

# In vitro anti-inflammatory effects of Mahanarayan oil formulations using dendritic cells based assay

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## Abstract

Mahanarayan (MN) oil is ayurvedic polyherbal oil for relieving joint and muscular pains. We investigated mechanism of action underlying the anti-inflammatory potential of MN oil at cellular level. Variants of MN oil (A, B, C, D and E) containing “kesar” and “haldi” in different ratios were evaluated for *in vitro* anti-inflammatory activity using key immune cells; Dendritic cells (DCs). At non-cytotoxic concentrations, all the 5 MN oil formulations significantly inhibited secretion of key cytokines; MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  from DCs against LPS-stimulated levels. Alterations in the morphology of LPS-treated DCs were also induced by MN oil formulations (A-E) indicating their anti-inflammatory activity. The data obtained suggested that MN oil exerts anti-inflammatory activity by inhibiting cytokines produced by DCs. MN oil formulations with varying ratios of kesar and haldi possess comparable anti-inflammatory effects.

**Key words:** Dendritic cells; mahanarayan oil; cytokines; anti-inflammatory; arthritis

## 1. Introduction

Ayurveda is the plant-based indigenous system of medicine practiced in India since ancient times (Ravishankar and Shukla, 2007). However, there is a growing need for scientific evidence for efficacy of various ayurvedic formulations. MN oil is a classical formulation which has been used as traditional ayurvedic medicine since ages for relieving joint and muscular pains. *Crocus sativus* Linn (commonly named as Kesar) and *Curcuma longa* (commonly named as haldi) are key ingredients of MN oil, with well-reported anti-inflammatory and antiarthritic properties (Aggarwal and Harikumar, 2009; Chahar *et al.*, 2012; Jalalpure *et al.*, 2011; Kumar and Rai, 2012; Ramadan *et al.*, 2011). Other major ingredients of MN oil and their pharmacological role are mentioned in Table 1.

Since kesar is expensive, it was replaced with haldi in different ratios and 5 different formulations of MN oil A, B, C, D and E were prepared with varying contents of kesar and haldi. The present study was conducted to explore the mechanism of action for anti-inflammatory activity of MN oil. Further, we compared the *in vitro* anti-inflammatory activity of 5 MN oil formulations; A, B, C, D and E with varying contents of kesar and haldi.

Bone marrow derived Dendritic cells (DCs) were employed to evaluate anti-inflammatory activity of MN oil formulations (A-E),

which are recognized as powerful antigen-presenting cells (Banchereau *et al.*, 2000; Liu *et al.*, 2008; Steinman, 1991). At non-cytotoxic concentrations (0.008%-0.025%), inhibitory effect of MN oil formulations (A-E) on secretion of key proinflammatory markers MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  in LPS stimulated DCs was assessed.

## 2. Materials and Methods

### 2.1 Chemicals

FBS (Tissue Culture Biologics), Penicillin/Streptomycin (Krishgens biosystems), RPMI-1640 medium (Lonza), rmGMCSF and ELISA kits for murine MIP-1- $\alpha$ , TNF- $\alpha$ , IL-1- $\beta$  (R&D systems), LPS (*E. coli* serotype 0127:B8) and MTT (Sigma).

### 2.2 Animals

Specific pathogen-free male C57BL/6 mice (20 to 25 g, 8-10 weeks) used in the study were obtained from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, India. Mice were kept in the in-house animal facility maintained at 22 $\pm$ 3°C and 55 $\pm$ 15% relative humidity with 12 hr light-dark cycle. They were given autoclaved pelleted feed and filtered drinking water ad libitum. All experiments employing the mice were performed under the protocols approved by the Institutional Animal Ethics Committee (IAEC) of Althea Life Sciences Limited.

### 2.3 MN oil formulations (A-E)

MN oil formulations (A-E) manufactured by Dabur India Ltd. were used in the study. The composition of kesar and haldi in MN oil formulations (A-E) is mentioned in Table 2.

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**Table 1:** Pharmacological properties and Chemical Constituents of Mahanaryan Oil

| Ingredient       | Botanicals                                       | Family                   | Pharmacological properties   | Chemical constituent  |
|------------------|--|--------------------------|--|---|
| Agnimantha       | <i>Clerodendrum phlomidis</i><br>Linn f          | Verbenaceae              | Inflammation.  | Sterols   |
| Bhringraj        | <i>Eclipta alba</i> Hassk.                       | Asteraceae               | Skin diseases, indicated in inflammation   | Alkaloids Ecliptine and nicotine  |
| Bilva Tvak       | <i>Aegle marmelos</i> Corr                       | Rutaceae                 | Anti-inflammatory, rheumatism  | Auraptin, coumarin, glycosides  |
| Devadaru         | <i>Cedrus deodara</i> (Roxb)<br>Loud             | Pinaceae                 | Pruritus, indicated in inflammation and rheumatism   | Terpenoids, flavonoids and glycosides   |
| Kantakari        | <i>Solanum xanthocarpum</i><br>Schrad and Wendle | Solanaceae               | Anti-inflammatory.   | Glucoalkaloids and sterols.   |
| Kushth           | <i>Saussurea lappa</i><br>C B Clarke             | Compositae               | Erysipelas, skin diseases, indicated in gout   | Essential oil, alkaloid saussurine, bitter resin  |
| Manjishtha       | <i>Rubia cordifolia</i> Linn                     | Rubiaceae                | Complexion promoting, glycosides. Skin diseases, erysipelas, anti-inflammatory                   | Glycosides  |
| Mashaparni       | <i>Teramnus labialis</i> Spreng                  | Fabaceae                 | Anti-inflammatory.   | -   |
| Mudgaparni       | <i>Phaseolus trilobus</i> Ait                    | Fabaceae                 | Edema, inflammation and gout   | Sterols   |
| Musta            | <i>Cyperus rotundus</i> Linn                     | Cyperaceae               | Anti-inflammatory, indicated in rheumatism   | Volatile oil  |
| Nagakeshara      | <i>Mesua ferrea</i> Linn                         | Guttiferae               | Pruritus, skin diseases, erysipelas, indicated in inflammation and edema                         | Essential oil and oleoresin   |
| Paribhadra       | <i>Erythrina indica</i> Lam                      | Fabaceae                 | Indicated in inflammation  | Alkaloids and resins  |
| Patala Tvak      | <i>Stereospermum suaveolens</i> DC               | Bignoniaceae             | Anti-inflammatory  | Bitter substances, sterols, glycosides and glycoalkaloids                               |
| Punarnava Shweta | <i>Trianthema portulacastrum</i><br>Linn         | Aizoaceae                | Indicated in inflammation  | Glycosides  |
| Pushkara         | <i>Inula racemosa</i> Hook f                     | Asteraceae               | Indicated in inflammation /edema.  | Essential oil   |
| Rasna            | <i>Pluchea lanceolata</i> Oliver and Hiern       | Asteraceae               | Indicated in inflammation, rheumatism and gout   | Quercetin , isorhamnetin,   |
| Shaileya         | <i>Parmelia perlata</i> (Huds) Ach               | Parmeliaceae             | Indicated in skin diseases   | Lichen acids like atranorin and lecanoric acid. Sterols                                 |
| Shalaparni       | <i>Desmodium gangeticum</i> DC                   | Fabaceae                 | Indicated in inflammation  | Alkaloids   |
| Shyonak Tvak     | <i>Oroxylum indicum</i> Vent                     | Bignoniaceae             | Indicated in rheumatism, stillness of thigh and leg, diseases of nervous system and inflammation | Flavonoids and tannins  |
| Yashti           | <i>Glycyrrhiza glabra</i> Linn                   | Leguminosae ( Fabaceae ) | Beneficial for eyes, complexion promoting, beneficial for hairs, edema, indicated in gout        | Glycyrrhizin, glycyrrhizic acid, glycyrrhetic acid, asparagin, sugars, resin and starch |

**Table 2:** Composition of kesar and haldi in MN oil formulations (A-E)

|          | Kesar   | Haldi  |
|----------|---|--|
| MN Oil A | 50% kesar of the original MN formulation                                    | No change  |
| MN Oil B | 25% kesar of the original MN formulation                                    | No change  |
| MN Oil C | 50% kesar of the original content   | 50% of more haldi than the original MN formulation |
| MN Oil D | 25% kesar of the original MN formulation                                    | 75% of more haldi than the original MN formulation |
| MN Oil E | Original MN oil formulation as per Bhaishajya ratnawali/vat Vyadhi Chikitsa |  |

#### 2.4 Preparation of MN oil stock solutions

MN oil formulations (A, B, C, D, E) were dissolved in DMSO to obtain a concentration of 10% (v/v) and sonicated in an ultrabath sonicator for 20 min. Stock solutions were further diluted in serum-free medium for preparation of working dilutions (10 times the final concentrations) in the range of 0.01% – 0.5% (v/v).

#### 2.5 Generation of DC cultures

DCs were generated from murine bone marrow, using a modified method (Lutz *et al.*, 1999). Femurs were excised from C57BL/6 mice and separated from the surrounding muscle tissue. The bone marrow was harvested by gently flushing the femur with RPMI 1640 medium using a 23-gauge needle. The cells were cultured ( $2 \times 10^6$  cells/10 ml) in 90-mm culture petridishes with growth medium (RPMI 1640 medium supplemented with 10% FBS, 20 mM penicillin-streptomycin and 20 ng/ml rmGM-CSF) for 6 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. On day 3, cells were replenished with additional 10 ml of growth medium containing rmGM-CSF. Semi-adherent, immature DCs were harvested on day 6 by gentle pipetting and used in experiments. Viable cells were counted using trypan blue exclusion method.

#### 2.6 Viability of DCs

Day-6 immature DCs were seeded at a density of  $5 \times 10^4$  cells/well in 96-well culture plates and treated with MN oil formulations (A-E) in the concentration range of 0.001% - 0.05% in triplicates. DCs treated with 0.1% DMSO were included as control cells. The cytotoxic effect of MN oil formulations on DCs was determined after 24 h of incubation by addition of MTT (0.5 mg/ml). After 3 h, cells were centrifuged at 250 g for 8 min. Supernatants were removed and cell pellets were resuspended in 150 µl of DMSO. Absorbance of samples was measured at 540 nm. Concentrations of MN oil formulations that retained > 80% viability of DCs were selected for subsequent experiments.

Viability of DCs = (100 - % cytotoxicity),

whereas, the percentage cytotoxicity of MN oil formulations was determined as:

$$\text{Cytotoxicity percentage} = \{(X-Y/X)\} * 100,$$

X = absorbance of control DCs at 540 nm,

Y = absorbance of DCs treated with MN oil formulations at 540 nm.

#### 2.7 Estimation of anti-inflammatory activity

Day-6 immature DCs were seeded at a density of  $0.16 \times 10^6$  cells in 24-well culture plates and stimulated with LPS (10 ng/ml) for 30 min at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. LPS-stimulated DCs were treated with MN oil formulations (A-E) at selected non-cytotoxic concentrations (0.008% - 0.025%) in triplicates. Cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24 h. DCs treated with LPS alone were included as control cells. After 24 h of incubation, culture supernatants were collected and centrifuged at 5000 rpm for 5 min. Cell-free supernatants were analyzed for secreted levels of MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  by ELISA (R&D Systems Inc, MN, USA).

Percent inhibition of cytokine secretion by DCs =  $\{(A-B)/A\} * 100$ ,

A = concentration of cytokine (pg/ml) in control (LPS-treated) DCs,

B = concentration of cytokine (pg/ml) in MN oil treated DCs.

Effect of MN oil formulations (A-E) on morphology of LPS-stimulated DCs was observed using inverted microscope (Nikon) and results were photodocumented.

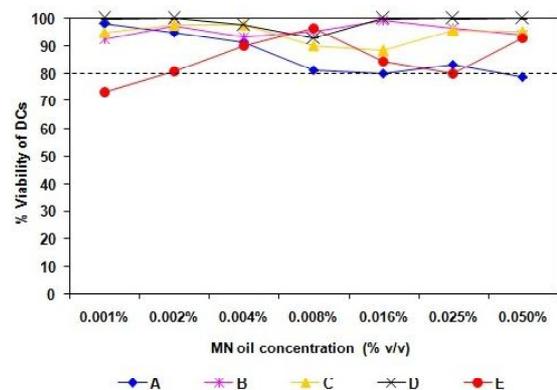
#### 2.8 Statistical analysis

Experimental values were expressed as arithmetic mean  $\pm$  SEM. Statistical differences between control and treatment groups were determined using one-way ANOVA with Dunnett's multiple comparison post test.

### 3. Results

#### 3.1 MN oil formulations (A-E) had no adverse effects on the viability of DCs

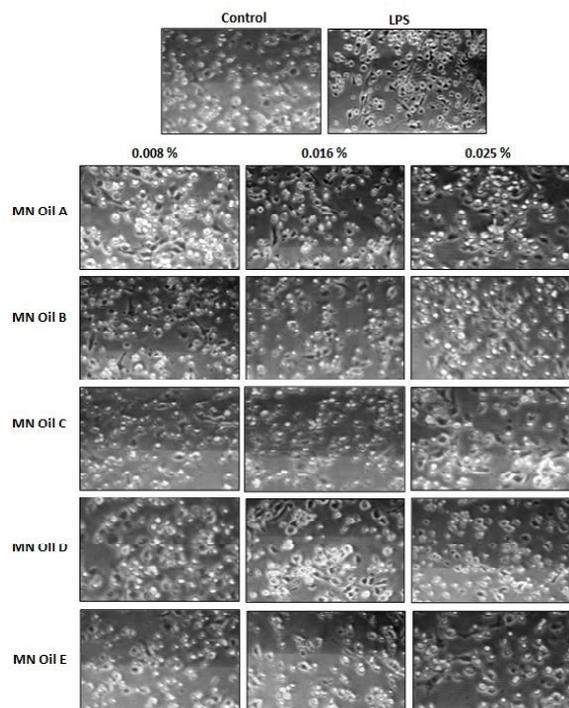
Effect of MN oil formulations (A-E) on the viability of DCs was determined at concentrations ranging from 0.001% - 0.05% after 24 h of treatment. No significant loss of DC's viability was observed at any concentration tested (Figure 1). 3 concentrations of MN oil formulations (0.008%, 0.016% and 0.025%) resulted in >80% viability of DCs and were selected for further evaluation of anti-inflammatory activity.



**Figure 1:** Effect of MN oil formulations (A-E) on viability of DCs. DCs were treated with MN oil formulations (A-E) in the concentration range 0.001% - 0.05% (% v/v) for 24 h. Cell viability was determined by MTT assay. n=3.

### 3.2 MN oil formulations (A-E) induced morphological changes in DCs

Immature DCs obtained on day-6 were treated with medium alone (untreated) or inflammatory stimulus LPS (*E. coli*, 10 ng/ml) alone for 24 h (control), or stimulated with LPS for 30 min and then treated with MN oil formulations (A-E) for 24 h. Morphological changes in DCs upon treatment with MN oil formulations were observed (Figure 2).



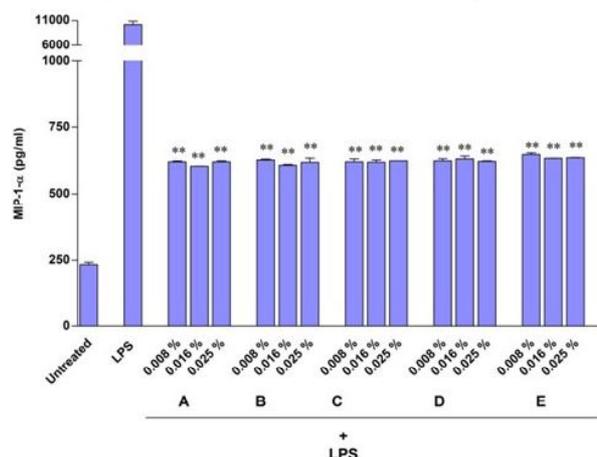
**Figure 2:** Effect of MN oil formulations (A-E) on morphology of LPS-stimulated DCs. DCs were treated with medium alone or inflammatory stimulus-LPS 10 ng/ml (control) or LPS (30 min) + MN oil formulations (A-E) (0.008%-0.025%) for 24 hr. (Magnification – 100X).

Untreated DCs showed formation of numerous colonies with semi-adherent cells in the suspension. Macrophages adhered to surface were also seen at the site of colony formation. Stimulation with LPS resulted in the maturation of DCs as evident by increased population of cells with branched morphology and adherent macrophages after 24 h of treatment. However, treatment of LPS-stimulated DCs with MN oil formulations (A-E) in the concentration range of 0.008% - 0.025% for 24 h resulted in reduced populations of adhered macrophages as compared to LPS-treated cells alone. This morphological change in DCs shows repairing potential of MN oil formulations (A-E) against LPS induced inflammatory damage. The anti-inflammatory effects of different MN oil formulations (A-E) on DC's morphology were comparable.

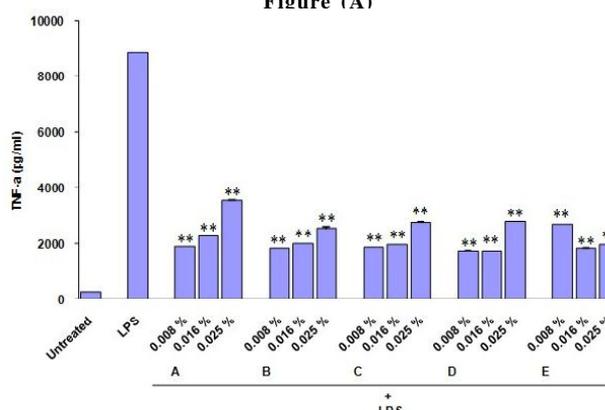
### 3.3 MN oil formulations (A-E) demonstrated inhibition of inflammatory cytokines secreted by DCs against LPS induced levels

Day-6 immature DCs were treated with MN oil formulations (A-E) at 0.008%, 0.016% and 0.025% for a time period of 24 h. DCs were induced with LPS-stimulation (10 ng/ml) for 30 min prior to

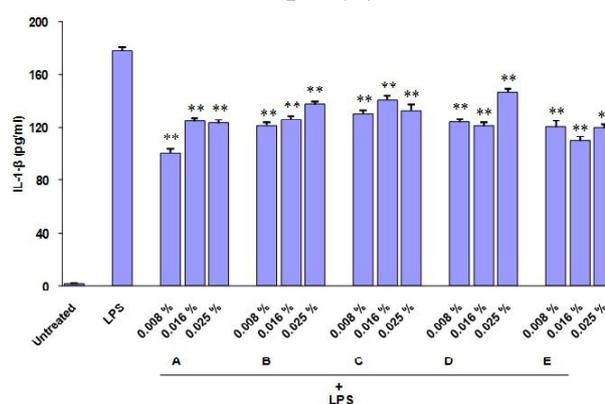
treatment with MN oil formulations. The expression levels of secreted MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  were estimated in culture supernatants by ELISA to evaluate the anti-inflammatory activity. MN oil formulations exhibited anti-inflammatory potential by down regulation of LPS-stimulated levels of MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  secreted by DCs in the extracellular medium (Figure 3).



**Figure (A)**



**Figure (B)**



**Figure (C)**

**Figure 3:** Effect of MN oil formulations (A-E) on secretion of LPS-induced cytokines. DCs were treated with LPS (10 ng/ml) alone (control) or MN oil formulations (A-E; 0.008% - 0.025%). After 24 h, levels of (A) MIP-1- $\alpha$  (B) TNF- $\alpha$  and (C) IL-1- $\alpha$ .  $n = 3$ ,  $**p < 0.01$  significant as compared with control LPS treated cells.

Treatment of LPS-stimulated DCs with MN oil formulations (A-E) in the concentration range (0.008% - 0.025%) resulted in significant ( $p < 0.01$ ) inhibition of MIP-1- $\alpha$  levels by 93% - 94% (Figure 3a). Similarly, TNF- $\alpha$  levels were suppressed significantly ( $p < 0.01$ ) by 60% - 80% against LPS induced stimulation (Figure 3b). Secretion of IL-1- $\beta$  was also significantly ( $p < 0.01$ ) down regulated by 18%– 43% (Figure 3c) against LPS-alone induced levels. However, the extent of inhibition of MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  secreted by LPS-treated DCs was comparable amongst 5 MN oil formulations.

#### 4. Discussion

Inflammation is a complex network of a variety of molecules, which is self-regulating through the balanced action of anti and pro-inflammatory cytokines (Feghali and Wright, 1997; Luster, 1998). An imbalance between anti and pro-inflammatory cytokines results in cellular damage in rheumatoid arthritis (RA), which is a common chronic inflammatory and destructive arthropathy (Agarwal and Malaviya, 2005; Isomäki and Punnonen, 1997). Excessive secretion and accumulation of pro-inflammatory cytokines and chemokines including MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  is a hallmark feature of inflammatory disorders (Choy and Panayi, 2001). Anti-inflammatory agents exhibit their protective effects by down regulation of excessively secreted pro-inflammatory molecules by immune cells (Calixto *et al.*, 2004). Dendritic cells (DCs) are professional antigen-presenting cells which play pivotal role in the induction of protective immunity by presenting antigens to naive T cells (Rescigno *et al.*, 1999). DCs also play important function in acute and chronic inflammation. DCs are identified as key cell populations in RA with specific implications in its pathogenesis (Lebre and Tak, 2008; Sarkar and Fox, 2005).

MN oil is a classical formulation and effective remedy for joint pain and arthritis. To reduce the cost factor on account of usage of kesar, MN oil formulations (A-E) containing different percentages of kesar and haldi were synthesized. Considering the crucial role of DCs and cytokines in inflammatory conditions such as arthritis, murine bone-marrow derived DCs stimulated with LPS were employed as a cell-based model to investigate anti-inflammatory effects of MN oil formulations (A-E).

There was no adverse effect on the cellular viability, when DCs were treated with 5 MN oil formulations (A-E). All the 5 MN oil formulations (A-E) imparted comparable repairing effects to LPS-stimulated DCs as shown by alterations in cellular morphology, which suggest their anti-inflammatory activity.

LPS (10ng/ml) is known to stimulate pro-inflammatory cytokines *in vitro* in immune cell populations such as macrophages and dendritic cells (Huang *et al.*, 2014; Madaan *et al.*, 2013). In the present study, stimulation of DCs with LPS resulted in substantial overproduction of proinflammatory cytokines. After 24 h of treatment, MN oil formulations (A-E) demonstrated significant down-regulation of MIP-1- $\alpha$ , TNF- $\alpha$  and IL-1- $\beta$  secreted by DCs against LPS-stimulated levels. MN oil formulations (A-E) demonstrated comparable inhibitory effect on secretion of cytokines.

A remarkable inhibition of key mediators of inflammation produced by DCs shows anti-inflammatory effects of MN oil. This finding deepens our understanding of mechanism of action of MN oil at cellular level. The results obtained substantiate the promising anti-inflammatory activity associated with the use of MN oil in joint pain and arthritic conditions. Different compositions of kesar and haldi in MN oil formulations did not have differential effects on the extent of anti-inflammatory activity. Therefore, it suggests that kesar can be replaced with haldi in MN oil formulation.

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