (UV)-purified water *ad libitum*. For all the experiments, IM-133N was administered as an oral aqueous suspension at a dosage volume of 10 mL/kg. The animals in the control group received water as a vehicle. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), and the animals used for this study were maintained in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest (Animal Welfare Division), Government of India.

Based on the outcomes of an acute oral toxicity study, we found IM-133N to be safe up to 5,000 mg/kg. Thus, for this study, we used doses of 1X/40 = 5,000/40 = 125 mg/kg, 1X/20 = 5,000/20 = 250 mg/kg, 1X/10 = 5,000/10 = 500 mg/kg and 1X/5 = 5,000/5 = 1,000 mg/kg for evaluating the therapeutic effect of IM-133N. The IM-133N formulation was suspended in 0.5% carboxy methyl cellulose (CMC) prepared in double-distilled water, and the formulation was prepared separately for each dose. In short, for the 125-mg/kg, p.o. dose, 125 mg of IM-133N was weighed and suspended in 0.5% CMC, and the volume was brought to 10 mL by using 0.5% CMC. Similarly for the 250-, 500- and 1,000- mg/kg, p.o. doses, 250, 500 and 1,000 mg of IM-133N were individually weighed and suspended in 0.5% CMC, and the volume was brought to 10 mL by using 0.5% CMC. The final prepared formulation was administered at a 10 mL/kg dose volume based on the animal's weight.

This study was carried out in two steps. In the first set of experiments, the strength of *Escherichia coli* (*E. coli*) required for inducing abdominal sepsis and 100% mortality was established, and based on the results, the effect IM-133N against *E. coli* induced abdominal sepsis and mortality was evaluated in the second set of experiments. In the first set, a pathogenic strain of *E. coli* was subcultured in

a nutrient broth by using a shaker water bath at 37°C, and the total viable-cell count was observed from the MacConkey agar plates [11, 12]. Different doses of *E. coli* were administered intraperitoneally to different groups of animals to elucidate the dose of viable *E. coli* cells required to cause abdominal sepsis with 100% mortality within 48 hours. The findings revealed that intraperitoneal injection of *E. coli* at 2.7×10^6 colony-forming units (CFU) corresponding to a 10^{-4} serial dilution was the minimum dose that could cause abdominal sepsis and 100% mortality within 48 hours in untreated mice.

Based on the findings in the first set of experiments, in second set, 30 male Swiss albino mice were selected and divided into five groups (G-I to G-V) of six (n = 6). G-I served as the vehicle control, and the animals in G-II, G-III, G-IV and G-V received IM-133N at doses of 125, 250, 500 and 1,000 mg/kg, p.o., respectively, for a period of 15 days. On the 15th day, after the last dose of drug administration, a 10^{-4} serial dilution corresponding to 2.7×10^6 CFU of *E. coli* was injected intraperitoneally to all the animals, after which the animals were observed for the induction of abdominal sepsis and mortality for the next 48 hours. The animals surviving 48 hours after the *E. coli* injection were observed for three more days. The results were expressed in terms of percentages [9, 13-15].

Next, 36 male albino Wistar rats (200 – 220 g) were randomized into six groups (G-I to G-VI, n = 6). The animals in G-I served as the vehicle control, and those in G-II received a dose of levamisole, 50 mg/kg, p.o., while the an-

imals in G-III, G-IV, G-V and G-VI received IM-133N at doses of 125, 250, 500 and 1,000 mg/kg, p.o., respectively. All assigned treatments were given for a period of 20 days. On the 21st day, 6 hours after the last dose, all the experimental animals were anesthetized using anesthetic ether, and blood samples were collected by using a retro-orbital puncture; subsequently, the blood samples were allowed to clot at room temperature, and serum was separated and used for estimating the immunoglobulin levels by using zinc sulphate turbidity (ZST) test. In brief, for each serum sample, a control tube containing 6 mL of saline (blank) and a test tube containing 6 mL of zinc sulphate solution (208 mg/L), pH 5.8, were prepared, and to each of these tubes 0.1 mL of serum was added. The tubes were inverted to enable complete mixing of the reagents and allowed to stand for 1 hour at room temperature; subsequently, the turbidity that developed in each tube was measured at a wavelength of 498 nm by using a spectrophotometer [11, 12].

In addition, 36 male albino Wistar rats (200 – 220 g) were randomized into 6 groups (G-I to G-VI) consisting of six animals each (n = 6). The animals in G-I served as the vehicle control, and those in G-II received levamisole 50 mg/kg, p.o. while the animals in G-III, G-IV, G-V and G-VI have received IM-133N at doses of 125, 250, 500 and 1,000 mg/kg, p.o., respectively, for a period of 14 days. On the 14th day, 6 hours after the drug administration, all the experimental animals were anesthetized using anesthetic ether, and blood samples were collected by using a retro-orbital

Table 1 Identification of various components from IM-133N by using the LC-MS/MS method

Sl. No	Constituent	Molecular formula	Actual mass (g/mol)	Obtained m/z value in LC-MS/MS spectra
1	Apigenin	C ₁₅ H ₁₀ O ₅	270.24	268.98
2	Chaulmoogric acid	C ₁₈ H ₃₂ O ₂	280.44	278.99
3	Mesquitol	C ₁₅ H ₁₄ O ₆	290.27	288.79
4	Quercetin	C ₁₅ H ₁₀ O ₇	302.23	300.85
5	Symphoxanthone	C ₁₈ H ₁₆ O ₆	328.31	327.29
6	Salireposide	C ₂₀ H ₂₂ O ₉	406.38	404.93
7	β-Sitosterol	C ₂₉ H ₅₀ O	414.7	412.84
8	Nonaeicosanol	C ₂₉ H ₆₀ O	424.78	424
9	β-Amyrin	C ₃₀ H ₅₀ O	426.71	424.9
10	Betulic acid	C ₃₀ H ₄₈ O ₃	456.7	454.9
11	Oleanolic acid	C ₃₀ H ₄₈ O ₃	456.71	454.9
12	Symplososide	C ₂₇ H ₂₆ O ₁₀	510.5	509.01
13	Symponoside	C ₂₇ H ₂₆ O ₁₁	526.5	525.07
14	Symploverside	C ₂₉ H ₃₂ O ₁₀	540.57	539.9
15	Symplocoside	C ₂₇ H ₂₆ O ₁₂	542.48	541.13
16	Symconoside A	C ₂₆ H ₃₂ O ₁₄	568.5	567.22
17	Locoracemoside B	C ₂₇ H ₃₂ O ₁₄	580.53	579.33

IM, immunomodulatory; LC-MS/MS, liquid chromatography-mass spectrometry.

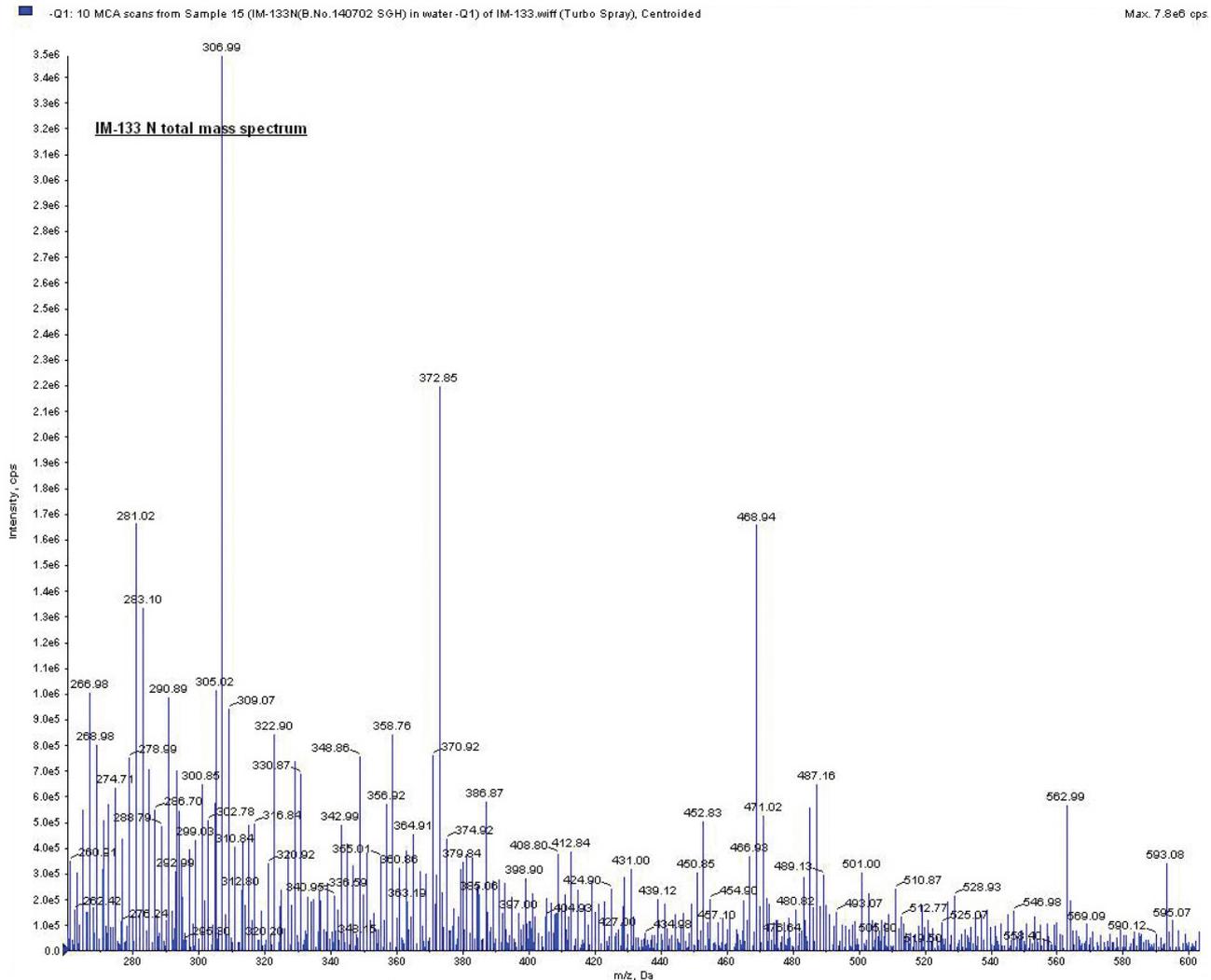


Figure 1 LC-MS/MS fingerprint of IM-133N.

LC-MS/MS, liquid chromatography-mass spectrometry; IM, immunomodulatory.

puncture. The blood samples collected at this stage were considered as blanks. Subsequently, a carbon ink suspension was administered intravenously to all animals at a dose of 0.5 mL/100 g body weight (example, an animal weighing 100 g received 0.5 mL of the carbon ink) after which blood samples were collected immediately and at 5, 10, 15, 20, 25 and 30 minutes thereafter. For the quantification of the carbon content in the blood, the erythrocytes were lysed by adding 4 mL of 0.1% sodium-carbonate solution to 50 μ L of the blood sample (80 volumes 0.1% sodium carbonate added to 1 volume of blood sample). Then, the absorbance of the supernatant of every samples was measured at a wavelength of 675 nm by using a spectrophotometer. The clearance value 'K' was calculated according to the following equation:

$$K = \frac{\log(C_1) - \log(C_2)}{t_2 - t_1}$$

Where t_1 and t_2 are time in minutes corresponds to sample C_1 and C_2 respectively, withdrawn at two different time intervals. The mean of the clearance values at different time intervals gives the granulopetic index per minute [9, 14, 16].

All data in this report are expressed as mean \pm standard error of the (SEM). The results were statistically analyzed by using the one-way analysis of variance (ANOVA), followed by Tukey's post test, with GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, California, USA). *P* values less than 0.05 were considered to be statistically significant.

3. Results

The present study demonstrates the IM activity of IM-133N, a herbal combination, and the benefit of using it in

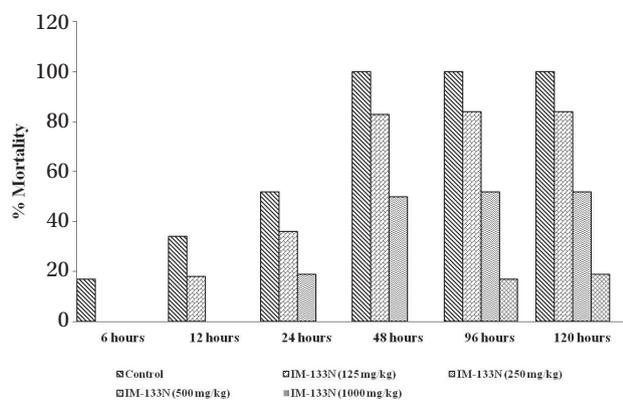


Figure 2 Effect of IM-133N on *E. coli*-induced abdominal sepsis and mortality in mice.

Values are expressed as percentage mortality for a total number of six animals in each group.

IM, immunomodulatory; *E. coli*, *Escherichia coli*.

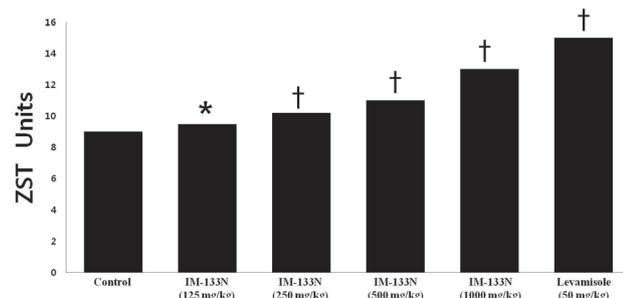


Figure 3 Effect of IM-133N on serum immunoglobulin levels in Wistar rats.

Values are expressed as mean \pm SEM (n = 6). All the groups were statistically compared by using an ANOVA followed by Tukey's multiple comparison test. * $P < 0.05$ and † $P < 0.01$ compared to control.

IM, immunomodulatory; SEM, standard error of mean; ANOVA, analysis of variance.

various immune compromised conditions in experimental models. A detailed LC-MS/MS analysis of IM-133N showed 17 bioactive phytochemical constituents: namely, apigenin, chaulmoogric acid, mesquitol, quercetin, symphoxanthone, salireposide, β -sitosterol, nonaeicosanol, β -amyrin, betulinic acid, oleanolic acid, symplioside, symponoside, symploveroside, symplocomoside, symconoside A and locoracemoside B. The LC-MS/MS spectrum and identified constituent are given in Fig. 1 and Table 1.

The first set of experiment was carried out to explore the dose of *E. coli* in normal male albino mice necessary to induce 100% mortality. The findings revealed that an intraperitoneal injection of *E. coli* into the mice at 2.7×10^6 CFU corresponding to a 10^{-4} serial dilution was the minimum dose that could cause abdominal sepsis and 100% mortal-

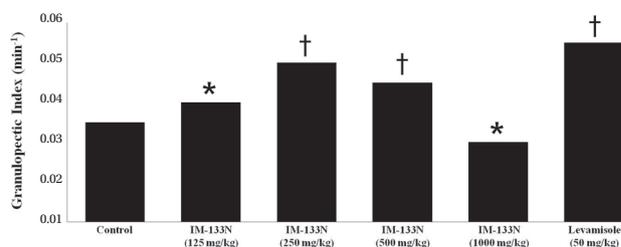


Figure 4 Effect of IM-133N on the carbon clearance rate in Wistar rats.

Values are expressed as mean \pm SEM (n = 6). All the groups were statistically compared by using an ANOVA followed by Tukey's multiple comparison test. * $P < 0.05$ and † $P < 0.01$ compared to control.

IM, immunomodulatory; SEM, standard error of mean; ANOVA, analysis of variance.

ity within 48 hours in untreated mice. In the second set of experiment, pretreatment with IM-133N showed significant, dose-dependent protection against *E. coli* induced mortality. Further, in the control group, 100% mortality was observed due to *E. coli* administration while the animals pretreated with IM-133N at 125 and 250 mg/kg, p.o. showed 17% and 50% inhibitions against *E. coli* induced mortality. Interestingly, the animals pretreated with 500 mg/kg, p.o. of IM-133N showed no mortality in the short term (till 48 hours), but 16% mortality was found 92 hours after injection of the *E. coli*. Exceptionally, the animals pretreated with IM-133N at a 1,000-mg/kg, p.o. dose showed no mortalities throughout the study period. The findings are given in Fig. 2.

In the present study, a 20-day pretreatment with IM-133N (125, 250, 500 and 1,000 mg/kg, p.o.) and with the reference standard levamisole (50 mg/kg, p.o.) showed enhanced serum immunoglobulin levels. In addition, the effect of IM-133N was found to be statistically significant and dose dependent. Noteworthy, the potency of high dose of IM-133N (1,000 mg/kg, p.o.) was comparable with that of reference standard levamisole. The results are given in Fig. 3.

In addition, the effect of IM-133N on the reticulo endothelial system (RES) was evaluated by using the carbon clearance test. In the outcomes, a 14-day pretreatment with IM-133N (125 and 250 mg/kg, p.o.) showed a significant, dose-dependent increase in the carbon clearance from the systemic circulation. However, the higher doses of IM-133N (500 and 100 mg/kg, p.o.) showed retrograde responses in clearing the carbon particles. Thus, based on the findings, one can conclude that IM-133N, a herbal medicine, acts as an immunostimulant and that it may be a useful agent for treating pathological conditions associated with immunosuppression. Furthermore, the effect of IM-133N at a dose of 250 mg/kg, p.o. was found to be comparable with that of the reference standard levamisole (50 mg/kg, p.o.). The rate of carbon clearance is expressed as the mean granulopoietic index and is depicted in Fig. 4.

4. Discussion

Septic shock is mediated by complex interactions of cells, cytokines and humoral factors, and the mortality due to septic shock is well known to be primarily due to a hyper-immune response in the host [9, 14]. Micro-organisms proliferate following an infection and produce endotoxins and exotoxins, which stimulate the primary immune system, endothelial cells, and other cells. Initially, nuclear factor- κ B (NF- κ B) is activated, which is followed by the productions of macrophage migration inhibiting factor (MIF), tumor necrosis factor- α (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6), free oxygen radicals, and reactive nitrogen species. The activation of the above factors results in the systemic inflammatory reactions and the myocardial depression observed during sepsis. In serious cases, this may lead to multi organ dysfunction (MODS) or even multi organ failure (MOF) [15, 17]. The *E. coli* induced abdominal sepsis model selected in the present study mimics the septic shock conditions observed in the humans. Thus, any agent that can selectively down-regulate the hyper-immune activation while enhancing the antimicrobial defenses should be beneficial in treating conditions like septic shock [9, 14].

In the above context, it is also the model to study the effect of drugs on opportunistic infections, the IM-133N (500 mg/kg and 1,000 mg/kg) was found to be an effective immunomodulator and an indirect anti-microbial agent which provided a significant protection in mice against *E. coli* induced abdominal sepsis. These findings were supported by a previous study, which stated that the predominant production of nitric oxide (NO) and an enhanced phagocytosis were the probable reasons driving the host immune system towards a Th1 type, akin to that of pro-host therapy [9, 14]. The leukocytosis, which probably occurs due to the secretions of IL-1 and colony stimulating factors (CSF) from activated macrophages, along with the increased number and functional capabilities of peritoneal macrophages, appears to be the underlying mechanism of protection offered by IM-133N [14]. Further, a recently published paper revealed that IM-133N could effectively up-regulate the expressions of inducible nitric oxide synthase (iNOS), TNF- α , IL-6, IL-10, IL-8 and interferon gamma (IFN- γ) in RAW264.7 and THP-1 cell lines in *in vitro* and thereby increase the production of NO and TNF- α [18].

The ZST test is the most widely used and highly accepted method for quantifying the specific immunoglobulin levels to determine the IM activity of test drugs. In the present study, the ZST test was modified to determine the correlation between the concentration of specific immune globulins present in the serum and the intensity of the ZST reaction that developed [11, 12]. The findings clearly showed that pretreatment with various doses of IM-133N (125, 250, 500 and 1,000 mg/kg, p.o.) could increase the serum immunoglobulin levels significantly and dose dependently.

In addition, the effect of IM-133N on the RES was evaluated by using the carbon clearance test. The RES is a diffuse system comprised of various types of phagocytic cells, such as mobile macrophages and fixed tissue macrophages, all of which constitute the MPS [19, 20]. Phagocytosis

represents an important innate defense mechanism against ingested particulates, including whole pathogenic micro-organisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. Once particulate material is ingested into phagosomes, the phagosomes fuse with lysosomes, and the ingested material is then digested [19, 20]. The various cells of the RES and the MPS are well-known for their biological functions, such as clearance of particles from the bloodstream. In the carbon clearance test, a colloidal ink containing carbon particles is injected directly into the systemic circulation, and the rate of clearance of carbon from the blood is quantified, which is a direct measure of immune function. In this model, among the various tested doses of IM-133N, the 125- and the 250- mg/kg, p.o. doses had a pronounced effect on the RES, increasing the rate of carbon clearance. The effect of IM-133N at a dose of 250 mg/kg was comparable with that of the reference standard levamisole (50 mg/kg, p.o.). However, further increases in the dose of IM-133N did not show a proportionate response. Because the immune response is not always directly related with the immune modulator concentration, the higher doses of IM-133N (500 and 1,000 mg/kg) showed a decrease in the rate of carbon clearance. This typical plateau effect may be partly due to the different constituents present in the fractions at different concentrations and to the saturation of the cells responsible for the immune response. Some of these constituents may have immunosuppressive activities whereas others possess immunostimulant activities [19]. Thus, the resultant effect of IM-133N treatment is immunostimulation at doses up to 250 mg/kg dose, with further increases in dose to 500 and 1,000 mg/kg resulting in a quenching of phagocytic activity.

IM-133N contains extracts of *Symplocos recemosa* and *Prosopis glandulosa*, and the LC-MS/MS analysis of IM-133N showcased the presence of various phytoconstituents, some of which, namely, oleanolic acid [21, 22], betulinic acid [23], β -sitosterol [22, 24], mesquitol [25], quercetin [22, 24, 26] and apigenin [27], are scientifically well demonstrated to have potent IM and anti-oxidant activities; therefore, these phytoconstituents are thought to act through multiple mechanisms, such as anti-oxidant and anti-microbial activities, increases in the expressions of cytokines, and increases in the productions of TNF- α and NO. Furthermore, studies are ongoing to explore the exact mechanism(s) of the action of IM-133N.

5. Conclusion

In conclusion, these findings suggest that IM-133N possess significant IM activity and, hence, could be useful for eradicating opportunistic disease-triggering pathogens via immunotherapeutic mechanisms. The findings also suggest IM-133N may also useful in other immunity disorders.

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Conflict of interest

The authors declare that there are no conflict of interest.

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