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Evaluation of *In-vitro* Immunomodulatory Potential of Siddha formulation *Naga Chendurum* using Murine Macrophage RAW 264.7 Cell Line Model

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Abstract

The immune system is known to be involved in the etiology as well as pathophysiological mechanisms of several diseases. Indian system of traditional medicines gives emphasis on promotion of health – a concept of strengthening host defenses against different diseases. The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. Immunomodulators are substances that help to regulate the immune system. The basic mechanisms by which the herbs (active principle) defend the body against infection have two probable ways one by destroying pathogens and other by enhancing the body immunity. Siddha formulations beings a multi-componential formulation offers greater relief by strengthening the host defense mechanism by its versatile pharmacological action. Macrophage cell line RAW 264.7 is widely utilized themodel for evaluating the *In vitro* immunomodulatory efficacy of the several Siddha formulations. *Naga Chendurum*(NC) is one such novel Siddha formulation comprised of a combination of herbs including *Baptisia bracfeata*, *Eclipta prostrata*,*Madhuga longifolia* and minerals such as zinc, sulphur, red orpiment etc.The present study was carried out to evaluate the immunomodulatory activity of Siddha formulation NC using Macrophage cell line RAW 264.7 using lipopolysaccharide (LPS) (1µg/mL) as a control. LPS induced nitrite production used an indicator for evaluating the level of phagocytosis. The concentration was measured using the spectrophotometric technique at 540nm. The result obtained from the present investigation indicates that the drug NC exhibit a significant decrease in the level of nitrites in the cell line medium from 952.38 µg to 483.61 µg. The results clearly indicate that the formulation NC possess immune boosting properties and suggest a usefulness in the disorder of immunological origin where the antioxidant system is adversely affected.

Keywords: Immune system, Siddha formulation, Naga Chendurum, Macrophages, RAW 264.7 cell line, Lipopolysaccharide

1. Introduction

Siddha formulations majorly act by enhancement of body resistance against infection acts as an adaptogen, immunomodulatory, and antimutagenic. Chemotherapeutic agents available today have mainly immunosuppressive activity. Most of them are cytotoxic and exert a variety of side effects. This has given rise to stimulation in the search for investigating natural resources showing immunomodulatory activity. Many herbs used in Indian system of traditional Siddha medicine were found to have immunomodulatory properties it could be because of effects of phytoconstituents such as phenolics, terpenoids, steroids, flavonoids, etc or because of minerals used in it. The endogenous antioxidant system prevents the deleterious influence of the free radical on the immune cells and preserves their normal function. Impairment in the immune system leads to overutilization of endogenous antioxidant [1].

Globally, it was estimated that about 870 million people were undernourished in the period from 2010 to 2012 and this represented 12.5% of the global population of which about 852 million people live in developing countries, where malnutrition is estimated at 14.9% [2]. However, malnutrition greatly affects the individual's immune system physiology and in most cases, there is need to stimulate it in circumstances of immunosuppression or suppress it in case of over exaggerated stimulation as in case of autoimmune disease conditions.

Herbs have unlimited capacity to the synthesis of bioactive compounds that are effective and have fewer side effects compared to synthetic drugs. Bioactive compounds from plants have shown over the years to have various biological activities. Scientists have developed a greater interest of using these compounds in the formulation of new and novel drugs, because of their biological activities and reliability [3].

Macrophages, important components in the human immune defense system, respond actively to inflammation by releasing pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6; high levels of these cytokines can cause systemic complications [4]. TNF-α is a pleiotropic inflammatory cytokine and can stimulate the production or expression of IL-1β and IL-6 [5]. IL-6 is a pivotal proinflammatory cytokine synthesized mainly by macrophages and plays a role in the acute-phaseinflammation response [6]. IL-1β is also considered to be another pivotal pro-inflammatory cytokine [7].

Naga Chendurum (NC) is a novel Siddha formulation comprised of a combination of herbs including *Baptisia bracfeata*, *Eclipta prostrata*, *Madhuga longifolia* and minerals such as zinc, sulphur, red orpiment etc. But still, now there is no proper documentary evidence available with respect to immune enhancing property hence the present investigation aimed at evaluating the immunomodulatory activity of Siddha formulation NC using Macrophage cell line RAW 264.7 using lipopolysaccharide (LPS) (1µg/mL) as a control.

2. Materials and Methods

2.1. Source of raw drugs

The required raw drugs for the trial medicineswill be purchased from a well-reputed country raw drug shop and drugs will be authenticated. Then the raw drugs will be purified separately then the trial drugs will be prepared in Gunapadam Laboratory of Arignar Anna government hospital,Government Siddha Medical College, Chennai, Tamil Nadu, India.

2.2. Ingredients

The Siddha formulation *Naga Chendurum* comprises of the following ingredients

Nagam (Zinc) - 35g

Manosilai (*Red* Orpiment)
Soodham (Mercury)
Veeram (Hydrargyrumper chloride) } Each 35 g

Gandhagam (Sulphur) - 70 g

Ilupainei (Oil of *Madhuga longifolia)*
Venkolinchiver (*Baptisia bracfeata*) - Q.S
Potralaikayanthagarai (*Eclipta prostrata*)

2.3. Purification

Nagam (zinc):Nagam (zinc) is melted and poured in the oil of *Madhuga longifolia* (illupainei). Repeatthis for 10 times. Now zinc is get purified

Rasam (Mercury): 35 grms of Mercury is triturated with brick powder and turmeric powder for one hour respectively and washed with water. Then the Mercury is boiled with the juice of Kuppaimaeni (1.3 litters) until it is detoxified[1].

Gandhagam (Sulphur): Sulphur is placed in an iron spoon. A small quantity of cow's butter is added and the spoon is heated till the butter melts; this mixture is immersed in an inclined position in cow's milk. This procedure is repeated for 30 times to get purified Sulphur. Each time, fresh milk is to be used.

Manosilai (RedOrpiment): Manosilai buried in limestone and poured by donkey urine to get purified.

Veeram (Mercury per Chloride): Camphor is mixed with tender coconut water and placed in a mud pot. Veeram is tied in a cloth and soaked in the pot without touching the water and the pot is burnt out for half an hour Then Veeram is taken out and washed

2.4. Formulation of Naga Chendhuram [8]

Nagam (zinc) is melted and poured in the oil of *Madhuga longifolia* (illupainei). Repeat this for 10 times. Now zinc is get purified, This purified Nagam (zinc) is placed inside the gugai and it is melted with the ulai. Here manosilai (Red orpiment) powder is added and stirred with the help of venkozhinjiroot (*Baptisia bracfeata*), then the whole content is turned into parpam. This prepared parpam, soodham (mercury),veeram (hydragramper chloride) are placed in astone mortar and grinded with milk for 4 samam(12 hours).Then sulphur is added and grinded well with potralaikarippan (*Eclipta prostrata*) and bring into incineration process with 10 cow dung cakes. Repeat this incineration process for 6 more times. Finally, it will turn into chendurum.

Dose: 130 mg. Twice a day for 48 days

Adjuvant : Honey

Indication: Vathadiseases, kasam, vallikunmamerikunmam, kalleeralkatti, pun, soolai, krani, megham, edappattuerralnoimanjalnoi , kaichal, kulir,mandharakasam.

2.5. Cell culture, measurement of cell viability [9]

Macrophage cell line RAW 264.7 was obtained from National Center for Cell Science (Pune, India) and cultured in DMEM supplemented with fetal bovine serum (10%) containing penicillin-streptomycin (10%) at 37°C in a humidified atmosphere containing 5% CO2. Cells were plated at a density of 1 × 104 cells/well in 25 or 75 cm2 flasks, or in 96-well plate overnight. RAW 264.7 were grown to 60% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1µg/mL). LPS stimulated RAW cells were exposed with different concentration (25, 50, 100 µg/mL) of the test sample and incubated for 24 hours. After 24 hours of incubation, the cells were digested and centrifugation was done at 6000 rpm for 10 minutes. Supernatant was discarded and cells were then resuspended in 200µl of cell lysis buffer (0.1M TrisHCl, 0.25M EDTA, 2M NaCl, 0.5 % Triton x-100). The samples were then kept at 4^{o}C for 20 minutes. After incubation, the Immunomodulatory response was performed by estimating nitrite levels in the cell lysate.

2.6.Estimation of Cellular Nitrite Levels [10]

The level of nitrite level was estimated by the method of Lee et al. (Lepoivre et. al. 1990) To 0.5 mL of cell lysate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

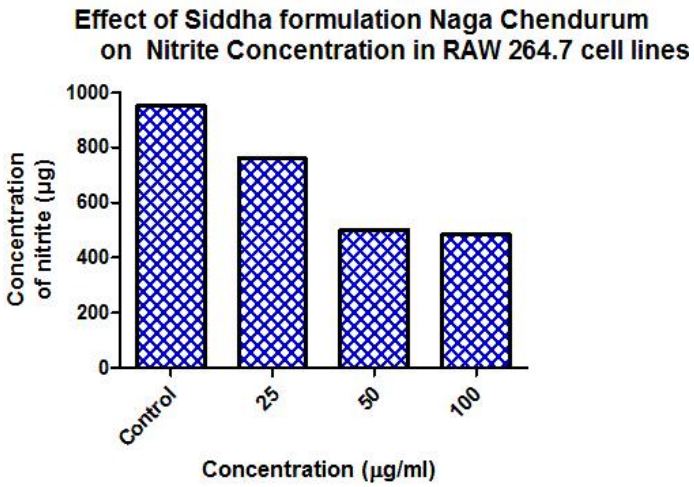
3. Results

It was observed that there was dosedependent decrease in the nitrite level in RAW 264.7 medium were observed at the concentration ranges from 25 to 100 µg/ml of the test drug NC. Lipopolysaccharide (LPS) (1µg/mL) treated well was served as a control with maximum nitrite level of about 952.38 µg. The formulation NC at the dose of 25 µg/ml shown a significant decrease in nitrite level of about 760.32 µg similarly at the concentration of 50 µg/ml it shows 501.93 µg and the maximum percentage decrease of nitrite level of about 483.61 µg were observed at 100 µg/ml. The results were tabulated in table 1 and shown in Figure 1.

Table 1: Effect of Siddha Formulation *Naga Chendurum*on Nitrite level RAW 264.7 Cell line.

Concentration (µg/ml)	Absorbance at 540nm	Concentration of Nitrites (µg)
Control (LPS1µg/mL)	0.1924	952.38
NC 25	0.1536	760.32
NC 50	0.1014	501.93
NC 100	0.0977	483.615

Figure 1: Effect of Siddha Formulation *Naga Chendurum*on Nitrite level in murine Macrophage RAW 264.7 Cell line



4. Discussion

High level of reactive oxygen species tends to attack macromolecules and this facilitates cells to undergo oxidative stress and inflammatory response. Antioxidant compounds scavenge the free radical molecules by donating one electron or proton to a molecule. The high intensity of the green colour is the indication of the high level of free radical molecules which can lead cells to undergo oxidative stress and inflammation. Reactive oxygen species act as a mediator to regulate cytokines production through activation of the transcription factors, such as NF-*κ*B. This suggests the direct link between ROS and other cytokines to initiate inflammatory response [11].

Cell-based immunotherapies are shown to be useful for some immune compromised diseases. Immune effector cells such as lymphocytes, macrophages, dendritic cells, natural killer cells (NKs) and cytotoxic T lymphocytes (CTL), operate concurrently to guard the body toward cancer by marking unusual antigens represented on the surface of the malignant cells due to mutation [12].The lipopolysaccharides (LPS)-treated Raw 264.7 cells have been widely used to study inflammatory responses. Exposure of Raw 264.7 cells to external bacterial toxins like LPS has been extensively shown to stimulate the secretion of nitric oxide (NO), which is produced by the inducible isoforms of nitric oxide synthase [13]. When the body is stimulated by pathologic injury, activated macrophages release numerous pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, and inflammatory mediators, such as NO, inducible NOS (iNOS) and cyclooxygenase- 2 (COX-2) [14]. COX-2 can convert arachidonic acid to prostaglandin and other eicosanoids. Aberrant functioning of COX-2 has been associated with carcinogenesis by promoting cell survival, angiogenesis, and metastasis [15,16]. NO is generated enzymatically by synthases (NOS) and is formed by iNOS in macrophages and other cells that play a role in the inflammatory response. Enormous amounts of NO can stimulate many proteins and enzymes crucial to inflammatory reactions, such as nuclear factor-kappa B (NF-κB) [17]. Therefore, various cytokines, NO, iNOS and COX-2 are important targets for immunomodulatory remedy.

Cytokines are immunological signals between cells and amplify both the local and systemic host responses; therefore, increase in interleukins is considered as a biomarker of increased immune response. Cytokines are necessary for stimulation of T and B lymphocytes. T-helper lymphocytes (Th) differentiate into Th1 and Th2 cells; Th1 are responsible for pro-inflammatory cellular immunity and express IL-2 and mediate humoral immunity while Th2 cells express IL-6 and mediate humoral immunity by differentiating B-cells to plasma cells to generate antibodies.It was observed that there was dose dependent decrease in the nitrite level in RAW 264.7 medium were observed at the concentration ranges from 25 to 100 µg/ml of the test drug NC. Lipopolysaccharide (LPS) (1µg/mL) treated well was served as acontrol with maximum nitrite level of about 952.38 µg. The formulation NC at the dose of 25 µg/ml shown a significant decrease in nitrite level of about 760.32 µg similarly at the concentration of 50 µg/ml it shows 501.93 µg and the maximum percentage decrease of nitrite level of about 483.61 µg were observed at 100 µg/ml.

5. Conclusion

Immunomodulation is a process which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulatory drug which primarily implies stimulation of specific and nonspecific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different

effector substances. The result of the present investigation clearly projects that the Siddha formulation *Naga Chendurum* significantly decreases the level of nitrites from 952.38 µg to 483.61 µg at the concentration 100 µg/ml which is considered to be one of the most important indicators of phagocytosis in the macrophage cell lines. Hence it may conclude that the formulation *Naga Chendurum* may be used as an Immunomodulators for clinical management of immune compromised diseases like Vathadiseases, Kasam, vallikunmamerikunmam, Kalleeralkatti, Pun, soolai, krani, Megham, Edappattuerralnoi, manjalnoi, Kaichal, kulir, Mandharakasam.

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