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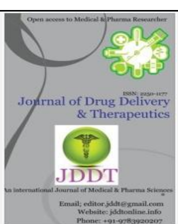

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Research Article

IMMUNOMODULATORY EFFECT OF *PARKIA JAVANICA* EXTRACT ON INTRACELLULAR EXPRESSION OF IL-6, IL-8, IL-12 AND TNF-α

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ABSTRACT

The crude methanol extract of *Parkia javanica* (crude MEPJ) was screened for immunomodulatory activity on macrophage cells, RAW 264.7, by cell proliferation, migration and qRT PCR based interleukin expression assays. The maximum proliferation and migration of macrophage cells were observed at the dose of 20µg/ml. The expression of proinflammatory cytokines, IL-6, IL-8, IL-12 and TNF-α were increased after treatment with crude MEPJ. This study clearly suggests the immune stimulant property of *Parkia javanica* on macrophage cells.

Keywords: *Parkia javanica*, macrophage cells, interleukins, immunomodulatory.

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INTRODUCTION

The immune system is a remarkably developed defence system that protects vertebrates from foreign bodies. The immune system produces many cells and molecules that distinguish and eliminate foreign and unwanted agents[1]. The modulation of the immune system refers to any alteration in the immune response, including stimulation, expression, amplification, or inhibition of any portion or stage of the immune response. Therefore, immunomodulators are substances used for their effects on the immune system. Immunomodulators are grouped into two types based on their effects: immunostimulators and immunosuppressors. These immunomodulators mount an immune response or defend against pathogens or tumors[2]. Immunomodulators are substances that modify the response of the immune system to a threat. Immunomodulators modulate and potentiate the immune system, keeping it highly prepared for any threat[3]. Currently, worldwide, there is an increase in diseases especially infectious diseases that requires efficient body defense mechanisms to control them through the process of immunomodulation[4]. Plant-derived compounds, bacterial products, synthetic drugs and marine compounds have been used as immunomodulation agents[5]. However, plant-derived compounds also have an important role as compared to many of the other immunomodulators because of their broad spectrum of therapeutic properties and low toxicity[6]. Among various immune system-related cells, macrophages are versatile cells that exist in almost all tissues and play important roles in immune responses[7,8]. In particular, macrophages are recruited in infection sites where they are activated to perform many functions through increasing phagocytosis. This process is the first line of defence against microbial and parasitic infections and in removing senescent and dead cells, immune mediator secretion, and antigen presentation [9,10]. In the innate immune system activated macrophages also prevent the invasion of pathogens by secreting inflammatory mediators, including nitric oxide (NO), prostaglandin E2 (PGE2), and cytokines, such as TNF-α and interleukins (ILs) [11-15]. Modulation of macrophage activity by drugs/exogenous substances can be used to counter many of these pathologic states[5]. *Parkia javanica* a plant

of leguminece family, has long ethnomedicinal history among different population of north eastern region of India[16,17,18]. This work has been undertaken to explore the effect of *Parkia javanica* extract on macrophage cells in *in vitro* system.

MATERIALS AND METHODS

Plant collection & Authentication

Fresh stem barks of *P. javanica* were collected from Suryamaninagar, Tripura, India. The plant was initially identified by Dr. B. K. Dutta, Taxonomist, Department of Botany, Tripura University and finally authenticated by Dr. H. J. Chowdhery, Joint Director, Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal and respective voucher specimen No. ≠BD-01/06 has been deposited in the Herbarium.

Preparation Plant Extract

After washing with water fresh stem barks of *Parkia javanica* were allowed to dry in shade. Then 500 gm of powdered bark was soaked in 2000 ml of methanol to prepare the crude methanol extract of *Parkia javanica* (crude MEPJ) and then kept in a shaker for 48 hours. After that the solution was filtered through Whatman filter paper no. 1 for 3 times. Then the solution was dried in rotary evaporator at 70°C. Finally these solutions were freeze- dried and stored at - 20°C [19].

Immunomodulatory effect *in vitro*

Cell lines

Mouse macrophage cells (Raw 264.7) were purchased from National Centre for Cell Science, Pune, India.

Cell culture

Macrophage cell lines, Raw 264.7, were maintained in DMEM and supplemented with 10% FBS, 100 IU/mL penicillin and 100µg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2 (ESCO, Singapore).

Cell Proliferation assay

Cells were cultured in 96 well plate tube with or without different concentration of crude MEPJ for 24 h time duration. The cells viability was assessed using MTT assay method[20]. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate-buffered saline at a concentration of 5 mg/mL. MTT was added to each well, and plates were incubated at 37°C for 3 h. The medium was replaced with 100 µL DMSO, and the absorbance for each well will be measured at 570 nm on a microplate reader. Total viable cell count was taken at different time periods of 24h, 48h and 72h.

Cell migration assay

Mouse macrophage (Raw 264.7) cells were treated with crude MEPJ and an artificial wound was created on culture plates by scratching the plates through microtips. Microscopic images were taken immediately after wounding and during an incubation period of up to 72 hours. Finally the activity of the crude MEPJ was compared with the control and measured by calculating the percentage of closed area [21,22].

In vitro stimulation of intracellular cytokines production

Total RNA was isolated using Qiagen RNeasy mini kit, according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using iScript reverse transcription supermix for RT-qPCR (Applied Biosystem) and the resulting cDNA was amplified by PCR using specific primers for the studied genes. The thermal cycling condition was set as denaturation for 30 s at 95°C, annealing for 30 s at appropriate Tm and elongation for 45 s at 72 °C with a final extension for 7 min at 72 ° C and it was ensured that amplicons were in the linear phase of amplification. PCR amplification cycles were performed using a Applied Biosystems, Waltham, MA, USA; 7500 Fast Real-Time PCR). Real-time PCR was done to quantify the amount of product using Syber green PCR master mix kit (Applied Biosystems, CA, USA). After each cycle a melting curve analysis was performed to check that no primer dimers or nonspecific products were formed. Fold induction of a gene was calculated using an equation: Fold induction = $2^{\Delta\Delta Ct}$, where Ct stands for threshold cycle number for a gene[23]. The sequences of the primers[24] are given in Table 1.

Table 1: Oligonucleotides used for qPCR

Gene	Forward Primer (5' - 3')	Reverse Primer (3' - 5')	Tm (°C)
Mouse Primers			
IL-6	GTTCTCTGGGAAATCGTGGA	GTACTCCAGGTAGCTATGG	55
IL-8	TGGCAGCCTTCCTGATTT	AGGTTTGGAGTATGTCTTTATGC	54
IL-12 p40	CAGAAGCTAACCATCTCCTGGTTTG	CCGGAGTAATTTGGTGCTCCACAC	60
TNFα	AAGCCTGTAGCCCATGTTGTA	TCAGCTCCACGCCATTG	50
β-actin	GTGGGCCGCTCTAGCCACCAA	TCTTTGATGTCACGCACGATTTC	60

Statistical analysis

All data were represented as mean ± SEM. Differences among groups were evaluated using one-way ANOVA to determine statistical significance. $P<0.05$ was accepted as statistically significant. $P<0.05$, $P<0.01$ are represented by *, ** and ***, respectively.

RESULTS

Parkia javanica stimulates Macrophage proliferation

MTT assay was used to examine the effect of Crude MEPJ on mouse macrophage cell line (Raw 264.7) proliferation. The cells were treated with different concentrations of crude MEPJ, and the cell viability was determined by MTT assay at 24 h, 48h and 72 h, respectively. As seen in Fig.1, after 24-h treatment, the proliferation of Raw 264.7 increased up to 140% in presence of crude MEPJ, compared to untreated control. Enhanced proliferation in macrophage cell line, after 48 h of crude MEPJ treatment was observed which increased slightly (Fig.1.). Thus crude MEPJ extract might have a significant role in the proliferation of macrophage cells.

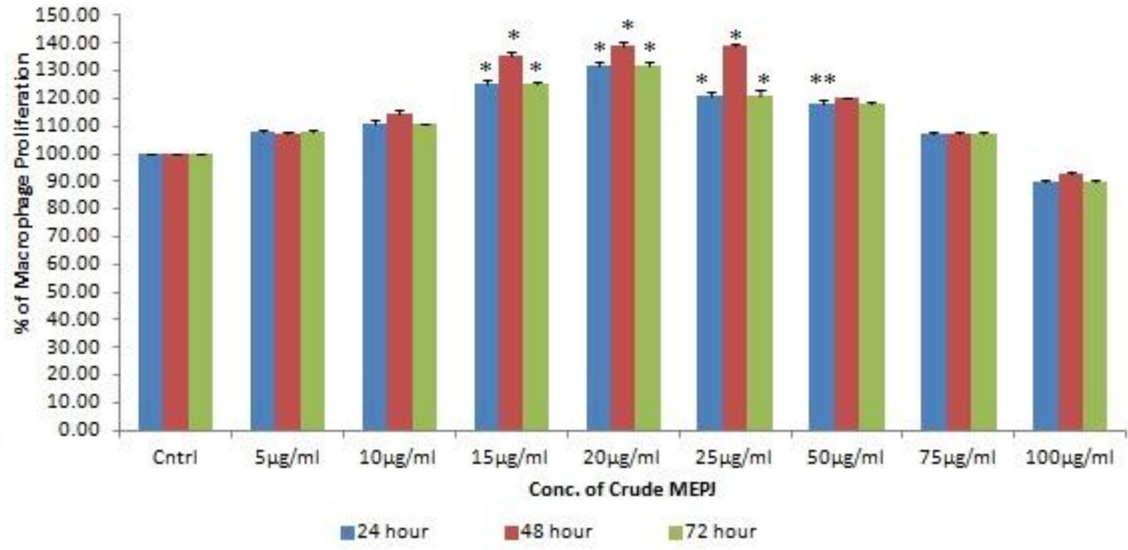
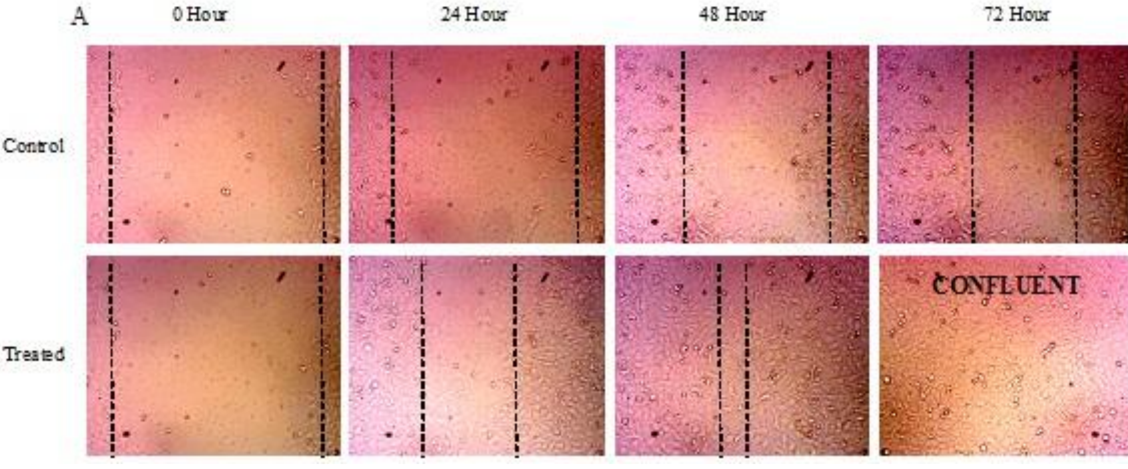


Figure 1: Histogram of % of cell proliferation in presence of crude MEPJ on macrophage cell line (Raw 264.7). Results were expressed as mean ± SD, n=3 and the statistical significance was calculated using one way ANOVA followed by Bonferroni test.* ($P<0.05$) and ** ($P<0.01$) in comparison to control (without extract).

Parkia javanica increases Macrophage Migration

As the proliferation of macrophage plays an important role in wound healing activity, we next explored the effect of crude MEPJ on the migration of Raw 264.7 cells in an in vitro wound healing model. In this model scrape wounds were generated in nearly confluent monolayer of cells, and the migration of the edge of the wound was monitored with a phase contrast microscope. Cells with or without crude MEPJ treatment were allowed to migrate into the void area for 0–72 h. The macrophage cells started to migrate into the void area at 24 h after crude MEPJ treatment and wound area clouses up to 80% compared to respective wound area, and the void area of the cells was almost closed at 72 h and 48 h, respectively. In contrast, the migration of untreated cells was slower at the corresponding time points. The gap width at each time point after treatment with crude MEPJ was measured and plotted in Fig 2.



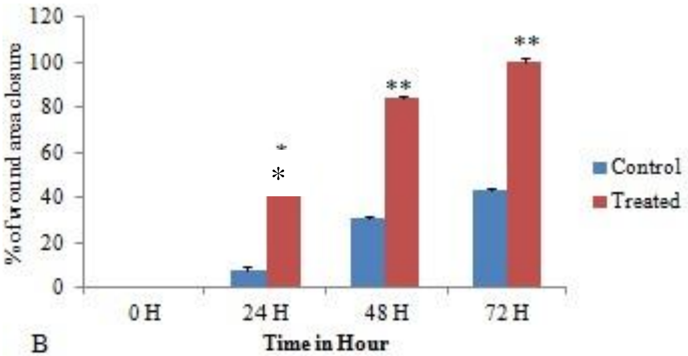


Figure 2: PJ (*Parkia javanica*) induced migration of macrophage cells in an *in vitro* wound model. (A) The wound is generated in monolayer cells and photographed with a phase contrast microscope (10X) immediately after wounding (0 h) and 24, 48 and 72 h later. (B) Gap of the wound areas were measured using ImageJ Software, and % of wound closure with time is calculated. Results were expressed as mean ± SD, n=3. * (P<0.05), ** (P<0.01) in comparison to control (without extract) and the statistical significance was calculated using one way ANOVA followed by Bonferroni test. h, hour.

Expression of IL-6, IL-8, IL-12 and TNF- α .

As various immune related cytokines, are produced by activated macrophages which are essential for the host survival of infection, modulation and orchestration of innate immunity, so next we explored the effect of crude MEPJ on such cytokines. In the present study induced expression of IL-6, IL-8, IL-12p[40], TNF-α, in treated macrophage cells compared to untreated cells was observed (Fig 3). The experiment revealed that, crude MEPJ treated cells showed increased ILs expression compared to untreated cells. The expressions of IL-6, IL-8 were increased up to 3 to 3.5 fold change. The expression of IL-12p[40] was also increased after treatment with crude MEPJ extract. In macrophage cell line the expression of TNF-α significantly increased after treatment.

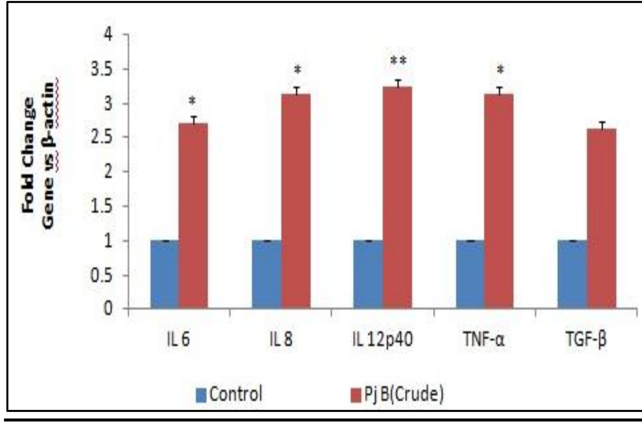


Figure 3: mRNA levels of IL6, IL8, IL12p40 and TNF-α were quantified by qPCR from the total RNA isolated from Raw 264.7 cells treated with crude MEPJ (20μg/ml). Results were expressed as mean ± SD, n=3. * (P<0.05), ** (P<0.01) in comparison to control (without extract) and the statistical significance was calculated using one way ANOVA followed by Bonferroni test.

DISCUSSION

The modulation of immune response to combat diseases has long been a topic of interest. Many recent studies made progress in the research on ethnomedicinal plants as immunomodulatory agents. It is an interesting area for inflammation, autoimmunity and anticancer therapy[25, 26]. Numerous plants are used in traditional medicinal system showed to stimulate or inhibit the immune responses[27]. Medicinal plants and their products are most useful in health and disease treatment through the involvement of various biological activities like anti-oxidant and anti-ulcerogenic activities[28]. Moreover several medicinal plants possess immunomodulatory activities as they impart anti-inflammatory, anti-microbial, and anti-tumor effects under varying experimental conditions.

Macrophages are the first line of defence in the innate immunity against microbial infection, and phagocytes engulf and kill microorganisms and present antigens that elicit adaptive immune responses[29]. Macrophages serve an important function in tissue remodelling through development, wound healing, and tissue homeostasis. Macrophages are essential to the innate immunity and pathology of tissue injury and inflammation[30] through phagocytosis. Macrophages secrete cytokines, such as interleukins, TNF-α, and TGF-β, as well as inflammatory mediators, such as nitric oxide[31]. Macrophages maintain homeostasis and serve an important function in the host defence against pathogens and attacking cells, such as cancer cells[32]. Different plant extracts such as *Poulteria cambodiana*, *Clausena excavate*, Nigella seed have shown to increase macrophage proliferation and migration[5,33]. In the present study the crude MEPJ found to increase macrophage proliferation and migration about 40 % and 72 % at the optimum dose of 20 μg/ml concentration.

Inflammation is the response of tissues to injury, characterized in the acute phase through increased blood flow, vascular permeability and the accumulation of fluid, leukocytes and inflammatory mediators, such as cytokines. The immune response in the chronic phase is characterized by the development of specific humoral and cellular immune responses to pathogens at the site of tissue injury[34]. Various immune-related cytokines, such as TNF- α, IL-6, and IL-8, are produced by activated macrophages for the modulation and

orchestration of innate immunity[35,36]. These cytokines can be produced from macrophages in response to bacterial LPS, infection, and inflammatory stimuli. They also play an important role in the immune system by aiding cytotoxic and cytostatic effects on infected or malignant cells [37,38]. The results of this study clearly reveal that the treatments using the plant enhance immune response and stimulate the production of essential mediator cytokines, such as IL-6, and IL-8, which are important in the acute and chronic inflammatory response. IL-6 is important in the stimulation of acute phase protein synthesis in the liver, acts as a growth factor for mature b cells, stimulates their final maturation onto antibody-producing plasma cells, is involved in T cell activation and differentiation, and affects the induction of IL-2 receptor expression. IL-8 is a potent neutrophil chemotactic factor and has a wide range of actions on the different types of cells, including endothelial cells, fibroblasts, monocytes, lymphocytes, and neutrophils. These functions suggest that IL-8 has important roles in different pathological disorders, such as chronic inflammation and cancer.

TNF- α is one of the earliest factors to be induced or activated in macrophages for eliciting tumor immunity. TNF- α plays as a key mediator of T lymphocyte and macrophage activation and exerts either beneficial or detrimental effects on mammalian cells by inducing the secretion of NO and PGE2 [39, 40]. IL-12 are produced by various immune cells, including macrophages. These cytokines are essential for host survival of infection, and they are required for the repair of injured tissue[41,42]. In the present study, crude MEPJ significantly stimulated the expression of these cytokines. Although further research is needed to identify the active pharmacological constituents of crude MEPJ and to understand the mechanisms of their actions, these findings clearly suggest immune stimulant property of *Parkia javanica* so far macrophage cells are concerned.

CONCLUSION

The findings of the present work showed that crude methanol extract of *Parkia javanica* activates macrophage cells in term of proliferation, migration, pro-inflammatory cytokines release. This strong immuno-modulatory activity of *Parkia javanica* provides a future basis for the development of this plant as a source of immunoregulating substance.

COFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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