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In Vitro Immunomodulatory Activity of Sagalanoi Chooranam (Poly Herbal Formulation) in RAW Macrophage Cell Line

Research Article

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Abstract

Within the realm of Siddha medicine, an ancient and venerable traditional healing system hailing from India, a comprehensive methodology is deployed to combat liver disorders. This approach is characterised by the utilisation of herbal remedies, accentuating the reinstatement of equilibrium in the body's vital life force, known as *"Vatham," "Pitham,"* and *"Kapham."* The utilisation of cell line studies is crucial in assessing the immunomodulatory properties of herbal medicine, offering invaluable insights into their capacity to influence the immune system and advancing our comprehension of their therapeutic benefits. The primary objective of this investigation was to elucidate the potential immunomodulatory properties of *sagalanoi chooranam,* a polyherbal Siddha formulation, through the execution of anti-proliferative assays conducted. On the RAW 264.7 macrophage cell line. Various test solutions were precisely prepared, encompassing a spectrum of concentrations (50, 100 and 200 µg/ml). The RAW 264.7 cells were diligently cultured and then carefully subjected to stimulation with lipopolysaccharide (LPS) to initiate cellular activation. Following this activation, the test formulation was introduced at varying concentrations, after which the cells underwent a 24-hour incubation period. The resultant nitrite levels, serving as indicators of immunomodulatory response, were evaluated within the cell lysate. The outcomes unveiled a notable decline in nitrite levels, correlating with the dosage of the test formulation during the incubation with RAW 264.7 cells. The outcomes of the study elucidate a conspicuous decline in nitrite levels in direct correlation with escalating concentrations of the test formulation, contrasting starkly with the control group exclusively subjected to LPS. Furthermore, the investigation meticulously probed the ramifications of the test formulation on cellular viability. The vitality of RAW 264.7 cells exhibited a discernible downward trajectory as the concentration of the test formulation ascended. Noteworthy is the fact that, at the concentration of 200 µg/ml, cellular viability registered at a mere 55.53 ± 3.567%. Collectively, these findings lend credence to the hypothesis that the test formulation possesses a dose-dependent capacity to attenuate both nitrite levels and cellular viability within the RAW 264.7 cell milieu, underscoring its auspicious immunomodulatory attributes. This research expands our insights into the potential immunological ramifications of the test formulation and underscores its plausible utility within the realm of immunomodulation.

Keywords: Immunomodulatory activity, Raw Macrophage cell line, Siddha Medicine, *Sagalanoi chooranam*.

Introduction

In Siddha medicine, liver diseases are referred to as *"KalleeralNoi."* These conditions are characterised by changes in the size of the liver and disruptions in its normal physiological functions, which can result in a range of diseases. In addition to *"KalleeralNoi,"* other terminologies used in Siddha medicine to describe liver diseases include *"Valapaateeralnoi," "Valapateeralviruthi," "Kaleeralvalarchi," "Maandhakatti," "Kalmaandham,"* and *"Yahrutham."*

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The utilisation of natural products holds particular appeal in the realm of medicine, given their established safety for human consumption. Although there are various medicines suggested to treat liver diseases, *SagalanoiChooranam,* (1) a renowned polyherbal Siddha formulation, holds a widespread reputation for its diverse applications, all centered around its capacity to bolster immune functionality. The medicinal efficacy of *Sagalanoi chooranam* extends to addressing *Kaleral Noigal*, a specific ailment explicitly outlined in the Biramamuni karukidai 800 text. *Sagalanoi chooranam* comprises essential elements such Cumin (*Cuminum cyminum Linn.*) (2), Licorice (*Glycyrrhiza glabra Linn)* (3), Madhana kamapoo (*Cycas circinalis Linn.),* Black cumin (*Nigella sativa Linn.*)(4), Cloves (*Syzygium aromaticum Linn.*) (5) (6), Dill *(Anethum graveolens Linn.)*(7), Coriander *(Coriandrum sativum Linn)* (8). These individual components have been substantiated

by research studies for their notable capabilities in augmenting immune functions. (9-11).

A multitude of research investigations have meticulously chronicled the immunomodulatory attributes inherent in extracts harnessed from natural reservoirs. A particularly notable instance is exemplified by a study delving into the effects of Fermented *Platycodon grandiflorum* (PG) extract (FPGE), wherein its prowess in orchestrating the modulation of nitric oxide (NO) expression and pivotal pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6, was demonstrated.

Moreover, the administration of FPGE exhibited a compelling association with heightened levels of activation and phosphorylation pertaining to the nuclear factor kappa B (NF-κB). Intriguingly, the engagement of AMP-activated kinase (AMPK) through 5-aminoimidazole-4-carboxamide ribonucleotide engendered a substantial reduction in NF-κB reporter gene activity, an effect observed in response to both lipopolysaccharides and the stimulation elicited by FPGE (12).

Undertaking medicinal investigations within the domain of traditional healing systems assumes paramount significance in establishing a robust evidentiary basis for the efficacy of such ancient practices. The utilisation of traditional medicine to augment immune functions across a spectrum of disease states constitutes a prevalent therapeutic approach. However, the precise intricacies of the immune-enhancing mechanisms inherent in *Sagalanoi chooranam*, particularly within the milieu of macrophage cells, remain conspicuously uncharted.

Hence, the current study endeavors to exquisitely evaluate cell viability within the confines of RAW 264.7 cells, thereby shedding light on the hitherto unexplored aspects of this traditional medicinal formulation.

Materials and methods (13) (14)

Preparation of test solutions

For anti-proliferative tests, the test formulation was serially diluted (to 50, 100, and 200 g/ml).

Culture: Macrophage cell line RAW 264.7

Cell Culture and Cell Viability Measurement

The RAW 264.7 macrophage cell line was sourced from the National Center for Cell Science in Pune, India, and cultivated in DMEM supplemented with 10% foetal bovine serum and 10% penicillin-streptomycin, under conditions of 37°C and 5% CO2 in a humidified environment. The cells were seeded at a density of 1 × 104 cells/well in either 25 or 75 cm2 flasks, or in a 96-well plate and allowed to incubate overnight. When the RAW 264.7 cells reached approximately 60% confluence, they were stimulated with 1 µL of lipopolysaccharide (LPS) at a concentration of 1 µg/mL. The LPS-activated RAW cells were then exposed to varying concentrations (50, 100, and 200 µg/mL) of the test sample and incubated for 24 hours. Following the 24-hour incubation period, the cells were harvested, and centrifugation was performed at 6000 rpm for 10 minutes. The resulting supernatant was discarded, and the cells were re-suspended in 200 µL of cell lysis buffer (comprising 0.1M Tris-HCl, 0.25M EDTA, 2M NaCl, and 0.5% Triton x-100). The samples were maintained at 4°C for 20 minutes. After incubation, the assessment of immunomodulatory response was carried out by measuring nitrite levels in the cell lysate.

Estimation of Cellular Nitrite Levels

The determination of nitrite levels was conducted following the method of Lee et al. (15). To 0.5 mL of cell lysate, 0.1 mL of sulphosalicylic acid was added and vigorously mixed for 30 minutes. The samples were subsequently centrifuged at 5,000 rpm for 15 minutes, and the protein-free supernatant was employed for nitrite level estimation. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer, and thoroughly mixed. Subsequently, 530 µL of Griess reagent was added, and the mixture was incubated in darkness for 10–15 minutes. The absorbance was then measured at 540 nm against a Griess reagent blank, with a sodium nitrite solution serving as the standard. The quantity of nitrite in the samples was determined from the standard curves obtained.

Results

It was observed that there was a dose-dependent reduction in nitrite levels in the RAW 264.7 medium incubated with the test drug within the concentration range of 50 to 200 µg/ml. A control group treated with Lipopolysaccharide (LPS) at a concentration of 1 µg/mL exhibited the highest nitrite level, approximately 1954 ± 47.15 µg. At a dose of 50 µg/ml, the SC formulation demonstrated a significant decrease in nitrite levels, approximately 1203 ± 181.5 µg, similarly at 100 µg/ml, it exhibited 866.7 ± 138.5 µg, and the maximum percentage decrease in nitrite levels of about 420.7 ± 119.4 µg was observed at 200 µg/ml. The study's findings reveal that the cell viability percentage of the macrophage cell line decreases as the concentration of the test drug SC increases, with the lowest cell viability observed at a concentration of 200 µg/ml, approximately 55.53 ± 3.567%.

Table 1: Effect of SC on Nitrite level in RAW 264.7 Cell line.

Concentration (µg/ml)	Concentration of Nitrites (µg)
Control (LPS1µg/mL)	1954 ± 47.15
SC 50 µg	1203 ± 181.5
SC 100 µg	866.7 ± 138.5
SC 200 µg	420.7 ± 119.4

Table 2: Effect of SC on Cell viability in RAW 264.7 Cell line

S.No	Concentration in µg/ml	% cell Viability
1	Control (LPS1µg/mL)	94.8 ± 1.425
2	SC 50 µg	77.64 ± 1.12
3	SC 100 µg	65.9 ± 2.304
4	SC 200 µg	55.53 ± 3.567

Figure 1: Effect of SC on Cell viability in RAW 264.7 Cell line

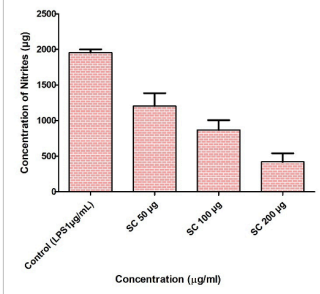


Figure 2 : Effect of SC on cell viability in RAW 264.7 cell line

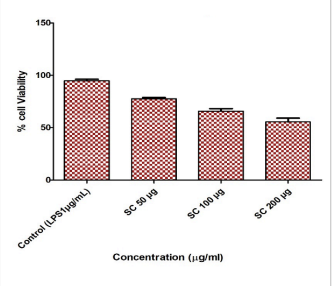


Figure 3: LPS induced proliferation in Macrophage cell line RAW 264.7 Control (LPS1 µg/mL)

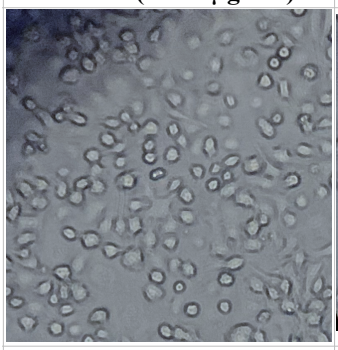


Figure 4: LPS induced proliferation in Macrophage cell line RAW 264.7 SC - 50 µg

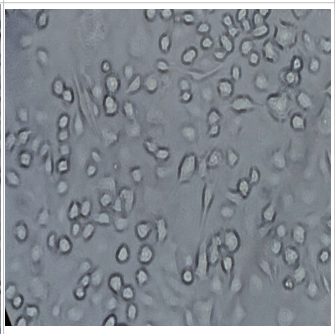


Figure 5: LPS induced proliferation in Macrophage cell line RAW 264.7 SC - 100 µg

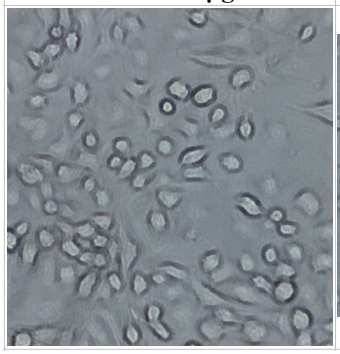
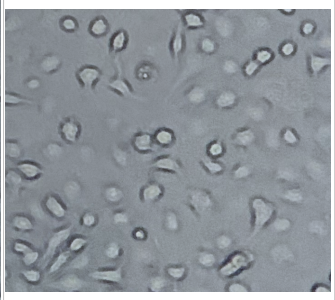


Figure 6: LPS induced proliferation in Macrophage cell line RAW 264.7 SC - 200 µg



Discussion

The Siddha system of traditional healing is a time-honored medicinal heritage with its origins deeply rooted in the southern regions of India (16).

One of the core components of Siddha medicine is the use of herbal combinations to formulate medicines that target various ailments and imbalances (17)

Cell lines play a crucial role in scientific research, particularly in disciplines like medicine and biotechnology. They serve as indispensable models in drug development, aiding in the evaluation of potential therapeutic compounds and ensuring the assessment of their effectiveness and safety. Herbal immune stimulants have been employed as agents to enhance the immune system (18) (19).

yet only a limited number of products have undergone scientific validation of their effects (20 - 26). The preparation of Siddha herbal medicine *Sagalanoi chooranam* involves intricate processes that aim to extract and preserve the active constituents of the herbs. These ingredients such as are carefully processed through methods such as grinding, sieving, to create potent medicine.

The preparation of Siddha herbal medicine *Sagalanoi chooranam* involves intricate processes that aim to extract and preserve the active constituents of the herbs. These ingredients such as are carefully processed through methods such as grinding, sieving, to create potent medicine.

The effectiveness of Siddha herbal combinations has been passed down through generations and is supported by empirical evidence. However, as with any traditional system, there is an increasing interest in scientific validation of Siddha medicines. Researchers are conducting studies to understand the mechanisms of action, active compounds, and potential interactions of these herbal combinations with modern medical practices.

Remarkably, certain natural compounds have demonstrated a dualistic propensity, wherein they enhance the immune response while concurrently manifesting anti-inflammatory attributes within RAW 264.7 cells (27) (28).

As an illustration, within a research inquiry involving the administration of β-glucan (polycan), it became evident that RAW 264.7 cells manifested conspicuous enhancements in the synthesis of nitric oxide (NO), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). This phenomenon was concomitant with a heightened expression of inducible nitric oxide synthase, TNF-α, IL-6, and IL-1β mRNA. It is noteworthy that these responses occurred at concentrations devoid of any cytotoxic effects, thereby underscoring the potential of these natural compounds to efficaciously modulate the immune response (29) (30).

Nitrites, frequently regarded as surrogates for immune responses and inflammatory processes, are recognised participants in a spectrum of cellular mechanisms, notably including the activation of immune entities such as macrophages. The marked decrease in nitrite concentrations suggests a plausible attenuation of immune reactions, potentially instigating a well-regulated and harmonised immune response (31).

In the present investigation, the observed diminishing trend in nitrite levels within the RAW 264.7 medium, following exposure to the test drug across a spectrum of concentrations spanning from 50 to 200 µg/ml, highlights the potential immunomodulatory attributes intrinsic to this therapeutic regimen. Notably, within this context, the lipopolysaccharide (LPS)-treated group, operating at a concentration of 1µg/mL, assumed a pivotal role as a pertinent control, distinctly showcasing a markedly elevated nitrite level, quantified at approximately 1954 ± 47.15 µg.

The findings stemming from this study distinctly delineate a dose-dependent relationship between the test formulation SC and nitrite levels within the RAW 264.7 cell line. At a concentration of 50 µg/ml, the formulation manifested a conspicuous reduction in

nitrite levels, registering an approximate value of 1203 ± 181.5 µg. A parallel pattern was observed at the dosage of 100 µg/ml, wherein nitrite levels descended to 866.7 ± 138.5 µg. However, the most pronounced decline in nitrite levels materialised at the highest dosage examined, namely 200 µg/ml, where nitrite levels reached an approximate nadir of 420.7 ± 119.4 µg.

Furthermore, the study embarked on an exploration of the test formulation's influence on the viability of the macrophage cell line. The data gleaned from this investigation underscored a direct correlation between the concentration of the test formulation SC and macrophage cell viability. It is noteworthy that the lowest cell viability was recorded at the zenith of the concentration spectrum, specifically at 200 µg/ml, at which point it plummeted to a mere 55.53 ± 3.567%.

The reduction in nitrite levels with an escalation in the concentration of the test drug implies a conceivable connection between the herbal formulation and the regulation of nitrite production. Nitrites, typically indicative of immune responses and inflammatory processes, are acknowledged participants in diverse cellular mechanisms, encompassing the activation of immune entities like macrophages. The substantial decline in nitrite levels alludes to a prospective attenuation of immune responses, potentially fostering a well-regulated and harmonised immune reaction.

The contrast between the LPS-treated control and the diminishing nitrite levels in the test groups supports the notion that the herbal formulation could be contributing to the modulation of immune responses within the RAW 264.7 cell line. However, further mechanistic studies are warranted to elucidate the precise pathways through which the herbal formulation may exert these effects on nitrite production. Moreover, investigating the potential involvement of specific bioactive compounds present in the herbal formulation could provide valuable insights into the observed immunomodulatory activity. The application of cell line studies in investigating immunomodulatory activity holds practical significance.

Conclusion

The formulation SC has demonstrated a notable decrease in nitrite levels in a dose-dependent fashion across the concentration range of 50 to 200 µg/ml. Consequently, the data lead to the conclusion that formulation SC exhibits compelling immunomodulatory potential.

Conflict of interest

The authors have no conflicts of interest regarding this study**.**

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