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EFFECT OF DESMODIUM GANGETICUM ON MACROPHAGES: AN IN VITRO STUDY

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ABSTRACT

Background- Desmodium gangeticum (DG) is commonly known as “shalparni” in Ayurveda. It grows as undershrub and categorised as “shothahara” meaning anti-inflammatory as per classical ayurveda texts. Macrophages are one of the main players involved in inflammatory conditions. This study aimed to study the effect of shalparni on inflammation in macrophages. Methods: Human monocytic cell line THP-1, was used for this study. THP-1 cells were stimulated using PMA and further differentiated into M1 using LPS and IFN-γ. Methanolic root extract of shalparni was used to study its effect on cells. Inflammatory mediators, surface proteins, chemokines and anti-inflammatory markers were studied using molecular biology tools. Results: The gene expression analysis showed DG treatment leads to reduction in transcripts level of IL-1β and IL-6 in M1 macrophages. CD38, a cell surface protein showed subdued expression following shalparni treatment. MCP-1, a chemokine showed downregulation while TGF-β expression level was found to be upregulated after treatment. Conclusion: The reduction in inflammatory mediators clearly validates the ayurvedic property of this plant. Alterations in cell surface markers suggests the phenotypic shift in classically activated macrophages. Reduction in chemokine associated with M1 macrophages confirms the reduction in inflammatory cytokine production. Increased TGF-β suggests increase in antiinflammatory response. These observations suggest shalparni has potential to be used in treating macrophage associated inflammatory conditions.

Key Words: M1 Macrophages, Inflammation, Desmodium gangeticum, Ayurveda Biology

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INTRODUCTION

Desmodium gangeticum (L.) DC., commonly called shalparni, is a perennial shrub of the Fabaceae family. It is found across tropical and subtropical regions of Asia and Africa. In Indian traditional medicine, it holds an important position as one of the ten roots of "Dashamoola". Dashamoola is a classical Ayurvedic formulation used for treating inflammatory disorders, respiratory ailments, and digestive issues. Its inclusion in remedies such as Dashmoolarishta and Dashmoolakwaath reflects centuries of reliance on its therapeutic properties (Govindarajan, Singh, et al., 2007).

Ethnobotanical surveys highlight its diverse applications in Ayurveda, Siddha, and Unani systems, where decoctions and extracts are prescribed for fever, bronchitis, diarrhoea and rheumatism. Shalparni is widely used for the treatment of ulcer, arthritis and ischemia among other indications (Dharmani et al., 2005; Govindarajan et al., 2003; Kurian & Paddikkala, 2012). A study in an animal model reports the flavonoid and alkaloid fraction of this plant exerts anti-inflammatory and antioxidant activity (Govindarajan, Vijayakumar, et al., 2007).

Immunity represents the body's sophisticated defense network, integrating innate and adaptive mechanisms to safeguard against pathogens. Upon activation, immune responses initiate inflammation that recruits protective cells, eliminates harmful agents, and promotes tissue repair. Thus, inflammation functions as the immediate effector arm of immunity, bridging defense and healing.

The human body governs inflammation in a context-dependent manner. Sometimes this tight regulation gets disturbed under prolonged activation or due to some external challenges. This dysregulation leads to hyperactivation of inflammatory pathways, resulting in self-damage and disease initiation and progression. Immune cells are the actual guardians of this sophisticated immune system, such as T cells, B cells, macrophages, lymphocytes and others. Macrophages are one of the immune cells that detect, engulf and clear the pathogens. Along with phagocytosis, macrophages regulate inflammation, secrete cytokines and present antigens to lymphocytes, showing a role in both innate and adaptive systems. Generally, macrophages are categorized in two groups: the tissue-resident, alternatively activated, and anti-inflammatory ones, called M2 macrophages. Whereas the classically activated ones, which are responsible for pathogen clearance, are called M1 macrophages (Mills, 2015). With the help of a diverse spectrum of cytokines and chemokines, M1 macrophages engulf the pathogen by a process called phagocytosis. TNF-α, IL-1β, IL-6 and IFN-γ are some of the inflammatory cytokines which are elevated during an immune response. However, sometimes they start damaging the host cells during uncontrolled high expressions causing damage to normal cells in vicinity. Any therapeutic agent reducing this excess cytokine is usually helpful in managing inflammatory diseases.

This study explores the effect of methanolic root extract of shalparni on M1 macrophages. Shalparni (DG) shows anti-inflammatory effect on M1 macrophage.

METHODOLOGY

Plant extraction

Roots of Desmodium gangeticum (DG) were collected and authenticated in our botany department herbarium.

Roots were washed thoroughly and air-dried, followed by coarse grinding. This powder was extracted in methanol [10%(w/v)] using the cold maceration method in a shaker for 72 h at 37^0C. The solution was filtered using Whatman filter paper, and the solvent was evaporated using rota-vapour. The methanolic root extract of DG and was kept in -20^0C for further usage.

Cell Culture

Human-derived THP1 cells were used in this study. These were cultured in RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum and 1% pen-strep antibiotic mixture. Cells were maintained at 37^0C with 5% CO_2.

MTT Assay

20000 cells were seeded per well in a 96-well plate format. Post 24 of cell seeding, PMA (Phorbol 12-myristate 13-acetate) was added to activate these cells. 24 hours after PMA treatment, media was removed, cells were washed with PBS and treated with different concentrations of DG extract along with vehicle control and positive control. After 24h, MTT (Methylthiazolyldiphenyl-tetrazolium bromide) was added to the media (0.5 mg/ml) and incubated

for 4h at 37^0C. After 4h, media was removed and DMSO was added to dissolve formazan crystals. Absorbance was read at 570 nm. Cell viability was calculated using the mentioned formula: Viability = (Reading- Min) / (Max-Min)* 100. qRT-PCR

500,000 cells were seeded per well in a 6-well plate, and PMA was added after 24 h to stimulate the cells. Media was removed after 24 hours, followed by a PBS wash, and differentiation media was added. Differentiation to M1 phenotype was done by treatment with LPS (5µg/ml) and IFN-γ (ng/ml) with or without the methanolic extract (10µg/ml) in complete media. After 24 h, media was discarded, Trizol was added and RNA was isolated manually using the phenol-chloroform method. RNA purity was checked using the 260/280 ratio and samples were used only when the ratio was >1.8. 2µg RNA was used for cDNA synthesis following manufacturer's protocol (iScript™ cDNA Synthesis Kit Biorad). Real-time PCR was performed to evaluate gene expression using SYBR green method (iTaq Universal SYBR Green Supermix Biorad). The 2^ddCT method was used to analyse relative gene expression using β-actin as a housekeeping gene. The primer sequences used are in Table 1.

Statistical Analysis

All the experiments were done at least 3 times. Data was expressed as mean ± SD. Student's t-test was used to calculate significance where p value < 0.05*.

Table 1

S.No	Gene	Forward (5'-3')	Reverse (5'-3')
1	β-actin	GACGACATGGAGAAAATCTG	ATGATCTGGGTCATCTTCTC
2	CD38	CAGACCTGACAAGTTTCTTC	GATGACATAAACCACAAGGAG
3	CD319	CATTGAAGAGAAGAAGAGAGTG	CTATTAGTGTGAGGGATTGTG
4	CD206	AAATTTGAGGGCAGTGAAAG	GGATTTGGAGTTTATCTGGTAG
5	TNF-α	AGGCAGTCAGATCATCTTC	TTATCTCTCAGCTCCACG
6	IL-1β	CTAAACAGATGAAGTGCTCC	GGTCATTCTCCTGGAAGG
7	IL-6	GCAGAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
	IFN-γ	GGTAACTGACTTGAATGTCC	TTTTCGCTTCCCTGTTTTAG
8	TLR4	GATTTATCCAGGTGTGAAATCC	TATTAAGGTAGAGAGGTGGC
9	IRF5	GTCAAGACCAAGCTTTTCAG	CAGTAATGAGCTTCTTCTCTC
10	TGF-β	CCCACAACGAAATCTATGAC	TGTATTTCTGGTACAGCTCC
11	IL-10	GCCTTTAATAAGCTCCAAGAG	ATCTTCATTGTCATGTAGGC
12	CCL18	CTATACCTCCTGGCAGATTC	CTCTCTTGGTTAGGAGGATG

RESULT

Desmodium gangeticum is non-cytotoxic and reduces inflammatory cytokines in M1 macrophages

We first assessed the effect of the Desmodium gangeticum (DG) on the viability of activated macrophages over a range of concentrations (Fig. 1B). No significant change in cell viability was observed, which shows that the drug is non-cytotoxic at the tested concentrations.

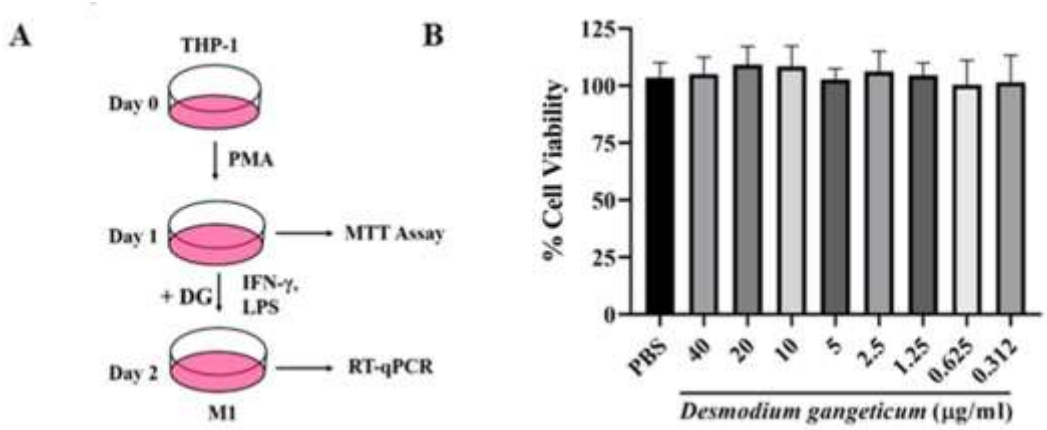

Figure 1: A) Cell culture treatment protocol and B) Cell viability assay of shalparni (DG) on M1 cells.

Next, we checked the effect of DG on M1 macrophages using gene expression analysis. M1 macrophages are known to have a high inflammatory state, and therefore we studied the expression of inflammatory genes. TNF-α expression level was reduced after DG treatment, though it did not reach significance (Fig 2A). IL-1β is known to have a direct correlation with fever, and its expression was significantly reduced upon DG treatment (Fig 2B). Similarly, IL-6 and IFN-γ levels also showed reduction upon DG treatment (Fig 2C, D). Overall, DG treatment reduced the inflammatory milieu in M1 macrophages.

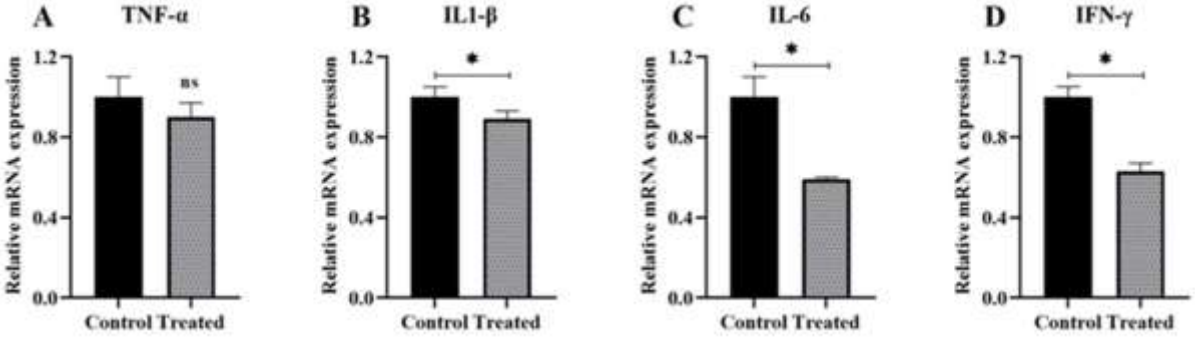

Figure 2: Effect Shalparni (DG) on inflammatory marker in M1 macrophages, A) TNF-$\boldsymbol{\alpha}$, B) IL 1-$\boldsymbol{\beta}$, C) IL-6 and D) IFN-$\boldsymbol{\gamma}$

Desmodium gangeticum modulates cell surface marker in M1 macrophages

Cell surface proteins are used as a marker to categorise the phenotypic state of macrophages. CD38 and CD319 are among the best studied surface proteins which we tracked. These are associated with M1 macrophages. DG treatment showed significant reduction in CD38 transcript level (Fig. 3A), whereas no change was found in the CD319 level (Fig. 3B). CD206 is another cell surface marker protein which plays an essential role in efferocytosis and is mostly associated with alternatively activated macrophages called M2. The CD206 level was found to be significantly decreased after DG treatment (Fig. 3C) suggesting that there is no tendency towards M2 macrophages. These results suggest that while the M1 macrophage markers and cytokines (Fig 2) are reduced, there is no tendency towards anti-inflammatory M2 macrophages.

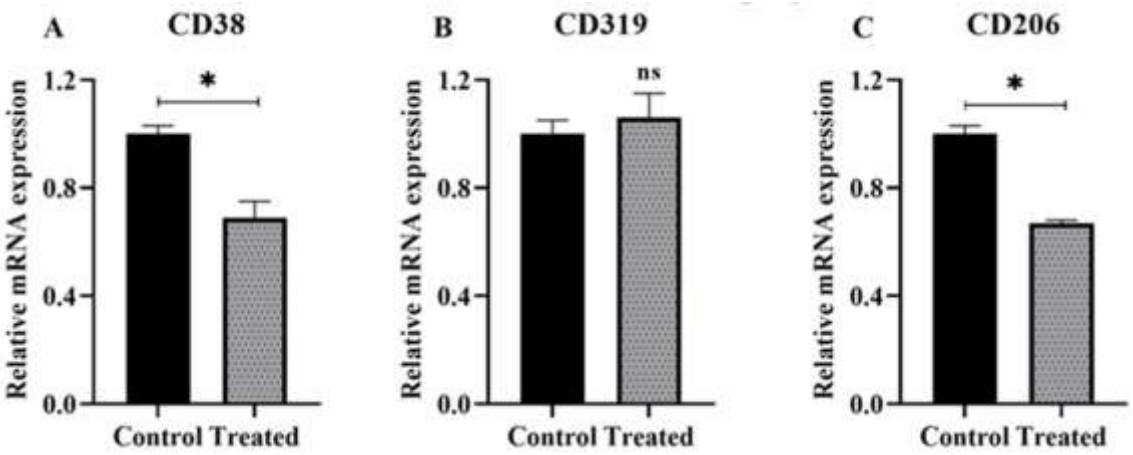

Figure 3: Effect of shalparni (DG) on cell surface marker ijn M1 macrophages, A) CD38, B) CD319 and C) CD206

Desmodium gangeticum shows differential effect on key functional genes

TLR4 is a pattern recognition receptor that functions as an innate immunity sensor as well as governs cross talk between innate and adaptive immune responses during infection (Mukherjee et al., 2016). DG treatment leads to an increase in the expression level of TLR4, suggesting an improved pathogen clearance (Fig. 4A). Monocyte chemoattractant protein-1 (MCP-1) belongs to the CC chemokine family, which either attracts other macrophages or enhances the inflammatory state. MCP1 transcript level was subdued after DG treatment (Fig. 4B). IRF5, interferon regulatory factor 5, is a transcription factor that plays an important role in inflammatory macrophage physiology (Krausgruber et al., 2011) and is considered important for inflammatory functions. DG treatment upregulated IRF5 level (Fig. 4C).

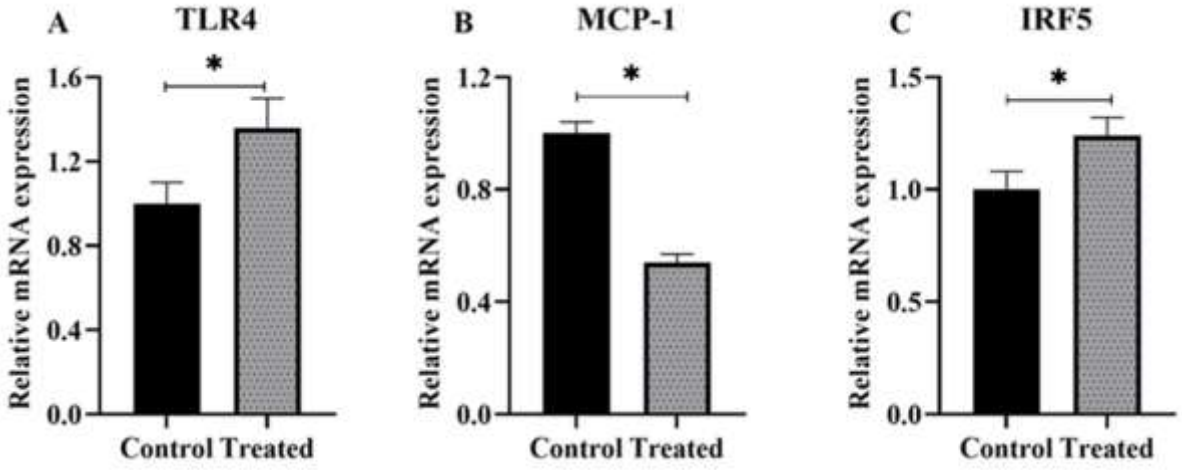

Figure 4: Effect of shalparni (DG) on functional genes in M1 marcrophage, A) TLR4, B) MCP-1 and C) IRF5

Desmodium gangeticum enhances TGF-β expression in macrophage

Transforming growth factor-β (TGF-β) is known to be an anti-inflammatory marker that is highly associated with alternatively activated macrophages (M2 type) (Travis & Sheppard, 2014). DG treatment enhances the TGF-β expression in M1 macrophages (Fig. 4A). Next, IL-10 and CCL18 transcript level were checked and the treatment showed significant reduction in both the markers (Fig. 4B & C). Both these cytokines are primarily secreted by M2 macrophages.

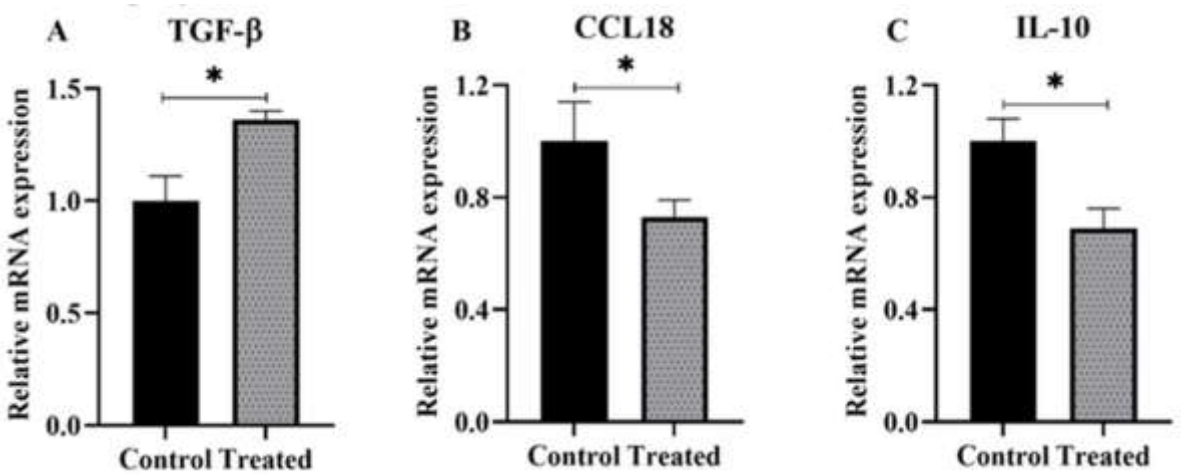

Figure 5: Effect of Shalparni (DG) on anti-intiflammatory market in MI macrophages, A) TGF-***β***, B) ccl 18 and C) IL-10

DISCUSSION

The findings of this study are very interesting because they hint towards a complex modulation of macrophage function rather than support the binary of M1 and M2 macrophage. Therefore, it shows that DG modulates immune response in a subtle way rather than acting and an anti-inflammatory agent.

The data suggests that while the phagocytic capabilities of macrophages increase, as evidenced by increased TLR4, these macrophages do not invite other immune cells as evidenced by lower inflammatory cytokines and MCP-1. Also, adding to the puzzle is the augmented expression of IRF5 which is required for pathogen clearance and enhanced activity of macrophages, usually associated with M1 macrophages.

The levels of M2 cytokines are also equally puzzling. While the classic cytokine IL-10 and CCL18 is reduced, more pleotropic TGF-β is enhanced. This means that DG is not anti-inflammatory but rather modulator which can enhance tissue remodelling (enhanced TGF-β without reducing the alertness of macrophages (no increase in antiinflammatory cytokines IL-10 or CCL18). The herb is known to reduce fever, oedema and helpful in myalgia. These changes validate it's Ayurvedic role in the classical preparation like Dasmoolarishta which is given to young mothers postpartum (Sastry, 2016). The data here suggest that the DG can remodel tissue with enhanced phagocytic capabilities of macrophages without raising inflammation.

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