Evaluation of anti-inflammatory effect of *Varanadi Kashayam* (decoction) in THP-1-derived macrophages

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Abstract

Background: *Varanadi Kashayam* is an Ayurvedic polyherbal decoction containing 16 ingredients, for which the mechanisms of action involved in controlling chronic inflammatory conditions have not been evaluated. The inhibition of release of proinflammatory cytokines by lipopolysaccharide (LPS)-stimulated monocytes/macrophages is an ideal *in vitro* model for identifying anti-inflammatory molecules. **Aim:** The aim of the study is to determine the anti-inflammatory effect of *Varanadi Kashayam* in THP-1-derived macrophages. **Materials and Methods:** The efficacy of *Varanadi Kashayam* on monocyte cell differentiation was determined by quantitative polymerase chain reaction to assess the expression of differentiation markers MMP-9, CD36, CD11b and CD14. Further *Varanadi Kashayam* treated THP-1 macrophages were induced with LPS and the production of proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) were measured and corresponding genes expressions were quantified. **Results:** The results indicate that *Varanadi Kashayam* reduced the differentiation of THP-1 monocytes to macrophages and downregulated the expression of cell surface markers. Furthermore, it could decrease the release of proinflammatory cytokines from LPS-induced THP-1 macrophages and downregulated the expression of TNF-α and IL-1β genes. **Conclusion:** The results obtained from this study suggest a possible mechanism of action of the herbal decoction in inflammatory processes and opens up the possibilities of identifying bioactive lead molecules with anti-inflammatory potentials.

Keywords: Ayurveda, inflammation, interleukin-1 β, THP-1, tumor necrosis factor-α, *Varanadi Kashayam*

Introduction

Inflammation is the body's immediate response to tissue damage by pathogens, chemical stimuli and physical injury and ultimately leads to restoration of normal tissue structure and function.[1,2] However, excessive and prolonged inflammatory response contribute to inflammatory diseases such as cancer,[3] atherosclerosis,[4] rheumatoid arthritis (RA),[5] obesity[6] and cardiovascular disease.[7] Macrophages play a critical role in the initiation of inflammation by releasing proinflammatory mediators and cytokines.[8] Overproduction of proinflammatory cytokines is involved in several disease states ranging from chronic inflammation to allergy.[9] Tumor necrosis factor-alpha (TNF-α) is a predominant proinflammatory cytokine synthesized and secreted from macrophages,[10] which plays a key role in many autoimmune diseases and induces production of other cytokines.[11] There are evidences for the role of macrophage-derived TNF-α in the development of atherosclerosis,[12,13] RA,[14] inflammatory bowel disease[15] and psoriasis.[16] Thus, drugs that block the release of TNF-α have proved to be useful in the treatment of ulcerative colitis and RA.[17,18] Interleukin-1beta (IL-1β) is an another proinflammatory cytokine secreted by macrophages,[19] which is a key mediators of the host response to infection and inflammation. Thus, the inhibition of release of proinflammatory cytokines by monocytes and macrophages in chronic inflammatory condition is a main target in the development of anti-inflammatory drugs.[20,21]

Varanadi Kashayam, also known as *Varanadi Kwatha*, is a well-known Ayurvedic herbal decoction used traditionally for the treatment of obesity, atherosclerosis, fatty liver disease, tumors and chronic arthritis.[22,23] It has been reported to have antioxidant and anti-lipase activity.[24] However, there is no documented evidence available for efficacy of this medication

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Access this article online

Quick Response Code:

Website: www.ayujournal.org

DOI: 10.4103/ayu.AYU_53_18

How to cite this article: Chinchu JU, Mohan MC, Devi SJ, Kumar BP. Evaluation of anti-inflammatory effect of *Varanadi Kashayam* (decoction) in THP-1-derived macrophages. AYU 2018;39:243-9.

in chronic inflammatory condition associated with obesity and lipid disorders. It is reported that in obese individuals, the adipose tissue infiltrated monocytes will differentiate into inflammatory macrophages and release proinflammatory cytokines such as TNF-α and IL-1β.[25,26] Therefore, the present study was carried out to determine anti-inflammatory property of *Varanadi Kashayam* on lipopolysaccharide (LPS)-stimulated THP-1-differentiated macrophages.

Materials and Methods

Chemicals

Human monocytic THP-1 cells were purchased from the National Centre for Cell Sciences, Pune. Roswell Park Memorial Institute medium (RPMI Medium), fetal bovine serum (FBS), antibiotic solution (10,000 units Penicillin and 10 mg Streptomycin), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DNase I (1 mg/mL) solution were supplied by HiMedia Ltd., India. Rolipram, dexamethasone, phorbol 12-myristate 13-acetate (PMA) and LPS (*Escherichia coli* 0111:B4) were bought from Sigma Aldrich, St. Louis, USA. ELISA max™ Human TNF-α and IL-1β quantification assay kits were procured from BioLegend, California. RNAiso Plus (total RNA extraction reagent), PrimeScript™ RT reagent kit (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kits were obtained from TAKARA BIO INC., Japan. All the forward and reverse primers were bought from Sigma Aldrich, St. Louis, USA. All other chemicals used were of analytical grade obtained from Merck Ltd., India.

Sample preparation

Varanadi Kashayam prepared in accordance with Ayurvedic text Ashtanga Hridaya[22] was obtained from authentic commercial source Kottakkal Arya Vaidya Sala, India (Batch Number: 512,540 and manufacturing date: 1/08/2014). The decoction contains 16 plants and the quantity of each plant parts used is given in Table 1. Details of quality control data as provided by Kottakkal Arya Vaidya Sala are attached as supplementary doc 1 (quality control certificate). For experiments, *Varanadi Kashayam* was fractionated using five solvents of increasing polarity: hexane, dichloromethane, ethyl acetate, methanol and water. Solvents from fractions were removed by rotary evaporation and water fraction was freeze-dried at −85°C using a Lyophilizer (Sub-Zero, India). All the dried fractions were stored at 4°C until analysis. For cell culture study, fractions were redissolved in dimethyl sulfoxide (DMSO) and serially diluted with cell culture medium and added to cells at varying concentrations. The final concentration of DMSO in samples was <0.01%.

Cell viability assay

Cell viability was measured by MTT assay according to protocol described earlier with slight modifications.[27] THP-1 cells were plated in a 96-well plate at a density of 1×10^4 cells/well. After 24 h of incubation, serial dilutions of (6.25, 12.5, 25, 50, and 100 μg/mL) fractions of *Kashayam* (dissolved in cell culture medium) were added and cells were incubated for next 24 h with 5% CO_2 at 37°C. 10 μL of MTT (5 mg/mL) in phosphate-buffered saline was added and incubated for 4 h. After incubation, the insoluble formazan crystals were dissolved in 100 μL/well DMSO and the absorbance was read at 570 nm. The absorbance value of the normal control group was represented as 100% and the absorbance of all other test groups was expressed as a percentage of the normal control group.

Cell viability percentage was determined as $([A_T/A_c] \times 100$, where A_T is absorbance of test and A_C is the absorbance of control.

Cell culture and differentiation of THP-1 monocytes

Human monocytic THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 0.1% antibiotic solution in a humidified CO_2 incubator at 37°C with 5% CO_2.

For the induction of cell differentiation, THP-1 cells were seeded at cell density of 1×10^6 cells per mL in RPMI medium and stimulated with 400nM PMA for 24 h in the presence of three different concentrations of *Varanadi Kashayam* fractions (100, 25 and 6.25 μg/mL). After incubation, images were taken using an inverted phase-contrast microscope. Cells stimulated with PMA without *Varanadi Kashayam* fractions treatment were taken as control.

Measurement of proinflammatory cytokines (tumor necrosis factor-alpha and interleukin-1 beta) production

THP-1 cells were seeded at a density of 1×10^6 cells/mL in 24-well plate with PMA (400nM) to induce differentiation and treated with three different concentrations of *Varanadi Kashayam* fractions (100, 25, and 6.25 μg/mL) for 24 h. The cells were then stimulated with LPS from *E. coli* (1 μg/mL) for 16 h and supernatants were collected. Concentrations of TNF-α and IL-1β were quantified using corresponding ELISA kits from Bio-Legend, USA, according to manufacturer's instructions. Dexamethasone and rolipram were used as standard controls for IL-1β and TNF-α production, respectively. Cells without drug treatment were considered as control and fresh medium was taken as blank.

Quantitative real-time polymerase chain reaction

THP-1 monocytic cells were treated with PMA (400nM) to induce differentiation in the presence of 100 μg/mL concentration of *Varanadi Kashayam* fractions. After 24 h of incubation, total cellular RNA was extracted for quantifying the expression of differentiation markers MMP-9, CD36, CD14 and CD11b. For quantification of proinflammatory cytokines genes expression, THP-1 cells were induced with 400nM PMA in the presence of 100 μg/mL concentration of fractions for 24 h. After that, cells were stimulated with LPS from *E. coli* (1 μg/mL) for 16 h and then, the total cellular RNA was extracted according to instructions in manufacture's kit (RNAiso Plus, total RNA extraction Reagent). The total RNA was quantified using UV/VIS Spectrophotometer and a quantity of 2 μg RNA was reverse transcribed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), Takara. Target cDNA levels were determined by SYBR green-based real-time

Table 1: Ingredients of *Varanadi Kashayam*

Sanskrit name	Botanical name	Part used	Form of ingredient	Quantity (g)
Varuna	*Crateva magna* (Lour.) DC.	Stem bark	Decoction	1.235
Saireyaka	*Barleria strigosa* Willd.	Root	Decoction	2.470
Shatavari	*Asparagus racemosus* Willd.	Root tuber	Decoction	1.235
Chitraka	*Plumbago zeylanica* L.	Root	Decoction	1.235
Murva	*Chonemorpha fragrans* (Moon) Alston	Root	Decoction	1.235
Bilwa	*Aegle marmelos* (L.) Correa	Root	Decoction	1.235
Kitamari	*Aristolochia bracteolata* Lam.	Whole plant	Decoction	1.235
Brihati	*Solanum anguivi* Lam.	Root	Decoction	1.235
Nidigdhika	*Solanum surattense* Burm. f.	Root	Decoction	1.235
Karanja	*Pongamia pinnata* (L.) Pierre	Stem bark	Decoction	1.235
Putikaranja	*Holoptelea integrifolia* Planch.	Root	Decoction	1.235
Agnimantha	*Premna corymbosa* Rottler and Willd.	Root	Decoction	1.235
Haritaki	*Terminalia chebula* Retz.	Fruit rind	Decoction	1.235
Akshiva	*Moringa concanensis* Nimmo	Root	Decoction	1.235
Darbha	*Desmostachya bipinnata* (L.) Stapf	Whole plant	Decoction	1.235
Bhallataka	*Semecarpus anacardium* L.f.	Fruit	Decoction	1.235

The ingredients used for *Varanadi Kashayam* (decoction) preparation. Each 10 mL of *Varanadi Kashayam* was prepared out of the given quantities of plant parts

polymerase chain reaction (PCR) (Light cycler 96 system, Roche Diagnostics) in which 20 µL reactions containing 10 µL SYBR Premix Ex Taq II (Tli RNaseH Plus) (2X), 2 µL cDNA, 0.8 µL 10 µM forward and reverse primer and 6.4 µL PCR grade water was used. The cycling conditions were initial denaturation at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. A dissociation curve was generated by adding a cycle of 95°C for 5 s, 60°C for 1 min and 95°C for 15 s, and cooling at 50°C for 30 s. Results were normalized using GAPDH and β-actin as reference genes.

The following primers were used: CD36: Forward: TGCAAAACGGCTGCAGGTCAA, Reverse: CGGGACGTAAGGACAGTAGGAGT, MMP-9: Forward: TTGACAGCGACAAG AAGTGG, Reverse: TATTCCTGCTGCACTTACCG, CD14: Forward: CGCTCCGAGATG CATGTG, Reverse: GACTTGAGGGAGTTAGACAGCAA, CD11b: Forward: CCCCCAGG TCACCTTCTCCG, Reverse: GCTCTGTCGGGAAGGAGCCG, TNFα: Forward: CTGCTGCAGTTTGGAGTGAT, Reverse: AGATGATCTGACTGCCTGGG, IL-1β: Forward: GTGGC AATGAGGATGACTTGTTC, Reverse: TAGTGGTGGTCGGAGATTCGA, GAPDH: Forward: TGCACCACCAACTGCTTAGC, Reverse: GGCATGGACTGTGGTCATGAG, and β-Actin: Forward: ATCCCCCAAAGTTCACAATG, Reverse: GTGGCTTTTAGGATGGCAAG.

Relative mRNA expression of each gene in control and sample treated cells was determined by ΔΔCq method,[28,29] where the Cq values are obtained from the expression of targeted genes normalized to non-targeted GAPDH and β-actin reference genes. ΔΔCq expression values were measured by taking the ratio of targeted gene ΔCq to that of control ΔCq.

Statistical analysis

Significance difference among control and sample treated groups were determined by one-way analysis of variance and the Dunnett's multiple comparison tests (GraphPad Prism® version 5.03). Data were presented as mean ± standard deviation (SD) of three independent experiments. $P < 0.05$ was considered to be statistically significant.

Results

Varanadi Kashayam fractions (6.25–100 µg/mL) were tested for cytotoxicity in THP-1 monocytes by MTT assay and none of the fraction was found to affect the viability of cells as given in Table 2. From the cytotoxicity data, three different concentrations 100, 25 and 6.25 µg/mL were selected for further experimental studies. Figure 1 clearly indicates that hexane and dichloromethane fractions of *Varanadi Kashayam* reduced the differentiation of THP-1 monocytes and induced cell proliferation more significantly when compared with control THP-1-derived macrophages. It is known that differentiated cells will lose proliferation capability and form adherent state and dendritic morphology.[30]

Hexane and dichloromethane fractions of *Varanadi Kashayam* suppressed the expression level of MMP-9 gene by 97.86% ± 1.76% and 90.67% ± 4.01%, respectively and the expression level was similar to that in undifferentiated THP-1 monocytes [Figure 2a]. Ethyl acetate, methanol and water fractions could also downregulate the expression of MMP-9 gene level by 78.619% ± 3.30%, 56.691% ± 9.41% and 34.68% ± 7.77%, respectively. CD36 is another surface marker gene expressed in macrophages. The treatment of THP-1 monocytes with 100 µg/mL concentration of hexane, dichloromethane, ethyl acetate and methanol fractions of *Varanadi Kashayam* downregulated the expression of CD36 transcript level by 98.18% ± 0.269%,

Table 2: Effect of *Varanadi Kashayam* fractions on cell viability

Samples	Percentage of cell viability (%)				
	100 μg/mL	50 μg/mL	25 μg/mL	12.5 μg/mL	6.25 μg/mL
Hexane fraction	86.88±1.81	88.09±1.84	93.64±4.78	95.45±4.29	96.97±0.761
Dichloromethane fraction	88.19±4.13	92.02±1.81	92.23±4.34	94.04±5.76	94.46±4.82
Ethyl acetate fraction	94.75±5.67	94.34±6.46	95.66±5.65	95.45±5.09	98.38±3.03
Methanol fraction	94.66±4.79	95.45±4.87	95.65±4.62	97.67±3.61	98.48±2.18
Water fraction	93.23±6.75	98.38±4.46	96.46±5.79	96.66±2.58	98.88±6.52

Cytotoxicity study of *Varanadi Kashayam* fractions in THP-1 cells. THP-1 cells were treated with varying concentrations of *Varanadi Kashayam* fractions for 24 h. Cell viability was assessed by MTT assay. All the values are expressed as mean±SD of three independent experiments in triplicate. MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SD: Standard deviation

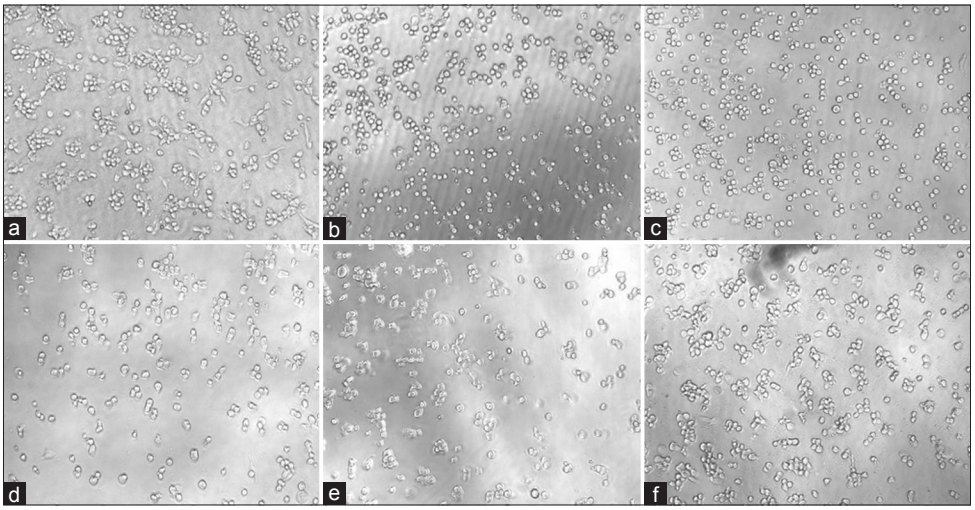

Figure 1: Microscopic images of phorbol 12-myristate 13-acetate-induced THP-1 cells treated with 100 μg/mL concentration of *Varanadi Kashayam* fractions (a) Differentiated control macrophage cells without drug treatment, (b) Hexane fraction, (c) Dichloromethane fraction, (d) Ethyl acetate fraction, (e) Methanol fraction and (f) Water fraction-treated cells. Images were taken at × 10 magnification using an inverted phase-contrast microscope (OLYMPUS 1 × 51)

86.31% ± 9.28%, 64.86% ± 9.84% and 61.73% ± 8.40% respectively, when compared with that of differentiated THP-1 macrophages [Figure 2b]. The expression of CD11b gene was decreased by hexane, dichloromethane, ethyl acetate, methanol ($P < 0.001$) and water fraction ($P < 0.05$) in a statistically significant level. 98.94% ± 0.830% of CD11b expression level was found in hexane fraction-treated cells and this level was similar to that in undifferentiated THP-1 monocytes (99.45% ± 0.769%) [Figure 2c]. CD14 is a marker for monocytes.[31] No change was observed in the expression level of CD14 gene in hexane fraction-treated cells. Whereas dichloromethane, ethyl acetate, methanol and water fraction-treated cells showed a 44.30% ± 8.29%, 57.87% ± 2.85%, 89.51% ± 7.02% and 96.47% ± 0.485% decreased expression of CD14 gene [Figure 2d].

Quantification of cytokines

The results obtained from the quantification of proinflammatory cytokines indicate that all the fractions of *Varanadi Kashayam* significantly reduced TNF-α and IL-1β levels in a dose-dependent manner [Figure 3]. TNF-α is a proinflammatory cytokine secreted by macrophages.[20] IC_{50} values for the inhibition of TNF-α production were determined through regression analysis using GraphPad Prism® Software. IC_{50} value, that is, 50% inhibition of TNF-α production by hexane, dichloromethane, ethyl acetate, methanol and water fractions of *Varanadi Kashayam* was found to be 0.1469 ± 0.0083, 0.1607 ± 0.0079, 2.015 ± 0.304, 15.56 ± 1.19 and 131.4 ± 2.11 μg/mL respectively and 0.1431 ± 0.0096 μg/ml by standard control rolipram. Upon treatment with *Varanadi kashayam* the level of IL-1β was decreased as compared to cells stimulated with LPS without drug treatment. Dexamethasone was used as a standard control for IL-1β production and an IC_{50} value of 1.083 μg/mL was obtained. 50% inhibition of IL-1β production by hexane, dichloromethane, ethyl acetate, methanol and water fractions were 0.105 ± 0.002, 1.965 ± 0.293, 2.23 ± 0.348, 238 ± 2.377 and 336.5 ± 2.527 μg/mL respectively. From the results, it was shown that only hexane and dichloromethane fraction showed inhibition in the production of IL-1β at levels comparable to standard control dexamethasone. Other fractions had no effect on IL-1β production.

Further expression levels of TNF-α and IL-1β genes were quantified through quantitative real-time PCR; results are given in Figure 4. All the fractions of *Varanadi Kashayam* at a concentration of 100 μg/mL inhibited TNF-α and IL-1β genes

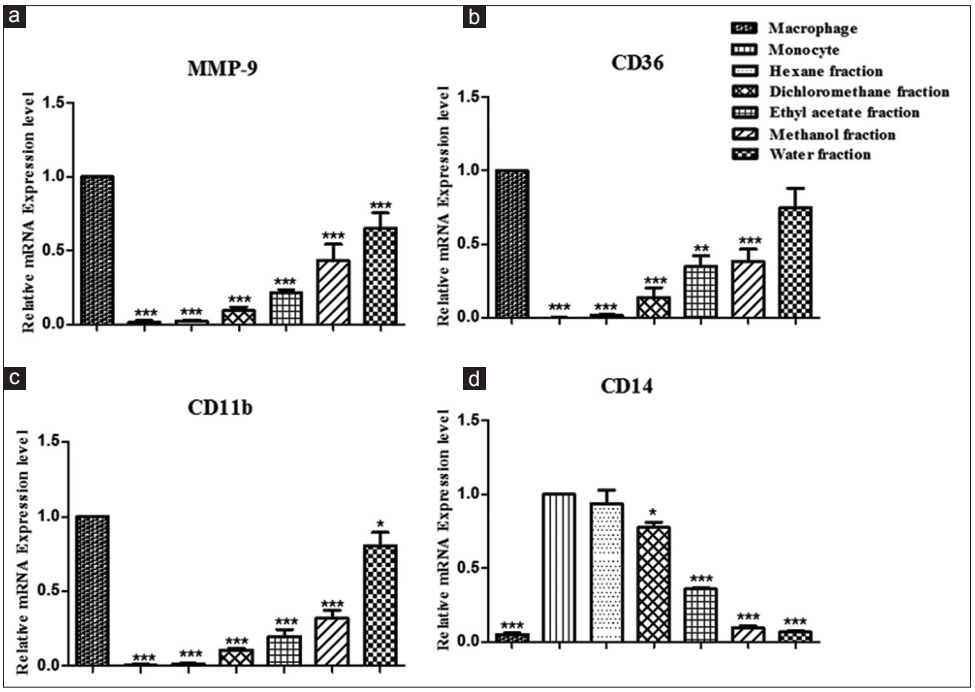

Figure 2: Effect of *Varanadi Kashayam* fractions on mRNA expression of differentiation markers. (a) MMP-9, (b) CD36, (c) CD11b, and (d) CD14 expression in differentiated macrophages, undifferentiated monocytes, and in sample treated cells. Results were normalized by GAPDH and β-actin reference genes and expressed as relative mRNA expression level. All values were expressed as mean ± standard deviation of three independent experiments in triplicate. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 of MMP-9, CD36, and CD11b are compared to the values of differentiated macrophages and that of CD14 is compared with undifferentiated monocytes

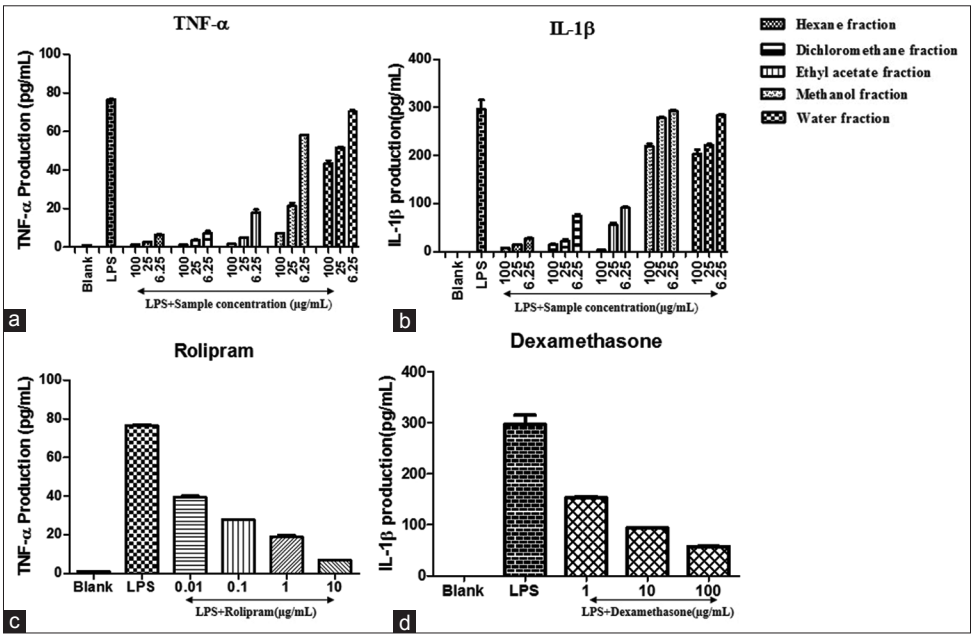

Figure 3: Effect of *Varanadi Kashayam* fractions on proinflammatory cytokines production by LPS-stimulated cells. (a) TNFs-α (b) IL-1β (c) Rolipram, standard control for tumor necrosis factor-alpha production, and (d) Dexamethasone, standard control for interleukin-1 beta production. All values were expressed as mean ± standard deviation of three independent experiments in triplicate. TNF-α: Tumor necrosis factor-alpha, IL-1β: Interleukin-1 beta, LPS: Lipopolysaccharide

expression at statistically significant level when compared with that of control macrophage cells stimulated with LPS. Hexane and dichloromethane fractions reduced TNF-α transcript level by 96.50% ± 2.33%, 94.21% ± 1.93% [Figure 4a] and IL-1β level by 94.04% ± 3.14%, 92.69% ± 1.04% [Figure 4b] respectively. The expression of proinflammatory cytokines found in hexane fraction-treated cells was similar to corresponding gene expression from cells without LPS stimulation.

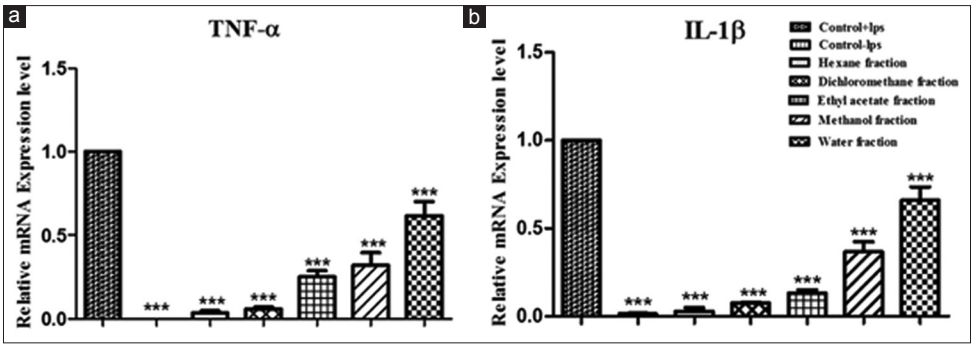

Figure 4: Effect of *Varanadi Kashayam* fractions on the expression of proinflammatory cytokines. (a) Tumor necrosis factor-alpha and (b) Interleukin-1 beta genes expression in fully differentiated macrophage cells stimulated with lipopolysaccharide, differentiated macrophage cells without lipopolysaccharide and in cells treated with *Varanadi Kashayam* fractions and stimulated with lipopolysaccharide. All values were expressed as mean ± standard deviation of three independent experiments in triplicate. ***$P < 0.001$ compared to the values of control macrophage cells stimulated with lipopolysaccharide

Discussion

Monocytes/macrophages are key mediators of inflammation and release proinflammatory cytokines such as TNF-α and IL-1β.[8] To establish immune response, THP-1 monocytic cells were differentiated to macrophages using PMA and stimulated with LPS. The inhibitory effects of *Varanadi Kashayam* on monocyte to macrophage differentiation and release of proinflammatory cytokines, TNF-α and IL-1β were investigated.

During the differentiation of THP-1 cells to macrophages induced by PMA, the THP-1 cells lose their proliferation activity and get attached to the culture plate surface.[32] Light microscopy revealed that hexane, dichloromethane, ethyl acetate and methanol fractions could prevent the morphological changes. Further analysis of cell surface marker genes MMP-9, CD36, CD14 and CD11b expression was carried out. MMP-9,[33,34] CD36 and CD11b[35,36] are markers expressed on the surface of differentiated macrophages, while CD14 is a marker expressed on monocytes.[31,37] During cotreatment of THP-1 cells with *Varanadi Kashayam* fractions and PMA, it was found that hexane, dichloromethane, ethyl acetate and methanol fractions downregulated the expression of MMP-9, CD36 and CD11b, while the levels of CD14 gene were similar to that of monocytes (undifferentiated THP-1 cells) as shown in figure 2. These results indicate that the fractions of *Varanadi Kashayam* have bioactive molecules capable of preventing the differentiation of monocytes to macrophages.

Inhibition of proinflammatory cytokine production in LPS-stimulated monocyte cells and regulation of proinflammatory cytokine gene expression in such cells are models for screening anti-inflammatory bioactive molecules.[38,39] Studies carried out in this work [Figure 4] showed that fractions of *Varanadi Kashayam* were capable of downregulating gene expression of TNF-α and IL-1β. However, only hexane fraction was capable of inhibiting the secretion/production of TNF-α and IL-1β at levels comparable to standard control rolipram and dexamethasone. While the fractions were able to downregulate TNF-α and IL-1β gene expression, it is quite possible that the molecules other than those present in hexane fraction would not be able to inhibit tumor necrosis factor-alpha converting enzyme and Caspase 1 which are involved in converting membrane-bound TNF-α to soluble TNF-α and in the maturation of pro-IL-1β. This requires further studies.

Conclusion

The study demonstrates that the polyherbal decoction *Varanadi Kashayam* effectively reduces the differentiation of monocytes to macrophages and the production of proinflammatory cytokines TNF-α and IL-1β in LPS-stimulated macrophages *in vitro*. Hence, *Varanadi Kashayam* can be used as an effective Ayurvedic formulation to control chronic inflammation and related disorders.

Acknowledgment

This study was carried out with the support from Kerala State Council for Science Technology and Environment (KSCSTE Order No: 863/FSHP/2015/KSCSTE dated 14/01/2015). The authors would like to thank DBT-MSUB-IPLSARE (BUILDER) program (BT/PR4800/INF/22/152/2012 dated 22/03/2012), School of Biosciences, Mahatma Gandhi University, Kerala, for providing Instrument facilities. The authors are grateful to Kottakkal Arya Vaidya Sala, Kerala, for their support.

Financial support and sponsorship

This study was carried out with the support from Kerala State Council for Science Technology and Environment (KSCSTE Order No: 863/FSHP/2015/KSCSTE dated 14/01/2015).

Conflicts of interest

There are no conflicts of interest.

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