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Immunomodulatory effects of *Tinospora cordifolia* (Guduchi) on macrophage activation

More P, ***Pai K**
Department of Zoology, Centre for Advanced Studies, University of Pune, Ganesh Khind, Pune 411007, Maharashtra (India).

***Corresponding Author:** kalpnapai@yahoo.co.in, morepm207@gmail.com

Abstract
Macrophages are the first line of defense and constitute important participants in the bi-directional interaction between innate and specific immunity. Macrophages are in a quiescent form and are activated when given a stimulus. In the present study, we have used *Tinospora cordifolia*, commonly known as Guduchi, to see its effect on macrophage activation. The direct drug treatment to J774A cells showed activation as assessed by biochemical assays. Enhanced secretion of lysozyme by macrophage cell line J774A on treatment with *Tinospora cordifolia* and lipopolysacharide was observed, suggesting activated state of macrophages. Enhanced lysozyme production was reported at different time intervals (24 hrs and 48 hrs). This led us to check the effect of the drug on the functional activity of macrophage with respect to microbicidal properties by disk diffusion antibiotic sensitivity test. The enhanced inhibitory effects of *T. cordifolia* (direct effect) and *T. cordifolia* treated cell supernatant (indirect effect) on the bacteria (*E. coli*) indicates the susceptibility of bacteria. This study is an attempt to check the potential significance of the *T. cordifolia* to be used as immunomodulator for activation of macrophages.

Keywords: BRM; LPS; Guduchi; macrophage activation; lysozyme; nitric oxide.

Introduction

Macrophages are quiescent cells which get activated when stimulated. Different types of agents such as antibiotics, antimetabolites and cytokines may exert an immunomodulating action that is expressed in the augmentation and/or inhibition of different immune responses (Pai et al., 1997). One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses (Tzianabos, 2000). Disc diffusion method is used to test the survival of bacteria to any antimicrobicidal activity. The results of disc diffusion tests depend upon a number of variables, of which media, antibiotic concentration on the disc and inoculum size are the most important. Many efforts have been made to standardise methodology within countries, such as various modified versions of the Kirby-Bauer method in the USA (NCCSL, 1984), and between countries, such as the WHO guidelines on susceptibility testing suggested in 1961 (WHO, 1961).

A number of natural products and synthetic immunopotentiators termed as Biological Response Modifiers (BRMs) are becoming increasingly popular for testing their potential for augmenting immune responses. Among the natural BRMs many herbs and medicinal plants have long been known for their immunoaugmentary potential, however, only recently scientists have recognized them for their possible BRM actions. The herb isolated from botanical sources – *T. cordifolia* or Guduchi has attracted a great deal of attention in the biomedical arena because of its broad spectrum of therapeutic properties and relatively low toxicity. While our understanding of the mechanism of action of these BRM is still developing, it appears that the primary mechanisms involve induction of the immune system. The basic mechanism of the immunostimulatory, antitumor, bactericidal and other therapeutic effects of BRMs is thought to occur via macrophage activation. We have focused this study on the role of the BRM (Guduchi) on macrophage functions. Macrophage activation with the BRM treatment can be measured by different markers. Analysis of the development of activation is facilitated when the operationally defined stages of activation is characterized using a library of markers for activation. In the present study, we have assessed the direct and indirect effects of Guduchi on macrophage activation.

Materials and Methods

Reagents: RPMI 1640 with L-glutamine and 25 mM HEPES buffer were purchased from HiMedia Pvt. Ltd. India. Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56^0C for 30 min. The drug (Guduchi) used was obtained from

Himalaya Drug Company, India. All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

Cells: The macrophage J774A.1 cell line, purchased from National Center for Cell Sciences (NCCS, Pune), was used as source of macrophages (Origin: BALB/c mouse; Nature: Mature), grown and maintained in the RPMI 1640 medium (pH 7.2-7.4) enriched with 10% Fetal Bovine Serum, at 37^0C and 5% CO_2 environment.

Viability assay: Cell viability was determined by the Trypan blue dye exclusion technique. Equal volumes of cell suspensions were mixed with 0.4% Trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for cytotoxicity assay in 2 x 10^6 density per ml in the 96 well tissue culture plate.

Stimulation of macrophages: The macrophage cells (cell line J774A) from late log phase of growth (subconfluent) were seeded in 96 well flat bottom microtitter plates (Tarsons, India) in a volume of 100µl under adequate culture conditions. Drugs were added in different concentrations in a volume of 100µl in triplicate. The cultures were incubated at 37^0 C and 5% CO_2 environment. After 24 hr and 48 hr incubation percent viability was checked and culture supernatants were collected and assayed for nitric oxide and lysozyme activity.

Cytotoxicity assay: In order to detect the toxicity of herbal preparation the cytotoxicity assay was standardized by using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at different time intervals for 24 hrs and 48 hrs (Mosmann, 1983) using the drug at various concentrations. After 24 hrs and 48 hrs of incubation, supernatants were collected and ten microlitres of MTT (3 mg/ml) was added to each well and plates were further incubated for 2 hrs. The enzyme reaction was then stopped by addition of 150ul of dimethyl sulphoxide (DMSO). Plates were incubated for 10 min under agitation at room temperature before the optical density at 570nm was read under an *ELISA* plate reader. Three independent experiments in triplicate were performed for the determination of sensitivity to each drug. Cells treated with medium alone were considered as Control. Percent viability was calculated by the given formula.

$$\text{Percent viability} = \frac{E}{C} \times 100$$

where E is the absorbance of treated cells and C is the absorbance of untreated cells.

Nitrite assay: The concentration of stable nitrite, an end product of the nitric oxide present in the supernatant of treated or untreated J774A macrophage cell cultures (2 x 10^6 cells/ml), was measured by the method of (Ding et al.,1988) based on the Griess reaction. Briefly, 50 ul of supernatant was incubated with an equal volume of Griess reagent (1% sulphanilamide in 2.5 % H_3PO_4 and 0.1% naphthyl-ethylene-diamine-dihydrochloride in distilled water; both solutions mixed in a ratio of 1:1 at room temperature) for 10 min. The absorbance at 550 nm was then measured in a microtitre plate reader. The standard curve for nitrite was prepared by using 10-100 µM sodium nitrite in distilled water.

Assay for lysozyme: Lysozyme assay was performed with culture supernatants from treated and untreated macrophage cultures by the method described by Litwack (1955). A substrate suspension of dried and killed *Micrococcus luteus* (0.1 mg/ml) was prepared in 0.1 M sodium phosphate buffer at pH 6.2 and 0.1 ml culture supernatant collected from treated and untreated macrophages were added to each tubes containing 2.5 ml of substrate buffer kept at 37^0C. Optical density at 600 nm was checked at zero hour of incubation. The tubes were then incubated at 37^0 C for 1 hr and decrease in optical density was checked at 600 nm. Experiments were repeated three times in triplicates and enzyme activity expressed in units/ml culture supernatant / 2 x 10^6 cells / ml.

Kirby-Bauer antibiotic testing or disk diffusion antibiotic sensitivity testing:
The antibacterial susceptibility has been checked by using the Kirby-Bauer method (Williams, 1990; NCCSL, 1984; WHO, 1961). This method is used for testing the antimicrobial susceptibility of bacteria based on the size of zones of inhibition of growth of a lawn culture around disks impregnated with the antimicrobial drug. A cell suspension of freshly grown *E. coli* by comparing with the 0.5 McFarland turbidity standard was prepared. A suspension in the range of 10^7-10^8 cells was prepared by adjusting, according to the 0.5 McFarland standard which was diluted by adding sterile water to obtain a suspension of upto 10^6 cells of *E. coli* (McFarland, 1907;

Finegold, 1986; Lennette, 1985). 100 µl of this suspension was spread on a sterile Luria-agar plate using a sterile glass spreader which was surface sterilized in absolute alcohol after every use. Two sterile paper discs made of Whatman paper no. 1 were placed in one plate to check the direct drug effect. 5 µl of the drug (Guduchi, 80µg/ml) and incomplete medium (RPMI 1640 as control) were then added on each disc. The indirect effect was checked with the drug treated or untreated cell supernatants at different time intervals (24h and 48h) in another plates. All the above steps were performed in sterile conditions. The plates were incubated at 37^0C in a BOD incubator for 18-24 hours. After incubation, the diameter of the zone of growth inhibition was measured and scored according to the size of the zone as sensitive intermediate or resistant. LPS treatment was considered as positive control.

Statistical analysis: Statistical significance of difference between the control and experimental samples was calculated by Student's t- test. All the experiments were done in triplicate samples. Conclusions were drawn from 3 independent experiments.

Results

Viability and proliferation of J774A cells: J774A cells showed 100% viability before the drug treatment by trypan blue dye exclusion test. The cells were treated with drugs initially on log scale and then on linear scale. J774A cells were incubated in medium alone or drug for 24 h and 48 h and checked for percent viability as described in Materials and Methods. Guduchi in concentration 80µg/ml showed maximum viability of macrophages as compared to medium alone, thereby proving that the drug was not cytotoxic to the cells.

Table 1: Effect of BRMs on the viability of J774A cells.

Treatment	Concentration	% Viability (±SD)	
		24 hrs	48 hrs
Medium alone		99 ± 0.75	99 ± 0.25
Guduchi	80µg/ml	94 ± 0.89	94 ± 0.89
LPS	10µg/ml	92 ± 1.00	93 ± 2.00

The values are mean ± S.D. and are representative of three independent experiments done in triplicate. Cells when treated with medium alone showed maximum viability.

Generation of nitrite: J774A cells were incubated in medium alone or Guduchi for 24 h and 48 h and the cell supernatant was checked for nitrite. Cell supernatant of macrophages treated with Guduchi 80µg/ml for 24h and 48h showed significantly increased levels of nitrite as compared to cells treated with medium alone. Similar results were obtained with the LPS treated cell supernatant.

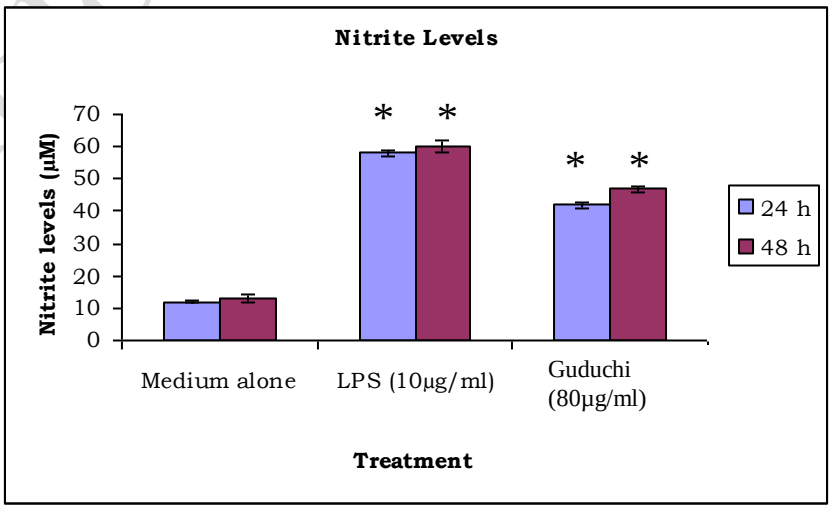

Fig. 1: The levels had increased significantly after 24 h and 48 h treatment. The values are mean ± S.D. and are representative of three independent experiments done in triplicate. *p<0.05; significantly different from respective controls.

Secretion of lysozyme: When macrophages were treated with the BRM, increased release of the lysozyme was observed as compared to untreated cells as assessed by their supernatants. Decreased turbidity of *Micrococcus luteus* in the substrate buffer (as a measure of lysozyme) was observed after treatment with the supernatants of drug treated cells. A significant level of lysozyme was observed in the supernatants after 24 h and 48 h of treatment. The lysozyme levels were measured in units/ml of culture supernatants/2 x 10^6 cells/ml.

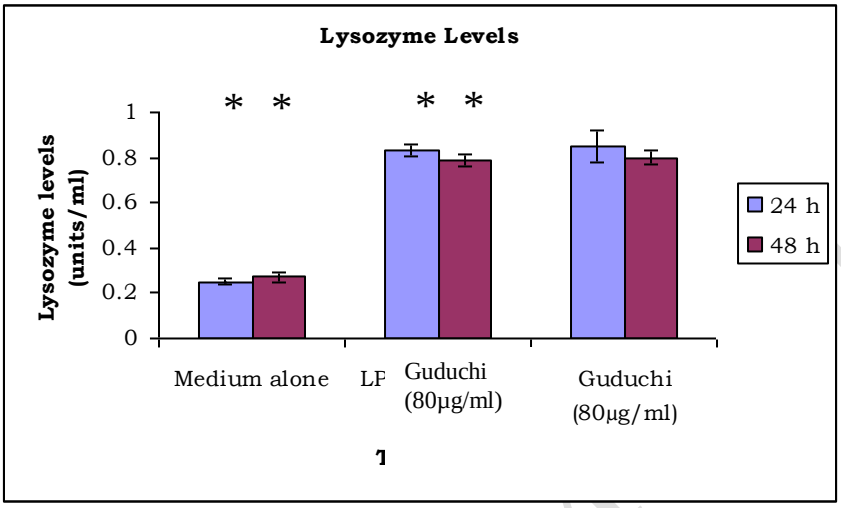

Fig. 2: The values are mean ±S.D. and are representative of three independent experiments done in triplicate. *p<0.05; significantly different from respective controls.

Disk diffusion antibiotic sensitivity testing

Direct effect: *E. coli* treated with LPS and Guduchi were found to be sensitive as compared to the *E. coli* treated with medium alone. Disc with the drug showed the zone of inhibition while no zone was observed with the disc impregnated with medium alone. The diameter of the zone of inhibition by the drug treated disc was 1.2 cm.

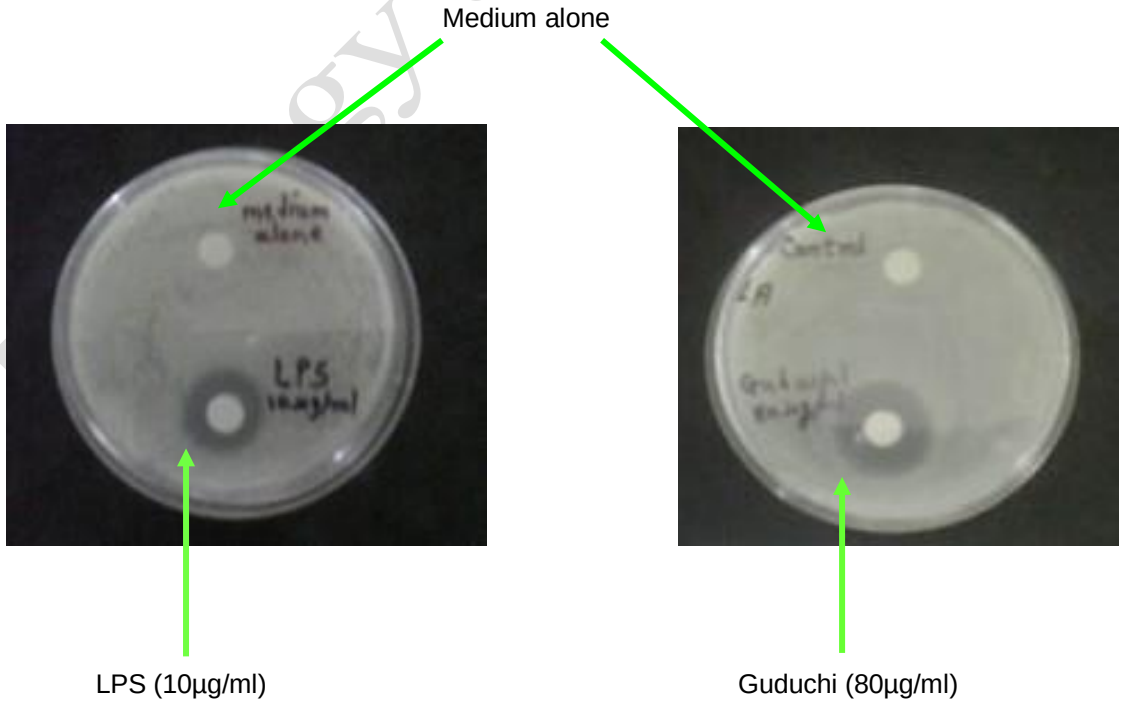

Fig. 3: Antibacterial susceptibility shown with the direct effect. Effective zone of inhibition can be observed.

Indirect effect: *E. coli* were treated with macrophage supernatants collected after 24 h and 48 h treatment of Guduchi and LPS. The 24 h and 48 h treated cell supernatant showed comparatively larger and clear zone of inhibition. The 48 h treated cell supernatant treatment to *E. coli* showed larger zone of inhibition which was 1.4 cm in diameter, while the medium treated cell supernatant showed 1.35 cm of zone of inhibition.

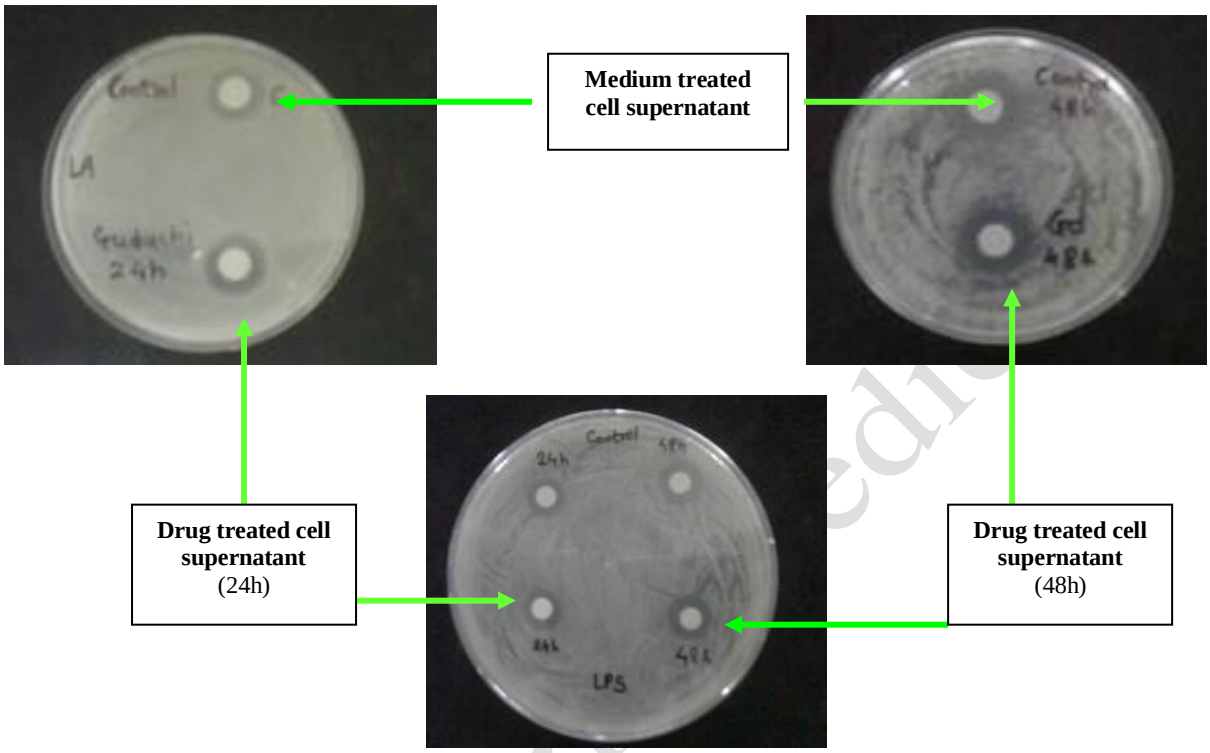

Fig. 4: Antibacterial susceptibility shown with the indirect effect. Effective zone of inhibition can be observed.

Discussion

Macrophage activation is being better understood with the biochemical and cytochemical assay. In actively respiring cells, superoxide and hydrogen peroxide can be activated which further generates reactive oxygen species (ROS). These ROS would cause extensive damage to DNA proteins and lipids. The drug treatment can activate the macrophages (phagosomes) which inhibit intracellular replication of the pathogens.

Among the natural BRMs, many herbs and medicinal plants have immunopotentiating capacity and are reffered to as 'Rasayan' in the Ayurvedic system of medicine. The chloroform & benzene extracts of Guduchi were found to possess significant antibacterial activity as compared with the standard. Guduchi leaf extract is useful in *Proteus vulgaris, Staphylocosus aureus, Streptococus pyrogens, Bacilus subtilis* and *Escherochia coli* infections (Nagvalli et al., 2006). The entire plant is regarded as a valuable alterative and stimulant. It is useful in eye conditions, as a tissue builder, helps development of intelligence, and retains youth by helping to prevent premature aging. In view of the known immunomodulatory properties of *T. cordifolia* (Dahanukar et al., 2000), we were interested in studying the antimicrobial potential by testing inhibition of bacteria by formation of a zone around a paper disc impregnated with the drug or drug treated macrophage cell supernatant placed on a mat culture of *E. coli*. Presence of nitrite and lysozyme in the cell supernatant of activated macrophages leads to inhibition of bacterial mats.

Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis and destruction of targeted organisms (Beutler, 2004). The spectrum of microorganisms kept on check by phagocytes includes fungi, bacteria and virus infected cells (Beutler, 2004). Production of nitric oxide and lysozyme (Fig. 1 and 2) appears to constitute one of the main microbicidal mechanisms of macrophages and has been implicated in the

elimination of viruses, bacteria, fungi and protozoa (De Groote and Fang, 1995). The direct effect of the drug to the bacteria is significantly different as compared to the supernatants from cells treated with medium alone as shown in Fig. 3. Though there is a little difference in the diameter of zone of inhibition in the indirect effect, the drug treated cell supernatant gives clear zone of inhibition as compared to untreated cell supernatant (Fig. 4). It appears that these are the primary mechanisms involving nonspecific induction of the immune system.

One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses (Tzianobos, 2000). Plant derived immunomodulatory compounds have also been used in traditional remedies for various medical problems and the investigation of these sources has grown exponentially in recent years. India has a rich tradition in the treatment of many diseases by therapy with 'Rasayans'. In Ayurveda, 'Rasayans' are concerned with nourishing body and boosting immunity. They are also modulators of the immune system and one such cell modulated by them is the macrophage. The macrophage is usually is in a quiescent state in a healthy individual, but in the presence of a pathogen or when treated with drug macrophage becomes activated (Singh et al., 2006). The effect of the BRM on the activation of quiescent resting macrophages was assayed by its secretory activity of factors like nitric oxide (assayed in the form of nitrite) and lysozyme which leads to the microbicidal activity of macrophages. In the present study, effect of Guduchi (*Tinospora cordifolia*) as immunomodulator and Lipopolysaccharide (LPS), a bacterial endotoxin as a positive activator, was investigated by standard biochemical parameters of macrophage activation.

Lysozyme, which is secreted constitutively and in large amounts by macrophages, was found to be further increased in BRM treated macrophages. Lysozyme is highly active against gram-positive species and deficiency in lysozyme production has been found to lead to susceptibility to *Streptococcus pneumoniae* (Shimada et al., 2008).

The alcoholic and aqueous extracts of *T. cordifolia* have been tested successfully for immunomodulatory properties (Thatte et al., 1987; Dahanukar et al., 1988; Thatte and Dahanukar, 1989; Rege et al., 1989, 1999; Dikshit et al., 2000; Manjrekar et al., 2000). Previous authors have reported findings that most of the therapeutic compounds in plants are polar in nature and require polar solvents for their extraction. However, the ethanolic extract failed to show significant activity in the present study. The herb is being consumed by people since a long time.

Today, it is becoming more and more clear that the products identified under the traditional or alternative medicines *in vitro* conditions also stimulate the natural *in vivo* conditions and thus may give the true direction to the problem. It would therefore suffice to extrapolate the *in vitro* observations to *in vivo* conditions. There is thus a great need to understand how exactly pathogen survives *in vivo* conditions and identify pathways unique to the intracellular environment that could be utilized for the development of new and better drugs.

Conclusion

The results of present study show experimental basis of immunomodulation by biological response modifier (BRM). This study addresses a very pertinent question of bio-medical sciences dealing with the scientific basis, particularly immunomodulatory effects, of the herbal medicine preparations on the macrophage activation, as macrophages are known to represent the first line of defense against invading microorganisms or in a state of altered self.

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