

# Homoeopathic medicines modulate inflammatory functions and adhesion receptor expression in human blood cells: An *in vitro* study

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## Abstract

**Background:** Homoeopathic medicines are used extensively for the treatment of many human diseases and are shown to affect immune cell functions. However, our understanding of the immunomodulatory effects of such medicines and associated mechanisms of action remains limited. **Objectives:** The present study aims to investigate the immunomodulatory effects of *Arsenic album*, *Rhus toxicodendron*, *Hepar sulphuris* and *Bryonia alba* on lipopolysaccharide (LPS) induced peripheral blood neutrophils and monocytes. **Materials and Methods:** In this work, neutrophils and monocytes were treated with different homoeopathic dilutions of 30C potency separately, followed by stimulation with the LPS, to examine the immunomodulatory effects of these medicines. Flow cytometry-based expression analysis of different surface markers was assessed, gene expression dynamics was determined using quantitative polymerase chain reaction and protein secretion was evaluated using enzyme-linked immunosorbent assay. **Results:** A significantly decreased levels of pro-inflammatory cytokines such as interleukin (IL-6) and tumour necrosis factor- $\alpha$  both at transcript levels as well as protein levels with reduced expression of the pathogen recognition receptors such as toll-like receptors (TLR)-2, and TLR-4 in LPS-stimulated neutrophils and monocytes were observed. Similarly, there was a reduction in reactive oxygen species production and expression of activation markers such as CD44, CD69 and CD62L in stimulated cells in the presence of the homoeopathic medicines. A differential level of expression of cell adhesion receptors (e.g., integrin  $\beta$ 1,  $\beta$ 3 and  $\alpha$ v) which mediate migration of immune cells in the tissues was observed in stimulated cells. **Conclusion:** We found that homoeopathic medicines have a significant effect against LPS-induced inflammatory response in innate immune cells and provide empirical support for their beneficial effects.

**Keywords:** Adhesion, *Arsenic album*, *Bryonia alba*, *Hepar sulphuris*, Homoeopathy, Inflammation, Peripheral blood neutrophils and monocytes, *Rhus toxicodendron*

## INTRODUCTION

Homoeopathic medicines are used to alleviate various clinical symptoms ranging from, cough, cold, headache, and sore throat, to complex and chronic diseases such as pneumonia, arthritis, atherosclerosis, and some cancers.<sup>[1-4]</sup> Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) solely caused a catastrophic COVID-19 pandemic that affected millions of people globally.<sup>[5,6]</sup> The SARS-CoV-2 produces pneumonia-like symptoms such as fever, dry cough and breathlessness in the infected individuals.<sup>[7,8]</sup> The immune cells such as neutrophils and monocytes/macrophages play a crucial role in the pathophysiology of COVID-19 and similar infection-induced systemic inflammatory conditions.<sup>[9-11]</sup>

Experiments in animal models have shown that systemic inflammatory responses could result in hyperactivation, altered migration and improper localisation of such cells in visceral organs leading to organ failure and death and that immunomodulatory reagents targeting adhesion receptors such as integrins (integrin  $\beta$ 1,  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 5 $\beta$ 1), expressed on such cells or genetic removal of such receptors could alleviate

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systemic inflammatory diseases such as sepsis.<sup>[12-14]</sup> The severity and high mortality rates due to COVID-19 and doubts regarding the vaccines' efficacies against new variants have led many to use a variety of herbal medicines as immune boosters and preventives, including homoeopathic medicines such as *Arsenic album* and *Bryonia alba*.<sup>[15-18]</sup> In this line, Ministry of Ayush, Government of India, had issued an advisory supporting *Arsenic album* 30C, one dose on an empty stomach daily for 3 days taken as preventive medicine against COVID-19.<sup>[19]</sup> However, there are limited scientific data to validate the prescription and use of such medicines.

Many inflammatory and infectious conditions, including multiple sclerosis and acute upper respiratory tract infections (rhinitis, tonsillitis and sinusitis), with symptoms such as sore throat and cough are frequently treated by homoeopathic practitioners with homoeopathic medications, such as *Arsenic album*, *Rhus toxicodendron*, *Hepar sulphuris* and *Bryonia alba*.<sup>[20,21]</sup> Evidence shows an anti-neoplastic activity of *Rhus Toxicodendron* and its beneficial effect in inflammatory diseases.<sup>[22-26]</sup> *Hepar sulphuris* is also used for tonsillitis, rhinitis, bronchitis, chronic urticaria and certain illnesses that are also frequently observed in COVID-19 patients.<sup>[27,28]</sup> Similarly, *Bryonia alba* has been shown to have anti-inflammatory properties and is used to treat illnesses such as synovial inflammation, acute appendicitis, intestinal ailments, lung diseases, arthritis and to prevent and treat infections.<sup>[29,30]</sup> Extracts of *Bryonia alba* have both therapeutic and preventative properties against SARS-CoV-2 and according to a different study, it has a positive effect on liver abscess in human patient.<sup>[31,32]</sup>

However, it is unknown how these homoeopathic drugs influence the body's immune cells and how they might be used as preventive medicines for acute inflammatory illnesses. In this paper, we have conducted a comparative analysis of the anti-inflammatory and immunomodulatory effects of *Arsenic album* 30C, *Rhus toxicodendron* 30C, *Hepar sulphuris* 30C and *Bryonia alba* 30C, on lipopolysaccharide (LPS) stimulated human peripheral blood neutrophils and monocytes *in vitro*.

## MATERIALS AND METHODS

### Homoeopathic medicines and reagents

Homoeopathic medicines *Arsenic album*, *Rhus Toxicodendron*, *Hepar sulphuris* and *Bryonia alba* in 30C potency were procured from a GMP-certified firm, SBL Pvt Ltd, India. Medicines were diluted in Roswell Park Memorial Institute (RPMI)-1640 media (Gibco, Thermo Fisher Scientific Waltham, Massachusetts, USA), to get different dilutions (1:5, 1:10, 1:25, 1:50 and 1:100) for use in various *in vitro* experiments. For all experiments, 90% v/v ethanol was taken as the vehicle control and cells stimulated with LPS were used as positive control. In addition to 1% antibiotic-antimycotic solution (penicillin, streptomycin and amphotericin), 1% sodium pyruvate solution, sodium bicarbonate (2.0 g/L) and 1% MEM non-essential amino acids, 10% fetal bovine serum

(FBS) was added to RPMI. All the supplements added in RPMI media were purchased from HiMedia (WC, USA).

For the data analysis and presentation, the guidelines for Reporting Experiments in Homoeopathic Basic Research were followed in every section.<sup>[33]</sup> The study protocol was approved by the Institute Human Ethics Committee (IHEC), Indian Institute of Technology, Roorkee, India, approval number BIOTECH/IHEC/AP/14/4 dated 28 January 2015.

### Isolation of human peripheral blood cells

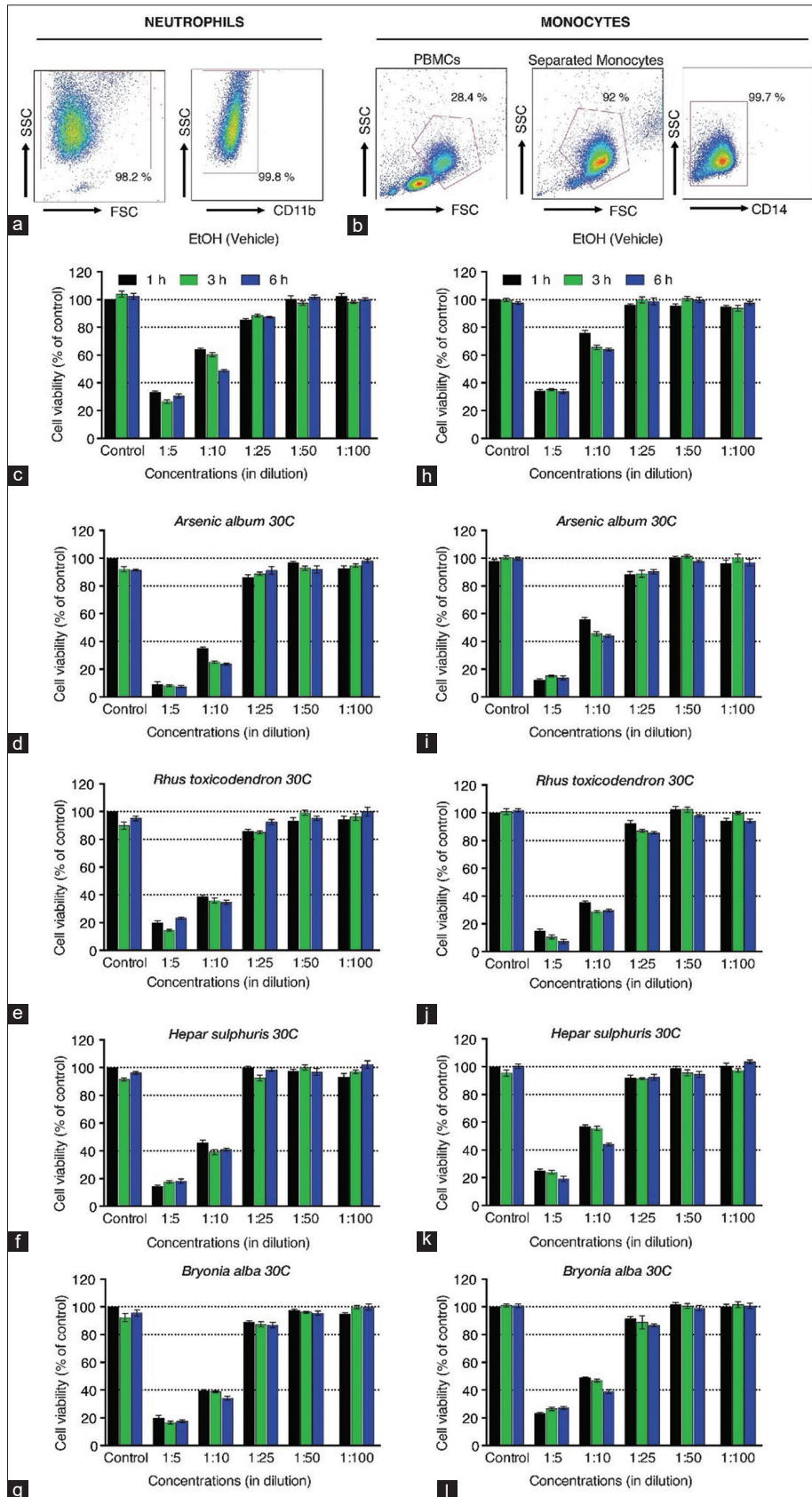
Blood was collected in heparin-coated vacutainers from healthy volunteers in accordance with the protocol, and for a single experiment, blood was collected from at least three donors. For neutrophil isolation, the whole blood was centrifuged through a 1-step polymorph prep density gradient (Axis-Shield, Dundee, UK) to separate granulocytes and erythrocytes as mentioned in the product datasheet. Once remaining erythrocytes were eliminated using hypotonic lysis, the purity of the neutrophils was determined to be >95% which was validated by flow cytometry, FACSVerse flow cytometer (BD Biosciences, San Diego, CA) by gating on the CD11b surface marker [Figure 1a]. Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) was used to separate the peripheral blood mononuclear cells (PBMCs). The plastic adherence approach was used to carefully separate the monocytes. Briefly,  $5 \times 10^5$  PBMCs were seeded for 1 h in a 48-well flat bottom plate using 400  $\mu$ L of RPMI 1640 media devoid of FBS. Tightly adherent monocytes were utilised in further experiments after being taken out of the media using ice-cold  $1 \times$  PBS. A purity of >90% was confirmed with flow cytometry by gating on the CD14 surface marker [Figure 1b].

### Determination of non-cytotoxicity of homoeopathic medicines

To determine the non-cytotoxic concentrations of EtOH (vehicle control) and different homoeopathic medicines,  $1 \times 10^5$  neutrophils or monocytes per well were seeded in 96-well plates separately. Several dilutions of EtOH and homoeopathic medicines (1:5, 1:10, 1:25, 1:50 and 1:100) were given to the cells for 1, 3 and 6 h at 37°C. After washing with  $1 \times$  PBS, Cell Counting Kit 8 (CCK8) solution (100  $\mu$ L/well) (Dojindo, Rockville, MD, USA) was added to each well. Following a 1h incubation period at 37°C for the cells, colour intensity was assessed at 450 nm with an EPOCH2 plate reader (BioTek, Winooski, VT, USA).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

The mRNA expression was measured using qRT-PCR. Briefly, neutrophils and monocytes were cultured in RPMI media containing 1:25, 1:100 homoeopathic dilutions or 90% v/v EtOH (vehicle control) for 1 h. LPS (100 ng/mL) (*Escherichia coli* 026: B6, Sigma-Aldrich, St. Louis, MO, USA) was then used to stimulate the cells. TRIzol (Life Technologies, Carlsbad, CA, USA) was used to extract the total RNA. The RNA was reverse transcribed using a



**Figure 1:** Representative dot plot showing purity of (a) neutrophils (b) monocytes isolated from healthy donor. The bar diagrams represent the percentage (%) of cell viability of (c-g) neutrophils and (h-l) monocytes after treatment with EtOH and homoeopathic medicines. Data are representative of three independent experiments performed in duplicates ( $n = 6$ )

High-Capacity cDNA reverse transcription kit (Applied Biosystems™, Foster City, CA, USA) and Real-time PCR was carried out using PowerUp™ SYBR™ Green master mix (Applied Biosystems™, Foster City, CA, USA) in accordance with the manufacturer's instructions. The StepOnePlus™ Real-Time PCR System was used for the qRT-PCR (Applied Biosystems™, CA, USA). Samples were standardised to the endogenous reference gene GAPDH and the relative level of gene expression was calculated using the  $2^{-\Delta\Delta CT}$  method. Table 1 contains a list of the primers utilised for the study.

### Antibodies and flow cytometry

Neutrophils and monocytes were treated with 1:25 dilutions of homoeopathic medicines or 90% v/v EtOH for 1 h, followed by LPS stimulation (100 ng/mL) for 15 min. To gate on living cells, BD Horizon™ Fixable Viability Stain 450 (FVS450; BD Biosciences, San Jose, CA, USA) was utilised. Anti-human APC-CD11b, PerCP-CD14, PerCP/Cy5.5-CD62L, PE-CD44, PE-CD69, FITC-  $\alpha$ v integrin and purified integrin  $\beta$ 1 antibodies were used to stain surface markers. For secondary staining, PE/Cy7 Goat anti-mouse IgG-HRP was used. The flow cytometry antibodies were procured from BioLegend (San Diego, CA, USA). After being fixed with 1% paraformaldehyde (HiMedia, WC, USA), the samples were acquired using a FACSVerse flow cytometer.

### Monocyte differentiation *in vitro*

Adherent monocytes were cultured for 6 days with 10% FBS, RPMI 1640 medium and 1:25 dilutions of homoeopathic medicines or EtOH (vehicle control) to differentiate macrophages. Cells were grown for 6 days with RPMI 1640 medium (including 10% FBS) as a control and with M-CSF (25 ng/mL) as the positive control. Differentiated cells were harvested at day 3 and 6 and surface markers were stained using anti-human APC-CD11b, PerCP-CD14 and PE-CD206 and the samples were acquired using a FACSVerse flow cytometer.

**Table 1: List of human primers used in qRT-PCR**

S. no.	Name of the primers	Sequence (5'- 3')
1	GAPDH forward	TCCTCTGACTTCAACAGCGACAC
2	GAPDH reverse	TCTCTCTTCTCTGTGCTCTTGC
3	IL-6 forward	GAAAGCAGCAAAGAGGCACT
4	IL-6 reverse	TTTACCAGGCAAGTCTCCT
5	hTNF- $\alpha$ forward	TATGGCTCAGGGTCCAACCT
6	hTNF- $\alpha$ reverse	CCTTGATGGTGGTGCATGAG
7	hITGB1 forward	GCACCAGCCCATTTAGCTACAA
8	hITGB1 reverse	TCGAAACCACCTTCTGGAGAATC
9	hITGB3 forward	AGTAACCTGCGGATTGGCTTC
10	hITGB3 reverse	GTCACCTGGTCAAGTTAGCGT
11	hCOX2 forward	AGGGTTGCTGGTGGTAGGAA
12	hCOX2 reverse	CGTTTGCGGTACTCATAAAAGAC
13	h TLR2 forward	ATCCTCCAATCAGGCTTCTCT
14	h TLR2 reverse	ACACCTCTGTAGGTCAGTGTG
15	h TLR4 forward	TGAGCAGTCGTGCTGGTATCAT
16	h TLR4 reverse	CTGTCTCCCACTCCAGGTAAG

qRT-PCR: quantitative real time-polymerase chain reaction

### Cytokine enzyme-linked immunosorbent assay (ELISA) analysis

For measuring the effect of homoeopathic medicines on the cytokine (IL-6) production from neutrophils and monocytes,  $1 \times 10^5$  neutrophils or  $2 \times 10^5$  monocytes per well were seeded in 96-well plates and treated with 1:25 dilution of homoeopathic medicines or 90% v/v EtOH (vehicle control) for 1 h at 37°C followed by LPS stimulation (100 ng/mL). Following 12 h, supernatants were collected and cytokine concentrations were assessed by sandwich ELISA in accordance with the manufacturer's instructions. Sandwich ELISA's capture and detection antibodies were bought from PeproTech (Rockey Hill, NJ, USA). The TMB liquid substrate system (BioLegend, San Diego, CA, USA) was utilised in the development of the colour reaction. 2N H<sub>2</sub>SO<sub>4</sub> (HiMedia, WC, USA) was used to stop the reaction and determined the absorbance at 450 nm using a plate reader.

### Reactive oxygen species (ROS) detection assay

The intracellular ROS generation was measured using the cell-permeable, oxidation-sensitive dye 2', 7'-Dichlorodihydrofluorescein diacetate (DCFDA) (Sigma-Aldrich, St. Louis, MO) as per the protocol.<sup>[14]</sup> Briefly, neutrophils and monocytes were separated as stated above and given a 1:25 dilution of various homoeopathic medicines or 90% v/v EtOH (used as vehicle control) for 1 h before being stimulated with LPS (100 ng/mL) for 15 min at 37°C. Following a 1X PBS wash, cells were incubated with 10  $\mu$ M DCFDA for a further 15 min at 37°C. The cells were then acquired using a FACS Verse instrument.

### Cell adhesion assay

A 96-well plate with a flat bottom was used to perform the cell adhesion experiment. 50  $\mu$ L of fibronectin (5  $\mu$ g/mL) (Merck, Sigma-Aldrich, Missouri, USA) were added to the wells to coat them overnight at 4°C. Thereafter, the wells were blocked up with 2% bovine serum albumin (BSA) for an hour at 37°C.  $1 \times 10^5$  cells neutrophils or  $2 \times 10^5$  monocytes per well were incubated for 1 h with 1:25 dilution of homoeopathic medicines or 90% v/v EtOH (vehicle control) before being stimulated with LPS (100 ng/mL) for 30 min. The treated cells were transferred to fibronectin-coated plates for an 1h incubation period at 37°C. The non-adhering cells were washed and the CCK8 cell counting kit was used to measure these. Before the addition of CCK8, images of the cells were captured with a bright field microscope (EVOS™ XL Core, Life Technologies, Carlsbad, CA, USA) at a magnification of 40 $\times$ . Using ImageJ's ROI tool, the size and perimeter of the cells from the recorded images were calculated.

### Data analysis

Each value is represented by its mean and standard error. The non-parametric Mann-Whitney test was used for data analysis in GraphPad Prism version 6.01 (GraphPad Software, La Jolla, CA) and one-way or two-way ANOVA was used for data involving more than two experimental groups. FlowJo software was used to analyse the flow cytometry data (Tree Star, Ashland, OR, USA).  $P \leq 0.05$  was considered significant in all the data sets.

## RESULTS

### Cytotoxicity study

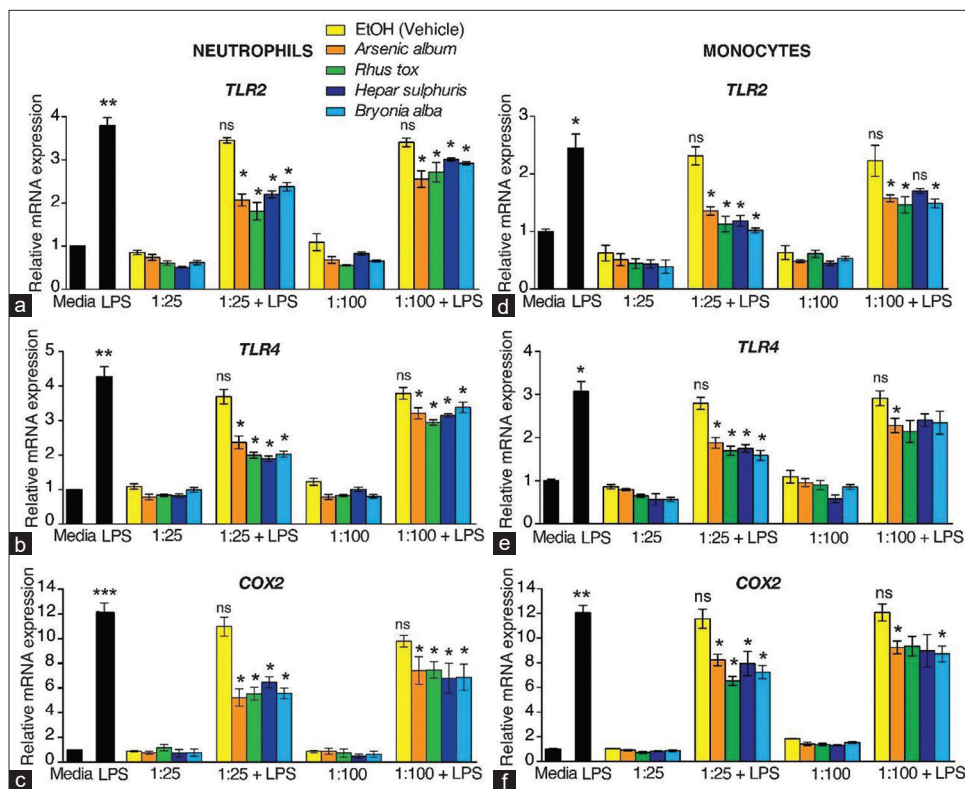
Since the homoeopathic medicines contain 90% v/v alcohol (EtOH); initially, we tested different concentrations of EtOH (solvent control) to check their cytotoxic effect on neutrophils and monocytes isolated from human peripheral blood. As shown in Figure 1, 1:5 and 1:10 dilutions of EtOH demonstrated cytotoxic effect in both neutrophils [Figure 1c] and monocytes [Figure 1h] and higher dilutions such as 1:25, 1:50 and 1:100, showed 95–100% viability in neutrophils and monocytes [Figure 1c and h]. Similar cytotoxicity experiments were also performed with each medicine and optimal doses for each medicine were tested in neutrophils and monocytes. At a concentration of 1:25, 1:50 and 1:100 dilution of *Arsenic album* 30C, *Rhus toxicodendron* 30C, *Hepar sulphuris* 30C and *Bryonia alba* 30C demonstrated 80–100% viability in neutrophils [Figure 1d-g] and monocytes [Figure 1i-l]. Thus, to investigate the inflammatory and immunomodulatory effects of homoeopathic medicines on neutrophils and monocytes, 1:25 or 1:100 dilutions were used in further experiments.

### Effects on pathogen recognition receptors and cell activation markers

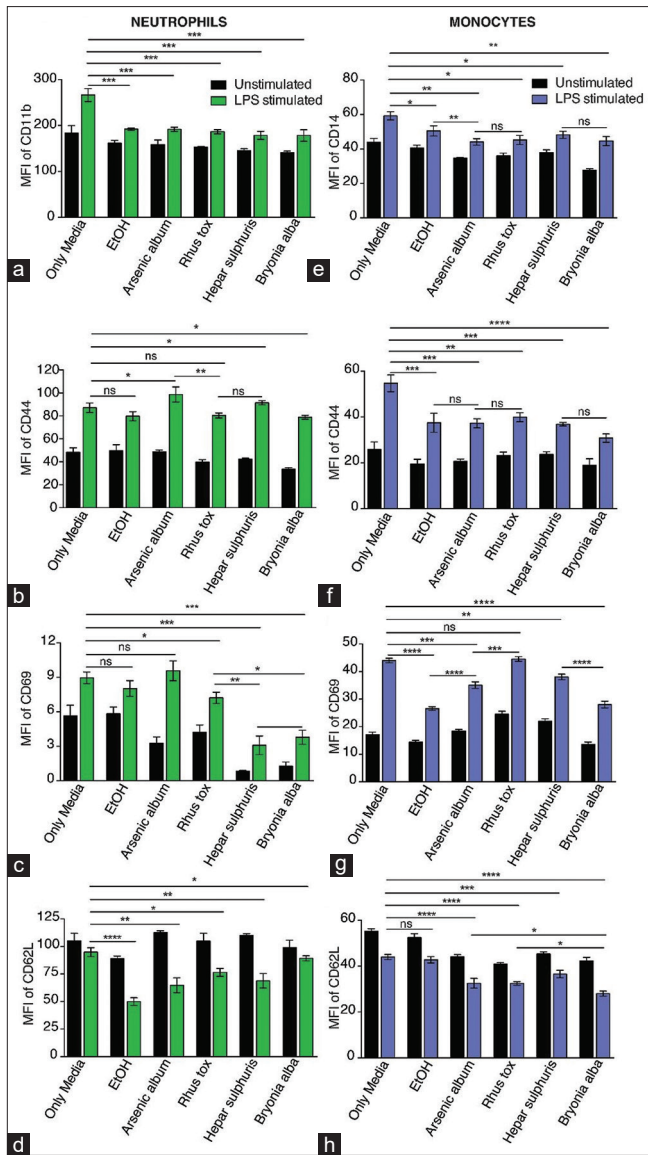
After optimising the concentrations, the inflammatory and immunomodulatory effects of these homoeopathic medicines were studied in neutrophils and monocytes. We measured the gene expression of toll-like receptors (TLR)-2 and TLR-4 and cyclooxygenase-2 in LPS-stimulated neutrophils

and monocytes. The gene expression of TLR2 and TLR4 was increased upon stimulation with LPS in neutrophils [Figure 2a and b] and monocytes [Figure 2d and e], compared to the unstimulated control cells. The increased gene expressions were reduced in the conditions where various homoeopathic medicines were added at 1:25 dilution along with LPS. EtOH does not show any significant difference in the mRNA expression of TLR2 and TLR4. Similarly, the expression of the COX2 in LPS-stimulated neutrophils [Figure 2c] and monocytes [Figure 2f] was also decreased with treatment with various homoeopathic dilutions.

As the homoeopathic medicines reduced the expression of pathogen recognition receptors in inflamed cells, these medicines might have some effects on these adhesion receptors in inflammatory conditions. We next used flow cytometry to examine how different homoeopathic medicines affected the expression of the surface markers CD62L, CD44 and CD69 in LPS-stimulated neutrophils and monocytes. The expression of CD11b in LPS-stimulated neutrophils [Figure 3a] and CD14 in LPS-stimulated monocytes [Figure 3e] were increased as compared to the unstimulated cells. In contrast to LPS-stimulated control cells, the cells treated with homoeopathic medicines displayed downregulation of CD11b and CD14. Furthermore, the expression of CD44 and CD69 in the LPS-stimulated neutrophils [Figure 3b and c] and monocytes [Figure 3f and g] were increased as compared to unstimulated cells. In comparison to the control group, there was a decrease in the expression of CD62L in



**Figure 2:** The bar diagram represents the expression kinetics of toll-like receptors 2 (TLR2), TLR4 and COX2 in (a-c) neutrophils and (d-f) monocytes, respectively. Data are representative of four independent experiments performed in duplicates ( $n = 8$ )



**Figure 3:** The bar graph represents mean fluorescence intensity of (a) CD11b, (b) CD44, (c) CD69, (d) CD62L in unstimulated and lipopolysaccharide-stimulated neutrophils and (e) CD14, (f) CD44 (g) CD69, (h) CD62L in monocytes followed by treatment with homoeopathic medicines. Data are representative of three independent experiments performed in duplicates ( $n = 6$ ).

both LPS-stimulated neutrophils [Figure 3d] and monocytes [Figure 3h] after homoeopathic treatments.

### Effect on cytokine production in LPS-activated immune cells

Having established that homoeopathic dilutions have no lethal effects on human neutrophils and monocytes, we investigated how homoeopathic medicines altered the immunological functions of these cells, including the production and release of inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor (TNF- $\alpha$ ) at the mRNA level. The LPS stimulation in neutrophils and monocytes increased the mRNA expression of both IL-6 and TNF- $\alpha$ . LPS stimulation induced

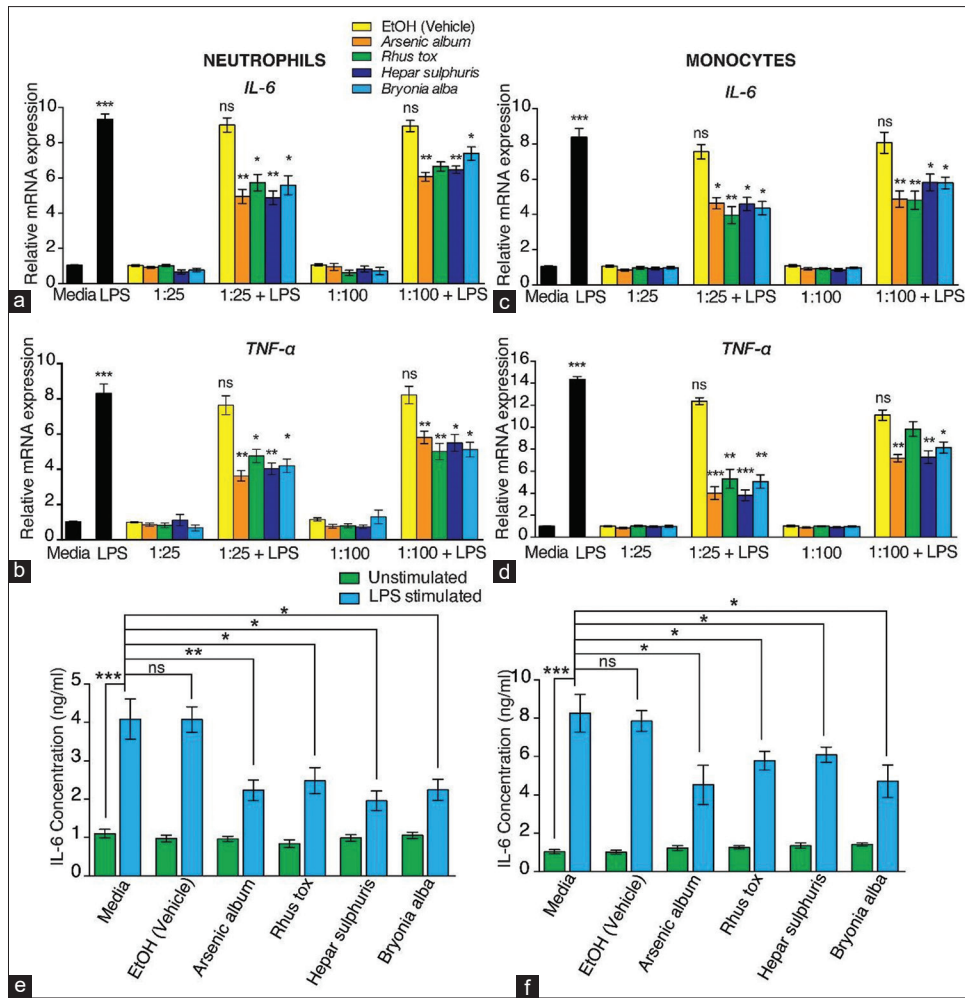
more than ten-fold and eight-fold in the production of IL-6 and TNF- $\alpha$  in neutrophils [Figure 4a and b] and more than eight-fold and fifteen-fold in IL-6 and TNF- $\alpha$  production in monocytes [Figure 4c and d], respectively. In contrast to control and EtOH-treated cells, homoeopathic treatments decreased the production of pro-inflammatory cytokines. The EtOH treatment does not have any significant effect on the LPS-induced cytokine production indicating that the effects observed in homoeopathic dilutions were not due to the presence of alcohol content. This result concluded that these homoeopathic dilutions (1:25 and 1:100) can significantly reduce LPS-induced pro-inflammatory cytokine production in neutrophils and monocytes. Furthermore, the reduced pro-inflammatory cytokine expressions did show dilution-dependent effect as the reduction of cytokine production by homoeopathic medicines was minimum in higher dilution (1:100) as compared to lesser dilution (1:25) in both neutrophils and monocytes [Figure 4a-d]. The release of IL-6 cytokines was higher in LPS-stimulated cells than in unstimulated (control) conditions and the use of homoeopathic medicines decreased the release of IL-6 cytokines in both neutrophils' [Figure 4e] and monocytes' [Figure 4f] supernatants. Like mRNA measurement, EtOH treatment did not affect the LPS-induced cytokine release.

### Antioxidant effect

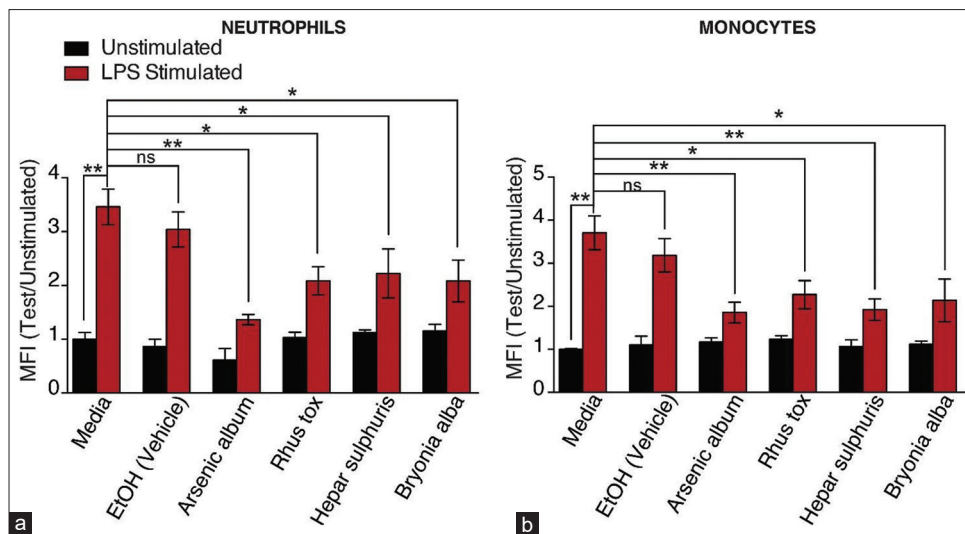
In addition to pro-inflammatory cytokines production, in inflammatory conditions, reactive oxygen species (ROS) production is elevated in innate immune cells to perform the antimicrobial and anti-inflammatory functions.<sup>[34]</sup> Therefore, the effects of homoeopathic medicines on the intracellular ROS production in LPS stimulated neutrophils and monocytes were measured. The generation of ROS from LPS-stimulated monocytes and neutrophils was increased as compared to the unstimulated cells [Figure 5]. These elevated ROS productions were significantly reduced in homoeopathic dilutions-treated neutrophils [Figure 5a] and monocytes [Figure 5b]. The solvent control (EtOH) treatment in immune cells had no effect on the production of oxidative stress, indicating that the effects observed in the case of homoeopathic dilutions were specific to the content of homoeopathic medicines only.

### Effects on cell adhesion receptors

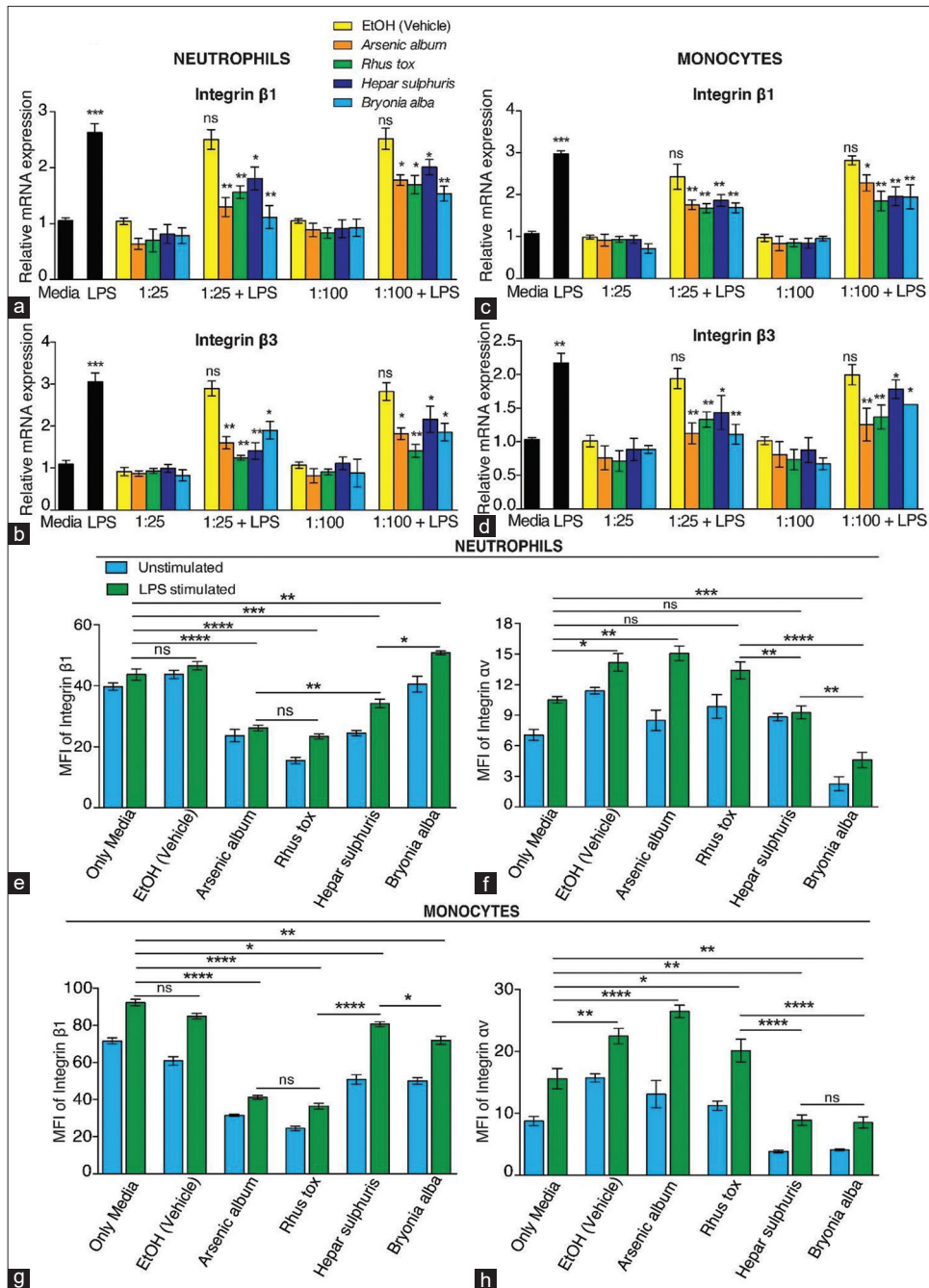
The effect of the four homoeopathic medicines (1:25 and 1:100) on the  $\beta$ 1 (ITGB1) and  $\beta$ 3 (ITGB3) integrin expression on the LPS-activated neutrophils and monocytes was measured at the transcript level. The stimulation of neutrophils and monocytes with LPS elevated the expression of both ITGB1 and ITGB3 mRNA [Figure 6]. LPS stimulation resulted in more than two-fold and three-fold in the expression of ITGB1 and ITGB3 mRNA in neutrophils [Figure 6a and b] and more than three-fold and two-fold in monocytes [Figure 6c and d], respectively. However, the treatment with homoeopathic medicines reduced the expression of both integrins as compared to the control and EtOH-treated cells. This result showed that the homoeopathic dilutions (1:25 and 1:100) of these medicines significantly reduced LPS-induced integrin expression in neutrophils and monocytes.



**Figure 4:** Expression of mRNA kinetics of interleukin (IL)-6 and tumour necrosis factor- $\alpha$  in homoeopathic treated (a and b) neutrophils and (c and d) monocytes, respectively. (e and f) The graphical plots are showing the IL-6 concentration (ng/mL) of (e) neutrophils and (f) monocytes quantitated using the sandwich enzyme-linked immunosorbent assay. Data are representative of four independent experiments performed in duplicates ( $n = 8$ )



**Figure 5:** The bar graphs represent the average mean fluorescence intensity of lipopolysaccharide-stimulated oxidative stress in (a) neutrophils and (b) monocytes. Data are representative of three independent experiments performed in duplicates ( $n = 6$ )



**Figure 6:** Expression of mRNA kinetics of integrin  $\beta 1$  and integrin  $\beta 3$  on homoeopathic medicines treated followed by lipopolysaccharide stimulated in (a and b) neutrophils and (c and d) monocytes, respectively. (e-h) Bar graph represents mean fluorescence intensity of integrin  $\beta 1$ , integrin  $\alpha v$  on (e and f) neutrophils and (g and h) monocytes. Data are representative of three independent experiments performed in duplicates ( $n = 6$ )

Many subfamilies of integrins that help in the binding of cells to ECM proteins contain the  $\beta 1$  or  $\alpha v$  subunits.<sup>[35-37]</sup> Thus, we checked the surface expression of integrin  $\beta 1$  and  $\alpha v$  in LPS-activated neutrophils and monocytes. The expressions of both the  $\beta 1$  and  $\alpha v$  integrin subunits are lowered after treatment with homoeopathic medicines, as illustrated in Figure 6e-h. The mean fluorescence intensity (MFI) of  $\beta 1$  integrin of *Arsenic album* and *Rhus tox* in both neutrophils and monocytes was reduced and the MFI of  $\alpha v$  was increased compared to the only-media control [Figure 6]. In contrast to these homoeopathic

medicines, *Hepar sulphuris* and *Bryonia alba* have opposite expression patterns. In addition, the MFI of  $\beta 1$  was increased in *Hepar sulphuris* and *Bryonia alba* treated cells and reduced in the expression in  $\alpha v$ .

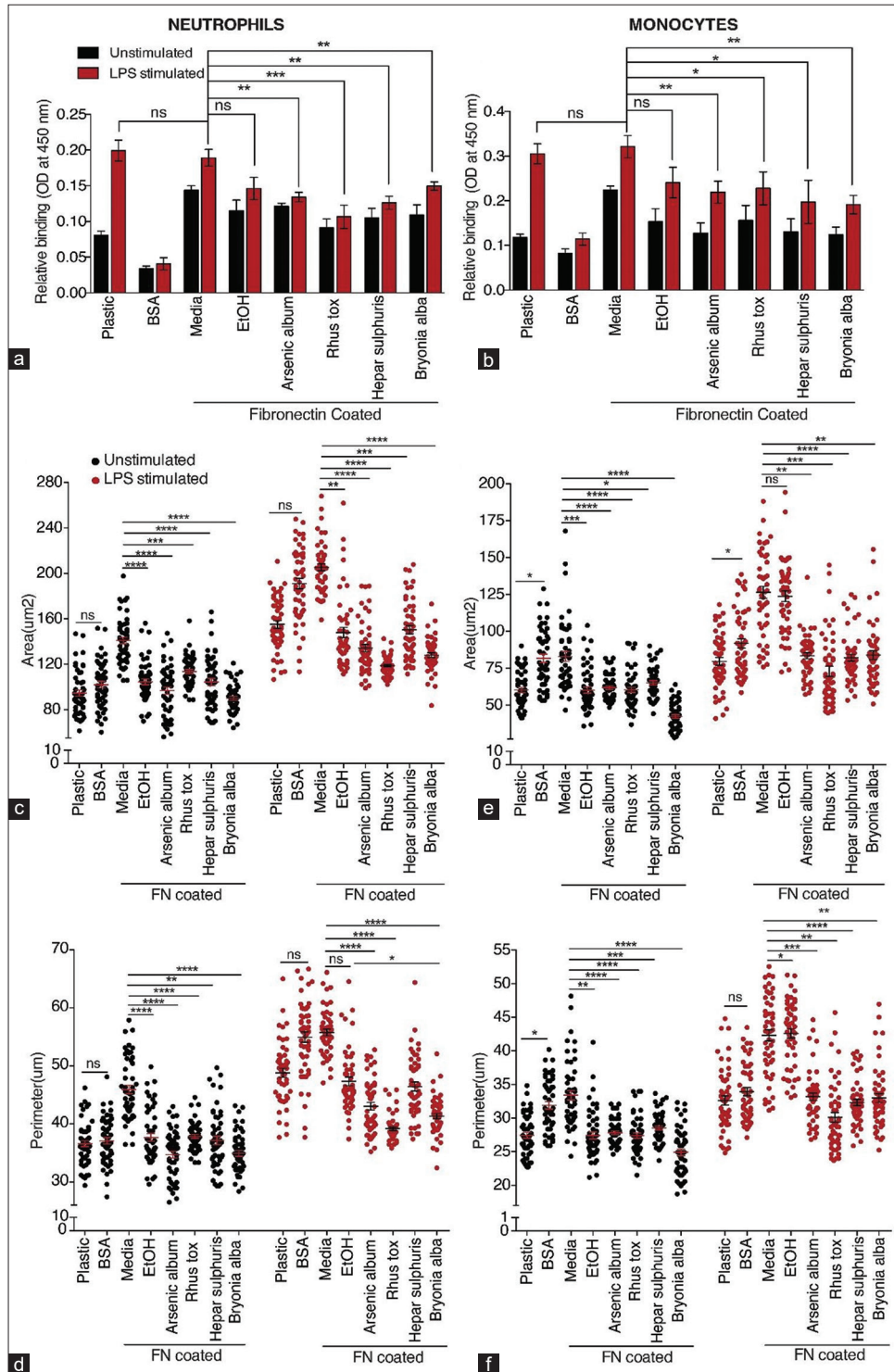
### Cell binding on extracellular matrix protein

In addition to the expression of the integrins, we also checked the adhesion of unstimulated and LPS-stimulated neutrophils and monocytes to the fibronectin with and without homoeopathic treatment. Treatment with LPS increased the



relative binding of neutrophils [Figure 7a] and monocytes [Figure 7b] to fibronectin-coated plates in contrast to cells that were not stimulated. However, the binding of cells treated with the homoeopathic medicines to the fibronectin-coated wells was significantly reduced as compared to the control

cells (media only). Supplementary figure 1a and b show the microscopic images of the binding of unstimulated and LPS-stimulated neutrophils and monocytes to fibronectin with or without homoeopathic treatments, respectively. Besides cell adhesion properties, integrins help in cell spreading and focal



**Figure 7:** The bar diagram shows the relative binding of unstimulated and lipopolysaccharide-stimulated (a) neutrophils and (b) monocytes to fibronectin w.r.t., media control. Data are representative of two independent experiments performed in triplicates ( $n = 6$ ). (c-f) Dot plots represent the results of the area ( $\mu\text{m}^2$ ) and perimeter ( $\mu\text{m}$ ) from 60 cells/group counted from 3–4 representative images taken at  $\times 40$  objective

adhesion formation. These findings have another postulation in the cell spreading of both homoeopathic-treated monocytes and neutrophils. The binding of neutrophils and monocytes to fibronectin-coated plates [Figure 7] enhanced the cells' adherence and spreading on the plates. This was further enhanced when the cells were activated with LPS. Neutrophils [Figure 7c and d] and monocytes [Figure 7e and f] treated with the four homoeopathic medicines had significantly smaller areas and perimeters than control (media only) cells.

### Monocytes differentiation

Monocytes show plasticity and can differentiate into M1 inflammatory and M2 anti-inflammatory macrophages during early inflammation.<sup>[38,39]</sup> As a result, we next looked at whether any of the four homoeopathic medicines may affect how monocytes differentiate. Figure 8 shows that on days 3 and 6 post-seeding, the expression of CD14, CD11b and CD206 on differentiated monocytes was considerably higher in the presence of macrophage colony-stimulating factor (M-CSF) compared to media control. However, not much difference was observed in the expression of these surface markers (CD14 or CD11b or CD206) in homoeopathic medicine-treated monocytes at day 3 and day 6 compared to media control [Figure 8a-c].

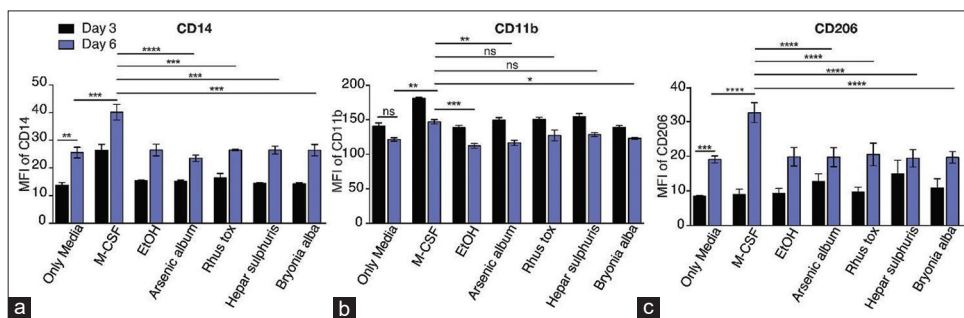
### DISCUSSION

*Arsenic album*, *Rhus toxicodendron*, *Hepar sulphuris* and *Bryonia alba* have been used for the treatment of various inflammatory conditions. To fully comprehend the mechanism of action of diverse homoeopathic medicines, further experimental data under varied *in vitro* and *in vivo* settings must be gathered. Using LPS-stimulated human peripheral blood neutrophils and monocytes, we conducted a comparative examination of the immunomodulatory and anti-inflammatory effects of the aforementioned medicines in this study. The presented data supports the hypothesis that the above-mentioned medicines modulate inflammatory and cell adhesion molecules in human peripheral blood neutrophils and monocytes.

TLRs are an essential subclass of pattern recognition receptors that recognise both pathogen-associated molecular patterns and damage-associated molecular patterns and are connected to the inflammatory responses of immune cells including

neutrophils and macrophages.<sup>[40-42]</sup> COX-2 overexpression is known to induce proinflammatory responses in neutrophils and macrophages.<sup>[43-45]</sup> In this study, all the four homoeopathic medicines reduced the expression of both TLR-2, TLR-4 and COX-2 in LPS-stimulated neutrophils and monocytes at the transcript level and this reduction in gene expression shows dilution dependent effects. Similarly, these homoeopathic medicines inhibit ROS generation as well as the secretion of proinflammatory cytokines such as IL-6 and TNF- $\alpha$  in LPS-stimulated neutrophils and monocytes. LPS stimulation boosted IL-6 and TNF- $\alpha$  production in neutrophils and in monocytes. However, when compared to control and EtOH-treated cells, treatment with these homoeopathic medicines reduced the production of IL-6 and TNF- $\alpha$ . The findings suggest that each of these medicines has anti-inflammatory properties and they are in line with those of a previous study that found mother tincture and 6C, 30C and 200C dilutions of *Arnica montana*, *Thuja occidentalis* and *Bryonia alba* reduced cytokine release (TNF- $\alpha$ , IL-1 and IL-6) and oxidative stress (glutathione, malondialdehyde, superoxide dismutase and catalase) in LPS-stimulated RAW-264.7, murine macrophage cells and human whole blood culture.<sup>[46]</sup> Our findings are also in line with a previous study where *Arsenic album* (3C, 5C, 7C, 9C, 15C and 30C) dilutions significantly decreased oxidative stress in inflammatory microglial cells and the *Arsenic album* 30C dilution had the best advantage in terms of lowering ROS generation when compared to the other dilutions.<sup>[47]</sup>

Integrins play a crucial role in immune cell adhesion, migration and leukocyte recruitment under inflammatory conditions.<sup>[12,13]</sup> The previous data have also shown that systemic inflammation could change the tissues' matrix environment, which can contribute to the functions of immune cells.<sup>[48]</sup> While there was a comparable and uniform reduction in the cytokine and ROS levels, a differential effect on integrin expression and binding to fibronectin was observed in the presence of different medicines. For example, the MFI of  $\beta 1$  in *Arsenic album* and *Rhus tox* treated cells shows the lower expression in both activated neutrophils and monocytes as compared to the control and the higher expression in *Hepar sulphuris* and *Bryonia alba*-treated cells. In contrast, the MFI of  $\alpha v$  is higher in *Arsenic album* and *Rhus tox* treated cells and lower in *Hepar sulphuris* and *Bryonia alba* treated cells. In addition,



**Figure 8:** Mean fluorescence intensity of (a) CD14 (b) CD11b and (c) CD206 at day 3 and day 6 post seeding of differentiated macrophage. Data are representative of two independent experiments performed in duplicates ( $n = 4$ )

the binding of neutrophils and monocytes on fibronectin after treatment with homoeopathic medicines was also reduced compared to the cells cultured only in media which were more prominent in *Rhus tox* treated cells in neutrophils and *Bryonia alba* treated cells in monocytes. The reduced cell spreading on homoeopathy treated cells was further substantiated by the lower neutrophil and monocyte cell area and perimeter measurements on fibronectin-coated plates compared to medium control.

While this study adds to our understanding of the immunomodulatory effects of these homoeopathic medicines, more research is needed to understand the effects on cell behaviour during inflammation, the reciprocal action and the mode of interaction with other immune cells. For example, in the presented results, there was a 25–40% reduction in the MFI levels of CD62L and CD69 expression on neutrophils and monocyte in the presence of homoeopathic medicines, while the level of CD14 and CD11b was not shown any relevant differences. In addition, these homoeopathic medicines do not have any significant effect on the differentiation of monocytes to macrophages, as confirmed by analysing the surface marker (CD14, CD11b and CD206) expression of monocytes on day 3 and day 6 post-seeding.

## CONCLUSION

Our data support the preventive benefits of homoeopathic medicines *Arsenic album*, *Rhus tox*, *Hepar sulphuris* and *Bryonia alba* against LPS-induced cytokine increases and oxidative stress in human peripheral blood neutrophils and monocytes. These medicines contain immunomodulatory properties that aid in the reduction of integrin-mediated cell binding to fibronectin. More research is needed to determine if these homoeopathic medicines engage with specific cell surface receptors or penetrate cell membranes through alternative pathways, as well as their function *in vivo*.

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## Declaration of patient consent

No patients were used for the experiment. Healthy participants voluntarily donated blood for the experiments as prior consent was approved by guidelines of IHEC, IIT Roorkee.

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## Conflicts of interest

None declared.

## Supplementary Figures

**Supplementary Figure 1:** Bright field microscopic images showing the binding of lipopolysaccharide stimulated or unstimulated neutrophils adhered to fibronectin following the treatment with different homoeopathic medicines ( $\times 40$  magnification, Scale bar = 50  $\mu\text{m}$ ).

**Supplementary Figure 2:** Bright field microscopic images showing the binding of lipopolysaccharide stimulated or unstimulated monocytes adhered to fibronectin following the treatment with various homoeopathic medicines ( $\times 40$  magnification, scale bar = 50  $\mu\text{m}$ ).

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## Les médicaments homéopathiques modulent les fonctions inflammatoires et l'expression des récepteurs d'adhésion dans les cellules sanguines humaines: une étude in vitro

**Contexte:** Les médicaments homéopathiques sont largement utilisés pour le traitement de nombreuses maladies humaines et ils affectent les fonctions des cellules immunitaires. Cependant, notre compréhension des effets immunomodulateurs de ces médicaments et des mécanismes d'action associés reste limitée.

**Objectif:** La présente étude vise à étudier les effets immunomodulateurs de l'album Arsenic, Rhus toxicodendron, Hepar sulphuris et Bryonia alba sur les neutrophiles et monocytes sanguins périphériques induits par le lipopolysaccharide (LPS).

**Matériaux et méthodes:** Dans ce travail, les neutrophiles et les monocytes ont été traités avec différentes dilutions homéopathiques de puissance 30C séparément, suivies d'une stimulation avec le LPS, pour examiner les effets immunomodulateurs de ces médicaments. L'analyse de l'expression basée sur la cytométrie de flux de différents marqueurs de surface a été évaluée, la dynamique d'expression des gènes a été déterminée en utilisant qPCR, et la sécrétion de protéines a été évaluée en utilisant ELISA.

**Résultats:** Une diminution significative des niveaux de cytokines pro-inflammatoires aux niveaux de transcription (IL-6, TNF- $\alpha$ ) et de protéine (IL-6) avec une expression réduite des récepteurs de reconnaissance des pathogènes (TLR-2, TLR-4) dans les neutrophiles et les monocytes stimulés par LPS a été observée. De même, il y avait une réduction de la production d'espèces réactives de l'oxygène (ROS) et de l'expression des marqueurs d'activation tels que CD44, CD69 et CD62L dans les cellules stimulées en présence des médicaments homéopathiques. Un niveau différentiel d'expression des récepteurs d'adhésion cellulaire (par exemple, l'intégrine  $\beta 1$ ,  $\beta 3$  et  $\alpha v$ ) qui négocie la migration des cellules immunitaires dans les tissus, a été observé dans les cellules stimulées.

**Conclusion:** Nous avons constaté que les médicaments homéopathiques ont un effet significatif contre la réponse inflammatoire induite par le LPS dans les cellules immunitaires innées et fournissent un soutien empirique pour leurs effets bénéfiques.

## Homöopathische Arzneimittel modulieren entzündliche Funktionen und die Expression von Adhäsionsrezeptoren in menschlichen Blutzellen: Eine in vitro Studie

**Hintergrund:** Homöopathische Arzneimittel werden in großem Umfang zur Behandlung vieler menschlicher Krankheiten eingesetzt und beeinflussen nachweislich die Funktionen von Immunzellen. Unser Verständnis der immunomodulatorischen Wirkungen solcher Arzneimittel und der damit verbundenen Wirkmechanismen ist jedoch noch begrenzt. **Zielsetzung:** In der vorliegenden Studie sollen die immunomodulatorischen Wirkungen von *Arsenic album*, *Rhus toxicodendron*, *Hepar sulphuris* und *Bryonia alba* auf Lipopolysaccharid (LPS) induzierte periphere Blutneutrophile und Monozyten untersucht werden. **Materialien und Methoden:** In dieser Arbeit wurden Neutrophile und Monozyten separat mit verschiedenen homöopathischen Verdünnungen der 30C-Potenz behandelt, gefolgt von einer Stimulation mit LPS, um die immunomodulatorischen Wirkungen dieser Arzneimittel zu untersuchen. Es wurde eine durchflusszytometrische Analyse der Expression verschiedener Oberflächenmarker durchgeführt, die Dynamik der Genexpression wurde mittels qPCR bestimmt und die Proteinsekretion wurde mittels ELISA bewertet. **Ergebnisse:** Bei LPS-stimulierten Neutrophilen und Monozyten wurde eine signifikante Verringerung der proinflammatorischen Zytokine sowohl auf Transkript- (IL-6, TNF- $\alpha$ ) als auch auf Proteinebene (IL-6) sowie eine verringerte Expression der Pathogenerkennungsrezeptoren (TLR-2, TLR-4) beobachtet. Auch die Produktion reaktiver Sauerstoffspezies (ROS) und die Expression von Aktivierungsmarkern wie CD44, CD69 und CD62L wurden in den stimulierten Zellen in Gegenwart der homöopathischen Arzneimittel reduziert. Eine unterschiedliche Expression von Zelladhäsionsrezeptoren (z.B. Integrin  $\beta 1$ ,  $\beta 3$  und  $\alpha v$ ), die die Migration von Immunzellen im Gewebe vermitteln, wurde in den stimulierten Zellen beobachtet. **Schlussfolgerung:** Wir fanden heraus, dass homöopathische Arzneimittel eine signifikante Wirkung gegen die LPS-induzierte Entzündungsreaktion in angeborenen Immunzellen haben und liefern empirische Unterstützung für ihre positiven Auswirkungen.

## होम्योपैथी दवाएं मानव रक्त कोशिकाओं में इन्फ्लेमेटरी फंक्शन और ऐड्‌हिसन एक्सप्रेशन को नियंत्रित करती हैं: एक इन विट्रो अध्ययन

**पृष्ठभूमि:** होम्योपैथी दवाओं का उपयोग कई मानव रोगों के लिए बड़े स्तर पर किया जाता है और प्रतिरक्षा कोशिका कार्यों पर इनका प्रभाव देखा गया है। हालांकि, इन दवाओं के इम्यूनोमॉड्यूलेटरी प्रभावों और कार्यवाही के संबंधित तंत्र के बारे में हमारी समझ सीमित है। **उद्देश्य:** मौजूदा अध्ययन का उद्देश्य लिपोपॉलीसैकेराइड (एलपीएस) प्रेरित पेरिफेरल ब्लड न्यूट्रोफिल्स और मोनोसाइट्स पर आर्सेनिक एल्बम (*Arsenic album*), रहुस टॉक्सिकोडेंड्रोन (*Rhus toxicodendron*), हेपर सल्फ्यूरिस (*Hepar sulphuris*) और ब्रायोनिया अल्बा (*Bryonia alba*) के इम्यूनोमॉड्यूलेटरी प्रभावों की जांच करना है। **सामग्री और विधियाँ:** इस कार्य में, इन दवाओं के इम्यूनोमॉड्यूलेटरी प्रभावों की जांच करने के लिए, न्यूट्रोफिल्स और मोनोसाइट्स को 30C पोटेंसी के विभिन्न होम्योपैथी विलयनों के साथ अलग-अलग मिलाया गया और एलपीएस (LPS) से उत्तेजित किया गया। विभिन्न संतही संकेतों का फ्लो साइटोमेट्री-आधारित एक्सप्रेशन एनालिसिस का मूल्यांकन किया गया, क्यूपीसीआर (qPCR) द्वारा जीन अभिव्यक्ति गतिशीलता निर्धारित की गई और एलिसा (ELISA) द्वारा प्रोटीन साव का मूल्यांकन

किया गया। **परिणाम:** एलपीएस उत्तेजित न्यूट्रोफिल्स और मोनोसाइट्स में रोगजनक पहचान रिसेप्टर्स (टीएलआर-2, टीएलआर-4) की कम अभिव्यक्ति के साथ ट्रांसक्रिप्ट (आईएल-6, टीएनएफ- $\alpha$ ) और प्रोटीन (आईएल-6) दोनों स्तरों पर प्रो-इंफ्लेमेटरी साइटोकाइन्स के स्तर में महत्वपूर्ण गिरावट देखी गई। इसी तरह, होम्योपैथी दवाओं की उपस्थिति में उत्तेजित कोशिकाओं में प्रतिक्रियात्मक ऑक्सीजन प्रजातियों (ROS) के उत्पादन और CD44, CD69 और CD62L जैसे सक्रियता संकेतों के एक्सप्रेशन में गिरावट हुई। कोशिका ऐडहेशन रिसेप्टर्स (जैसे कि, इंटेग्रिन  $\beta 1$ ,  $\beta 3$  और  $\alpha v$ ) के एक्सप्रेशन का एक डिफरेंशियल स्तर, जिसके माध्यम से ऊतकों में प्रतिरक्षा कोशिकाओं का स्थानांतरण होता है, उत्तेजित कोशिकाओं में देखा गया। **निष्कर्ष:** हमने पाया कि होम्योपैथी दवाओं, जन्मजात प्रतिरक्षा कोशिकाओं में, एलपीएस-प्रेरित इंफ्लेमेटरी फंक्शन पर महत्वपूर्ण प्रभाव डालती हैं और उनके लाभकारी प्रभावों के लिए अनुभवी सहायता प्रदान करती हैं।

### Los medicamentos homeopáticos modulan las funciones inflamatorias y la expresión de los receptores de adhesión en las células sanguíneas humanas: Un estudio in vitro

**Antecedentes:** Los medicamentos homeopáticos se utilizan ampliamente para el tratamiento de muchas enfermedades humanas y se ha demostrado que afectan a las funciones de las células inmunitarias. Sin embargo, nuestra comprensión de los efectos inmunomoduladores de dichos medicamentos y de los mecanismos de acción asociados sigue siendo limitada. **Objetivo:** El presente estudio tiene por objeto investigar los efectos inmunomoduladores de Arsenic album, Rhus toxicodendron, Hepar sulphuris y Bryonia alba sobre los neutrófilos y monocitos de sangre periférica inducidos por lipopolisacáridos (LPS). **Materiales y métodos:** En este trabajo, se trataron neutrófilos y monocitos con diferentes diluciones homeopáticas de potencia 30C por separado, seguidas de estimulación con el LPS, para examinar los efectos inmunomoduladores de estos medicamentos. Se evaluó la expresión de diferentes marcadores de superficie mediante citometría de flujo, se determinó la dinámica de la expresión génica mediante qPCR y se evaluó la secreción de proteínas mediante ELISA. **Resultados:** Se observó una disminución significativa de los niveles de citocinas proinflamatorias tanto a nivel de transcripción (IL-6, TNF- $\alpha$ ) como de proteína (IL-6) con una expresión reducida de los receptores de reconocimiento de patógenos (TLR-2, TLR-4) en neutrófilos y monocitos estimulados por LPS. Del mismo modo, se produjo una reducción de la producción de especies reactivas de oxígeno (ROS) y de la expresión de marcadores de activación como CD44, CD69 y CD62L en las células estimuladas en presencia de los medicamentos homeopáticos. En las células estimuladas se observó un nivel diferencial de expresión de los receptores de adhesión celular (por ejemplo, integrina  $\beta 1$ ,  $\beta 3$  y  $\alpha v$ ) que median la migración de las células inmunitarias en los tejidos. **Conclusión:** Encontramos que los medicamentos homeopáticos tienen un efecto significativo contra la respuesta inflamatoria inducida por LPS en las células inmunitarias innatas y proporcionan apoyo empírico a sus efectos beneficiosos.

### 势法物 人血 胞中的炎症功能和粘附受体表达：体外研究

**背景:** 势法物被广泛用于治 多人疾病, 并被 明会影响免疫 胞功能。然而, 我 对此类 物的免疫 作用和相 作用机制的了解仍然有限。

**目的:** 本研究旨在探 砷、毒、硫肝和白叶藻对脂多糖 (LPS) 的外周血中性粒 胞和单核 胞的免疫 作用。**材料和方法:** 在这 工作中, 中性粒 胞和单核 胞分别用30C效力的不同 势法稀 液处理, 然后用LPS刺激, 以 这些 物的免疫 作用。估基于流式 胞术的不同表面 物的表达分析, 使用qPCR 定基因表达动力学, 并使用ELISA 估蛋白 分泌。

**果:** LPS刺激的中性粒 胞和单核 胞中, 促炎 胞因子的 录物 (IL-6、TNF- $\alpha$ ) 和蛋白 (IL-6) 水平 著降低, 病原体 别受体 (TLR-2、TLR-4) 的表达降低。类似地, 在 势法物存在的情况下, 受刺激 胞中活性氧 (ROS) 的 生和活化 物如CD44、CD69和CD62L的表达减少。在受刺激的 胞中 察到介 免疫 胞在 中迁移的 胞粘附受体 (如整合素  $\beta 1$ 、 $\beta 3$  和  $\alpha v$ ) 的表达水平不同。 : 我 势法物对LPS 的先天免疫 胞炎症反 具有 著作用, 并 其有益作用提供了 支持。