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Immunomodulatory activity of Siddha Herbo-Mineral formulation of Rasa Chendhuram in RAW Cell line 264.7.

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Abstract

The term 'immunity' has traditionally referred to the resistance exhibited by the host towards injury caused by microorganisms and their products. The immune response to an antigen, whatever its nature, can be of two broad types-Humoral mediated immunity and Cell mediated immunity. Macrophages, important components in the human immune defense system, respond actively to inflammation by releasing Pro-inflammatory cytokines, such as TNF- α , IL-1,6, high levels of these cytokines can cause systemic complications. Chronic inflammatory disorders such as Rheumatoid arthritis, Psoriasis, SLE, asthma diseases result in the production of several cytokines. These can recruit activated inflammatory and immune cells to the involved site and thereby amplify and perpetuate the inflammatory process. B cells and T cells are the major types of lymphocytes and are derived from haematopoietic stem cells in the bone marrow, Thymus(T-Lymphocytes) and Spleen. To evaluate the immunomodulatory activity of RCM using Macrophage cell line RAW264.7 using lipopolysacchrides (LPS)(1 μ g/ml) as a control.¹While the concentration level is decreased, nitrate level increased. Hence 25 μ g/ml of RCM has rich level of nitrate and thus proven to be an Immunomodulator.

Keywords: Rasa Chendhuram, Immunomodulator activity, RAW Cell line 264.7, Nitrate concentration.

Introduction

The immune system is a host defense system comprising many biological structures and processes within an organism that protects against disease. To function properly, an immune system must detect a wide variety of agents, known as Pathogens, viruses, parasitic worms, and distinguish them from the organism's own healthy tissue. The lymphoreticular system is a complex organization of cells of diverse morphology

distributed widely in different organs and tissue of the body responsible for immunity. The lymphoid cells, lymphocytes, and plasma cells are primarily concerned with specific immune response. The phagocytic cells, forming part of the reticuloendothelial system, are primarily concerned with the "scavenger" functions of eliminating the effete cells and foreign particles. They also plays a role in specific immunity, both in the afferent and efferent limbs of the immune response.^[7]

The cells of the adaptive immune system are special types of leucocytes, called lymphocytes. B cells and T cells are the major types of lymphocytes and are derived from haematopoietic stem cells in the bone marrow, Thymus(T-Lymphocytes) and Spleen.

Free radical generation in the immune system:

During normal biochemical reactions in our body there is a generation of Reactive Oxygen and nitrogen species (ROS and RNS). This gets enhanced during patho-physiological conditions creating "Oxidative stress". During this phenomenon cellular constituents get altered resulting in various diseased states. This may be efficiently neutralized, by enhancing the cellular defenses, in the form of antioxidants. Reactive species are also generated during 'Phagocytosis' a manifestation of innate immunity. The migration of leucocytes at an inflammatory site results in phagocytosis with the release of enzymes and cytokines from both macrophages and neutrophils. The free radical nitric oxide (NO), first described as endothelium-derived relaxation factor(EDRF), is produced from arginine by nitric oxide synthase (NOS). An inducible nitric oxide synthase (i NOS) is capable of continuously producing large amounts of NO. In activated immune cells, it acts as a "killer molecule". Cellular components of immune system are rich in polyunsaturated fatty acids and these are very much susceptible to oxidative attack resulting in highly damaging lipid peroxidation and it is highly cytotoxic. This phenomenon may result in increased prostaglandin levels that are strong immunomodulators^[1]

Effect of immunomodulatory agents:

Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms. These may be an antigen independent and may directly induce production and effector molecules by the immunocompetent cells. This type of antigen independent immunity is thus distinct from the one achieved by conventional immunization or by passive immunization using antibodies^[1]

The immunomodulatory agents may selectively activate either cell mediated or humoral immunity by stimulating either T-helper cells(1 or 2) type of cell response respectively. Oxidative stress may influence the immune system either by hyperexcitation to cause autoimmune disorders or suppress it, resulting in higher susceptibility to infections^[1]

Materials and Methods

Details regarding sample:

Rasa chendharam (RCM) is a classic Siddha herbo-mineral formulation mentioned in Sikicha Rathna Deepam.^[2]

Ingredients:

Purified Rasam (Mercury)
Purified Gandhagam (Sulphur)
Purified Paalthutham (Sulphate of zinc)
Mirabilis jalapa-Q.S

Drug collection:

All the ingredients were obtained from country drug shop, Ramasamychetti, Parrys Chennai, Tamilnadu, India.

Identification and Authentication:

All the raw drugs were identified and authenticated at Siddha Central Research Institute (SCRI), Chennai and medicinal botany department, Govt Siddha Medical college, Arumbakkam, Chennai.

Preparation of the drug:

The flower juice of the yellow variety of Mirabilis jalapa is to be grinded well with the above mentioned raw drugs in the stone mortar for 6 hours(2 saamam) till the juice and the drugs gets spreaded well in the mortar on all sides. Then it is to be collected using the spatula without any wastage. Next the collected medicine is to be placed in a mud jar and is closed with a proper lid and sealed up tightly with 7 layers of mud pasted cloth. After the sealing is dried, the mud jar is

placed in the vaalugaendiram. Then it is to be ignited with kamalakini for 6 hours (2 saamam) then for kaadakini for next 6 hours. Then it is to be left aside for the whole night to allow it to cool. Then the settled medicine is to be collected safely and placed in the mortar for grinding to get a fine chendhuram.

Then it is to be collected and placed in a air tight container^[2]

Significant effect on Rasa Chendhuram:

All the ingredients of RCM have antioxidant properties and free radicals scavenging and immunomodulatory effects as follows, Purified Sulphur has proper immune response to enhancing proliferation of lymphocytes, cytotoxic, T cells and Natural killer cells. Sulfasalazine also used in the treatment of rheumatoid arthritis and some other auto immune conditions^[4]. Purified Zinc sulphate plays an important role in maintaining immune status and wound repair via regulation of DNA and RNA polymerases, thymidine, kinase, and ribonuclease. It maintains macrophage and neutrophil function, NK Cells activity, and complement activity. It activates natural killer cells and phagocytic function of granulocytes and stabilize the plasma subcellular membranes especially the lysosomes. It inhibits the expression of integrins by keratinocytes and modulates the production of inflammatory mediators like nitric oxide. It has also been demonstrated to decrease the serum levels of TNF- α and inhibit the TNF- α induced apoptosis of peripheral blood mononuclear cells that helps in controlling the diseases activity and reactional states.^[3] *Mirabilis jalapa* revealed that the methanolic extract is a potent immunostimulant on both specific and nonspecific immune mechanisms. Thus, it might be served as an effective natural immunomodulatory agent.^[5]

Immunomodulator activity-cell line study

The evaluation of the immunomodulatory activity of Rasa chendhuram was carried out in cultured raw cell line in Biogenix Research Center.

Determination of in vitro immunomodulatory effect of extracts on cultured raw cell lines

RAW 264.7 cells will be grown to 60% confluence followed by activation with 1 μ L lipopolysaccharide (LPS) (1 μ g/mL). LPS stimulated RAW cells were exposed with different concentration (25, 50, 100 μ g/mL) of 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 200 μ l of cell lysis buffer (0.1M TrisHCl, 0.25M EDTA, 2M NaCl, 0.5 % Triton x-100). The samples were then kept at 4⁰C for 20 minutes. After incubation, the immunomodulatory response was performed by estimating nitrite levels in the cell lysate.

Estimation of Cellular Nitrite Levels

The level of nitrite level was estimated by the method of Lepoivre 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 μ L of the supernatant, 30 μ L of 10% NaOH was added, followed by 300 μ L of Tris-HCl buffer and mixed well. To this, 530 μ L of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against 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.

Results

While the concentration level is decreased, nitrate level increased. Hence **25 μ g/ml** of RCM has rich level of nitrate and thus proven to be an Immunomodulator.

Standard – nitrite level

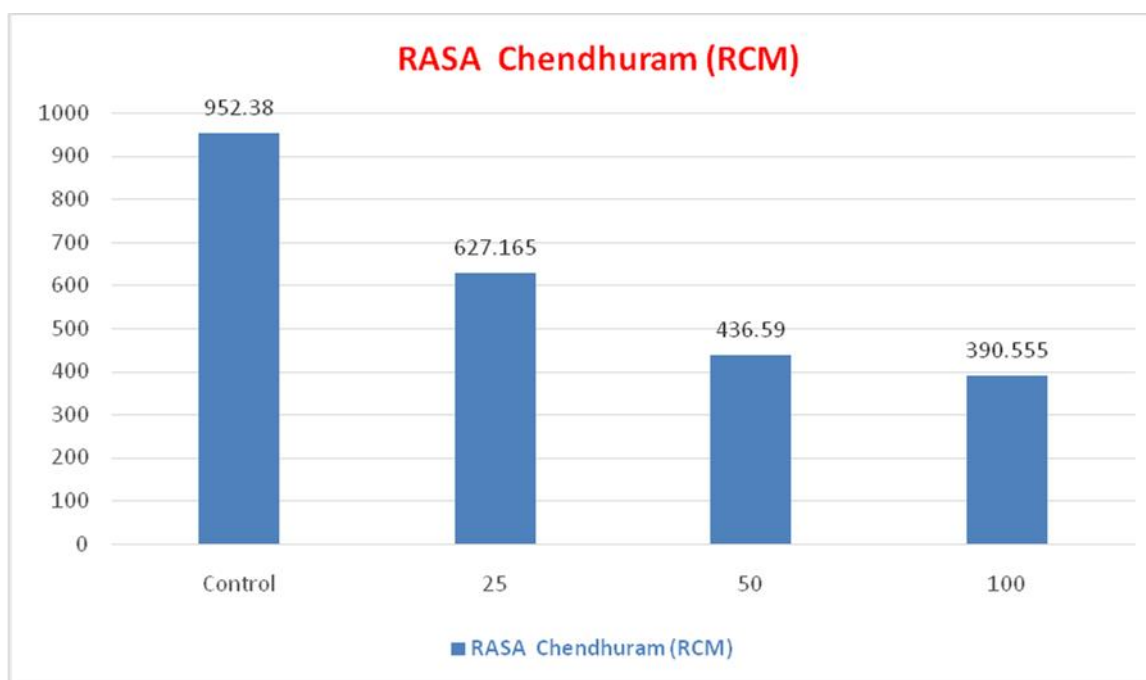
Table—1- Standard nitrate level for Immunomodulatory activity

Concentration (µg)	OD (540 nm)
100	0.021
200	0.42
300	0.06
400	0.08
500	0.17

Table-2-Immunomodulatory effect on Rasa Chendhuram(RCM) in RAW Cell line.

Sample Concentration (µg/ml)	OD at 540nm	Concentration (µg)
Control	0.1924	952.38
25	0.1267	627.165
50	0.0882	436.59
100	0.0789	390.555

Chart I –Immunomodulatory result of RCM



LD₅₀ value of Rasa Chendhuram(RCM):25µg/ml.

Discussion

Siddhars had the knowledge of converting inorganic substances into nano and ionic form which is easily absorbed by the human cells. And it is easily penetrating and targeting the cells.

The primary target of the immunomodulatory compounds is believed to be the macrophages, which plays a major role in the generation of immune response. It is known that the activated macrophages display not only increased phagocytosis and intracellular killing of

pathogens by producing effector molecules like free radicals and nitric oxide, but also produce cytokines like tumor necrosis factor(TNF- α), and interleukin(IL-1,IL-6,IL-12)etc..These cytokines may, in turn, activate T cells or NK cells.^[1]While the concentration level is decreased, nitrate level increased. Hence 25 μ g/ml of RCM has rich level of nitrate and thus proven to be an Immunomodulator.

Conclusion

To evaluate the immunomodulatory activity of RCM using Macrophage cell line RAW264.7 using lipopolysaccharides (LPS)(1 μ g/ml) as a control. It is widely utilized the model for evaluating the *in-vitro* immunomodulatory effect of the several siddha formulations. LPS induced nitrite production used an indicator for evaluating the level of Phagocytosis. The concentration was measured using the spectrophotometric technique at 540nm.While the concentration level is decreased, nitrate level increased. Hence 25 μ g/ml of RCM has rich level of nitrate and thus proven to be an Immunomodulator.

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