Original Research Article

Effect of Crude Extract of Ethno Botanical Mixture Used in Indigenous Treatment of Tuberculosis on the Release of TNF α, IFN Ɣ and IL 10 by LPS-Stimulated THP-1 Macrophage Cell Line

Jyoti Tomar[1*], Vijaylatha Rastogi[2], Tarun Patni[3], Chhavi Vijay[4]

[1]Ph. D. Scholar, Department of Microbiology, J.L.N. Medical College, Ajmer, Rajasthan, India
[2]Senior Professor, Department of Microbiology, J.L.N. Medical College, Ajmer, Rajasthan, India
[3]Microbiologist, Intermediate Reference Laboratory, KNSTDC, Ajmer, Rajasthan, India
[4]Technical Officer, Intermediate Reference Laboratory, KNSTDC, Ajmer, Rajasthan, India

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Abstract

Introduction: In-vitro study of pro inflammatory cytokine IFN Ɣ, TNF α and anti-inflammatory cytokines IL-10, production by LPS sensitized THP-1 cell line with response to different concentrations of hawan samagri (HS) extract, has not been documented in literature *impeding* its possible use in TB treatment. This study attempts to fill the above knowledge gap in an effort to explore possibility of effective ethno botanical alternative therapy. IFN Ɣ response is well established as an immune correlate of *M. tuberculosis* infection. Here in this study, we studied the immune response profile of three cytokines believed to influence immunity to TB- IFN Ɣ, TNF α and IL-10. **Materials and Methods: Hawan samagri:** Two different hawan samagri HS1 & HS2 were used as test samples. **Cell culture:** THP-1, a promonocytic cell line was obtained from the ATCC. **Treatment for Cytokine gene expression:** In RPMI complete medium $1x10^5$ cells/well was seeded to each well of the 96 well micro-titer plate. After 24 hrs. incubation, PMA (10ng/ml) added to 96 well plates to differentiate THP1 cells. **Sample Preparation and RNA Isolation:** Total RNA from THP-1cells was extracted using TRizol Reagent. **RT-PCR:** A semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out to determine the levels of TNF-α, IL-10 and IFN and GAPDH mRNA expressions. **Gel Electrophoresis:** Ten microliters of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed. **Results:** Test samples showed dose dependent increase in, IFN-gamma, anti-inflammatory cytokine mRNA levels. Highest expression of IFN-gamma was observed in HS2 at 62.5µg/mL with mRNA expression of 2.94-fold, followed by HS1 with IFN mRNA levels of 1.98-fold compared to control cells. **Conclusion:** All test samples show dose dependent anti-inflammatory activity as demonstrated with suppression of proinflammatory cytokines (TNF-alpha and IFN gamma) and stimulation of anti-inflammatory cytokine (IL-10), LPS induced inflammatory THP-1 cell based model.

Keywords: Proinflammatory, Anti-inflammatory, Cytokines, TNF-alpha, IL-10, IFN gamma, LPS, THP-1.

Introduction

Cytokines are soluble, small proteins that are produced by cells and act in a largely paracrine manner to influence the activity of other cells. Currently, the term "cytokine" describes proteins such as the tumour necrosis factor family, the interleukins, and the chemokines. Virtually every nucleated cell can produce and respond to cytokines placing these molecules at the centre of most of the body's homeostatic mechanisms[1-3].*Mycobacterium tuberculosis* is successful as a pathogen because of its ability to persist in an immunocompetent host. This bacterium lives within the macrophage, a cell whose function is the elimination of microbes. Recent advances have improved our understanding of how *M. tuberculosis* evades two major antimicrobial mechanisms of macrophages: phagolysosome fusion and the production of toxic reactive nitrogen intermediates. *M. tuberculosis* also modulates antigen presentation to prevent the detection of infected macrophages by $CD4^+$ T cells[4].

**Correspondence*
Dr.Jyoti Tomar
Ph. D. Scholar, Department of Microbiology, JLN Medical College, Ajmer, Rajasthan, India
E-mail: jyotibst1998@yahoo.com

One recent development in our understanding of TB stems from theories of co-evolution between modern humans and MTB[5]. Evolutionary patterns based on genetic analyses suggest that Mtb and humans coexisted for tens of thousands of years in Africa but that when humans left Africa and developed a more urban lifestyle TB developed into a substantial health problem[6].During co-evolution between humans and Mtb, Mtb likely evolved tools and stratagems with which to manipulate the human immune response to ensure effective transmission[3]; this manipulation has been so successful that it is thought that over one third of the world's population harbours some form of Mtb infection]7\.The use of medicinal plants, or extracts from them, has been traditionally practiced worldwide in the prevention and treatment of several chronic diseases such as cardiovascular diseases, intestinal inflammatory diseases, inflammatory bowel disease, arthritis, diabetes, allergies, multiple sclerosis, Parkinson's and Alzheimer's diseases, and others[8].The biological activity of medicinal plants strongly depends on their composition.As an alternative medicine the ancient traditional therapies for TB seem to be worth exploring. *Hawan* is an ancient fumigation method devised and used by sages (scientists of ancient India), that involves the use of clarified cow's butter and a mixture of odoriferous and medicinal plant parts (known as *Hawan Samagri*, HS) for oblation onto consecrated fire of specific medicinal wood[9].

Inhalation of medicinal fumes emanating from this is proved to be effective in treatment of TB.[10] The extract of this same HS mixture is also used as oral medicine in such patients as advised by *Charak*, the famous physician of ancient India, in his treatise *Charak Samhita[11]*.However due to lack of scientific validation, the use of these has not been attempted in modern medicine. Recently few studies have tried to prove the depolluting and antibacterial effect of *hawan[12]*. Also, In-vitro anti-tubercular effect of *hawan* and HS extract was reported by Rastogi et al. against H37Ra strain of MTB[13,14]. However, In-vitro study of pro inflammatory cytokine IFN Ɣ, TNF alpha and anti-inflammatory cytokines IL-10, production by LPS sensitized THP-1 cell line with response to different concentrations of HS extract, has not been documented in literature *impeding* its possible use in TB treatment. This study attempts to fill the above knowledge gap in an effort to explore possibility of effective ethnobotanical alternative therapy

Materials and Methods

Herbal Mixture

Two different mixture of herbs HS1 and HS2 were used in study. HS1 was prepared as per standards mentioned in literature as used by V Rastogi et al.,(14) and HS2 was obtained from "Rishi Udhyan" Arya Samaj, Ajmer, where *hawan* is being performed daily since ages using the fixed formula mixture.

Extraction of Essential Oil

Extraction of essential oil (EO) from both herbal mixtures was done by hydro distillation using Clevenger apparatus[14].

Removal of Water and Storage

Water constituents in the oil collected after hydro distillation was removed by using anhydrous sodium sulphate. Obtained oil was stored at -4°C in a dark colored bottle[15,16].

Cell Culture

THP-1, a promonocytic cell line was obtained from the ATCC. Cells were cultured in RPMI -1640 supplemented with 10% inactivated fetal bovine serum, 50µM 2-mercaptoethanol, 2mM L-Glutamine penicillin (100 IU/ml) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent.

Treatment for Cytokine Gene Expression

Table 1: Treatments and controls

Cell	Treatments	Concentration
THP-1	Control	Media with 1% DMSO
	LPS Control	100 ng/mL
	HS1	31.25 µg/ml
	HS1	62.5 µg/ml
	HS2	31.25 µg/ml
	HS2	62.5 µg/ml

supernatant is aspirated from each of the wells into sterile micro centrifuge tubes and centrifuged at 1000rpm for 2-3mins to settle any cells if present. The cell supernatant is then used for evaluation of cytokine expression levels.

Sample Preparation and RNA Isolation:

Total RNA from THP-1cells was extracted using TRizol Reagent (Invitrogen,) according to manufacturer's instruction. THP-1cells were washed twice with PBS and centrifuged at 2000 rpm for 5 min. To the cell pellet, 1ml of TRIzol (per p35 dish) was added in 1.5 ml eppendorf tube and vortexed. Samples were allowed to stand for 5 minutes at room temperature. To the reaction mixture 0.2ml of chloroform is added and vigorously mixed for 15 seconds. The tube was allowed to stand at room temperature for 5 minutes, centrifuged the resulting mixture at 10,000rpm for 15min at 40ºC. Upper aqueous phase is transferred to a new clean eppendrof tube and treated with 0.5ml of isopropanol. The resultant mixtures mixed gently by inverting the sample 5 times and incubated at room temperature for 5 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes at 40ºC. Supernatant was discarded and the RNA pellet was washed by adding 1ml of 70% ethanol. Mix the sample gently by inverting few times. Centrifuged for 5min at 14,000 rpm at 40ºC. Supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5min at 550ºC. The pellet was then re-suspended in 25 µl of DEPC treated water.

RT-PCR

A semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels of TNF-α, IL-10 AND IFN and GAPDH mRNA expressions. The cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20µl and cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 85°C for 5 min.

Primers

Table 2: Primer details

Gene	Primer pair	Sequence	Tm	Product size (bp)
GAPDH	FP	GTCCAGTTAATTTCTGACCT	47	155
	RP	GCTTTGTACATGGTATTCAC	47	
TNF- α	FP	CAGAGGGAAGAGTTCCCCAG	61.40	169
	RP	CCTTGGTCTGGTAGGAGACG	61.40	
IL-10	FP	TGAGAACCAAGACCCAGACA	57.30	150
	RP	TCATGGCTTTGTAGATGCCT	55.25	
IFN	FP	GCAGAGCCAAATTGTCTCCT	57.30	166
	RP	ATGCTCTTCGACCTCGAAAC	57.30	

PCR

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1µM of each complementary primer specific for TNF- α , IL-10 AND IFN and GAPDH (internal control) sequence. The samples were denatured at 94°C for 5 minutes and amplified using 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for TNF- α, IL-10 AND IFN renaturation was set to 56 and 58 ºC and for GAPDH the renaturation was set to 52°C for 30 seconds followed by a final elongation at 72°C for 10 minutes. The optimal numbers of cycles have been selected for amplification of these genes experimentally so that amplifications were in the exponential range and have not reached a plate. Ten microliters of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed. Quantification of the results was accomplished by measuring the optical density of the bands, using the computerized imaging

program Image J. The values were normalized to GAPDH intensity levels.

Results

Analysis of Amplicons:

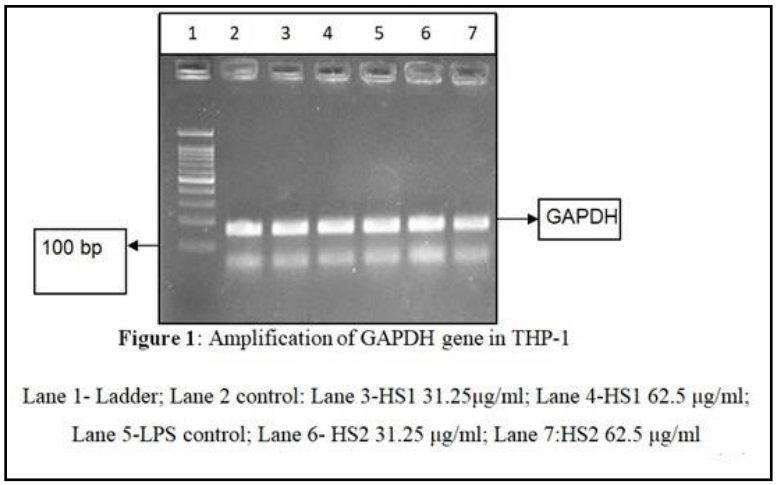

Figure 1: Amplification of GAPDH gene in THP-1

Lane 1- Ladder; Lane 2 control: Lane 3-HS1 31.25µg/ml; Lane 4-HS1 62.5 µg/ml; Lane 5-LPS control; Lane 6- HS2 31.25 µg/ml; Lane 7:HS2 62.5 µg/ml

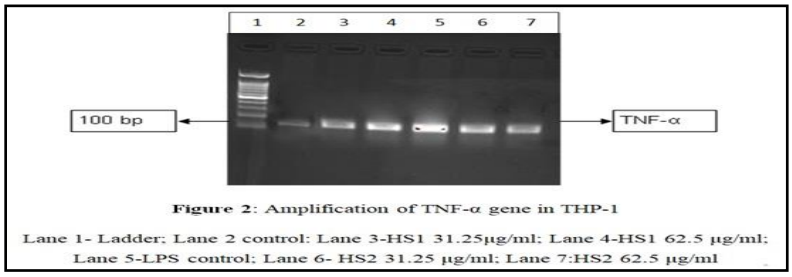

Figure 2: Amplification of TNF-α gene in THP-1

Lane 1- Ladder; Lane 2 control: Lane 3-HS1 31.25µg/ml; Lane 4-HS1 62.5 µg/ml; Lane 5-LPS control; Lane 6- HS2 31.25 µg/ml; Lane 7:HS2 62.5 µg/ml

Table 3: Relative expression TNF-α gene in THP-1

Samples	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
	GAPDH	TNF α		
Control	16647.02	5944.811	0.36	1.00
LPS control	18291.25	15453.589	0.84	2.37
HS1 31.25 µg/ml	17196.37	7638.447	0.44	1.24
HS1 62.5 µg/ml	17267.51	7481.095	0.43	1.21
HS2 31.25 µg/ml	18399.08	11271.004	0.61	1.72
HS2 62.5 µg/ml	14259.59	6831.125	0.48	1.34

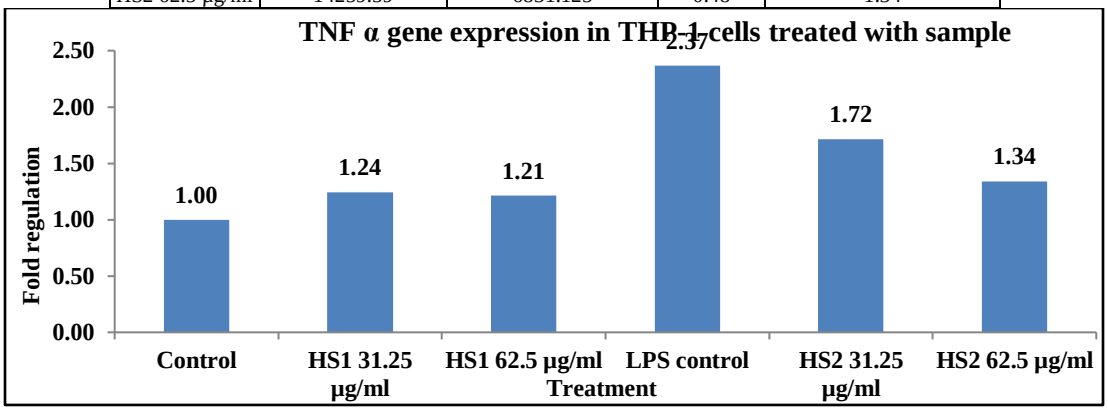

Fig 3: Relative expression TNF α gene in THP-1

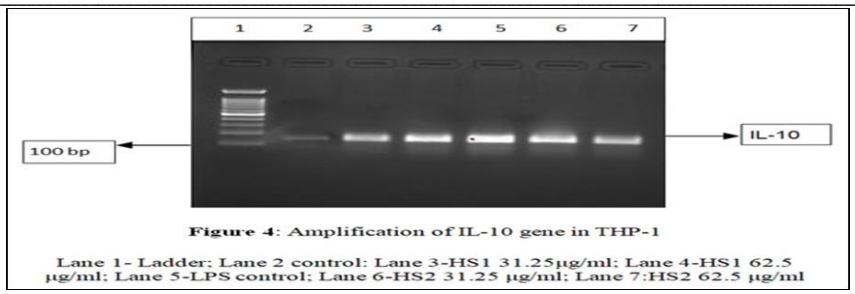

Figure 4: Amplification of IL-10 gene in THP-1

Lane 1- Ladder; Lane 2 control: Lane 3-HS1 31.25µg/ml; Lane 4-HS1 62.5 µg/ml; Lane 5-LPS control; Lane 6-HS2 31.25 µg/ml; Lane 7:HS2 62.5 µg/ml

Table 4: Relative expression IL-10 gene in THP-1

Samples	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
	GAPDH	IL-10		
Control	16647.02	4444.811	0.27	1.00
HS1 31.25µg/ml	17196.37	11107.731	0.65	2.42
HS1 62.5µg/ml	17267.51	10946.154	0.63	2.37
LPS control	18291.25	14520.054	0.79	2.97
HS2 31.25µg/ml	18399.08	10907.782	0.59	2.22
HS2 62.5µg/ml	14259.59	7195.66	0.50	1.89

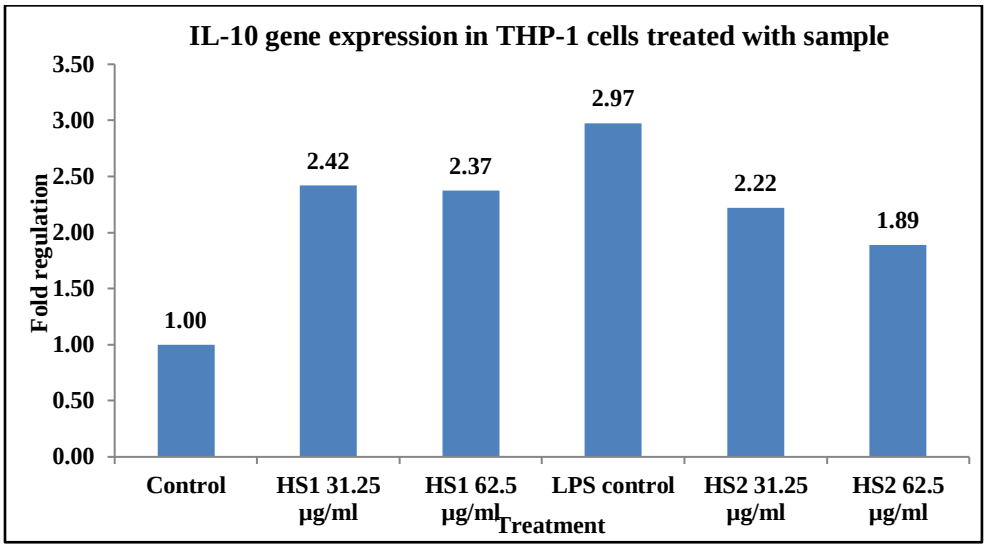

Fig 5: Relative expression IL-10 gene in THP-1

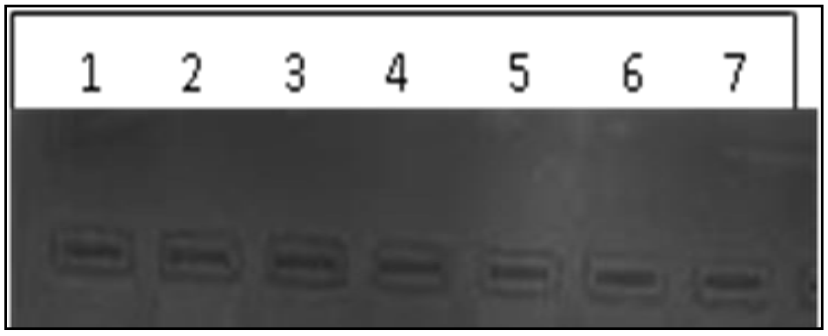

Fig 6: Amplification of IFN gene in THP-1

Lane 1- Ladder; Lane 2 control: Lane 3-HS1 31.25µg/ml; Lane 4-HS1 62.5 µg/ml; Lane 5-LPS control; lane 6- HS2 31.25 µg/ml; lane 7:HS2 62.5 µg/m

Table 5: Relative expression IFN gene in THP-1

Samples	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
	GAPDH	IFN		
Control	16647.02	3544.811	0.21	1.00
LPS control	18291.25	7011.69	0.38	1.80
HS1 31.25 µg/ml	17196.37	7131.74	0.41	1.95
HS1 62.5 µg/ml	17267.51	7281.004	0.42	1.98
HS2 31.25 µg/ml	18399.08	8326.175	0.45	2.13
HS2 62.5 µg/ml	14259.59	8922.518	0.63	2.94

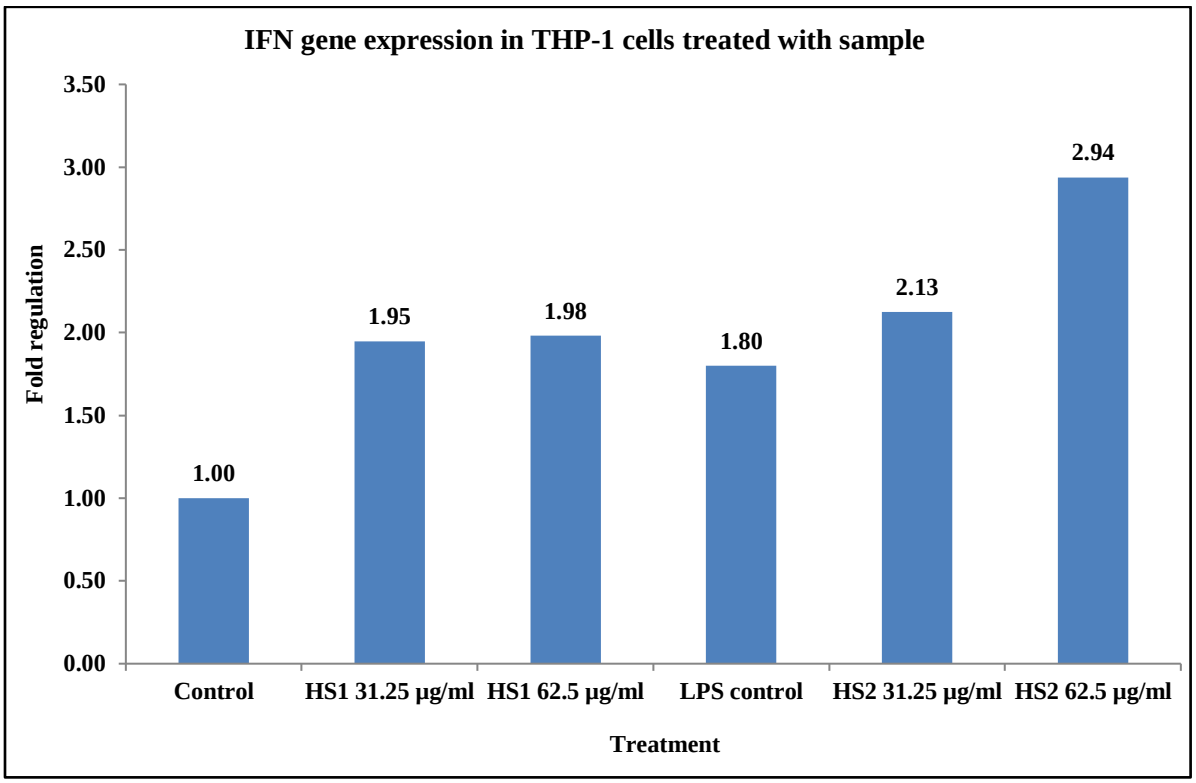

Fig 7: Relative expression IFN gene in THP-1

Discussion

Inflammation in THP-1 cells was induced with LPS (100ng/mL). Compared with no treatment control, 2.37 and 2.97 fold increase in TNF-alpha and IL-10 mRNA levels were observed in LPS treatment. Test samples tested at 31.25 and 62.5µg/mL showed a dose dependent suppression of TNF-alpha mRNA and IL-10 cytokine levels. Sample HS1 showed highest suppression of TNF- alpha m-RNA levels to 1.21 fold compared to 2.37 fold observed with LPS control. Sample HS2 showed relatively higher suppression of IL-10 mRNA levels of 1.89 fold compared to 2.97 fold expression observed in LPS control. Both samples showed dose dependent increase in, IFN-gamma, anti-inflammatory cytokine mRNA levels. Highest expression of IFN-gamma was observed in HS2 at 62.5µg/mL with mRNA expression of 2.94 fold, followed by HS1 with IFN mRNA levels of 1.98 fold compared to control cells. Overall, both samples show anti-inflammatory activity as demonstrated with LPS induced inflammatory THP-1 cell based model.

IFN-Y response is well established as an immune correlate of *M. tuberculosis* infection. According to Sia et al., 2019 Interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF- α) are essential for protection against mycobacterial infections.(17) In this study both test samples HS1 and HS2 showed increase in relative gene expression of IFN-γ, with increase in concentration of test samples. Relative gene expression of IFN-γ is more for test sample HS2 as compared to test sample HS1 **(Table 5)**. IFN-Y possesses unexpected properties as a master regulator of immune responses and inflammation. TNF- α acts in synergy with IFN-γ, stimulating the production of reactive nitrogen intermediates (RNIs), thus mediating the tuberculostatic function of macrophages[18,19]. So we expected that TNF- α will also show similar pattern but in this study both test samples HS1 and HS2 showed decrease in relative gene expression of TNF- α with increase in concentration of test samples. Relative gene expression of TNF- α is more for test sample HS2 as compared to test sample HS1 **(Table 3)**. This decrease in relative gene expression of TNF- α, might be due to production of anti-inflammatory cytokines such as IL-10 by activated THP-1 cells. IL-10 is a potent inhibitor of the production of TNF-α by human monocytes and macrophages as mentioned by Barsig et al., 1995.[20] Both test samples HS1 and HS2 showed decrease in relative gene expression of IL-10 with increase in concentration of test samples. Relative gene expression of IL-10 is more for test sample HS2 as compared to test sample HS1 **(Table 4)**. Thus our study reveals that crude extract of ethno botanical mixture used in indigenous treatment of tuberculosis, its effect on the release of TNF α, IFN Y and IL 10 by LPS-Stimulated THP-1 macrophage cell line. Further studies are

needed to validate these findings and to explore the role of other cytokines and chemokine's.

Conclusion

Both test samples HS1 and HS2 showed dose dependent anti-inflammatory activity as demonstrated with suppression of pro-inflammatory cytokines (TNF-alpha and IFN gamma) and stimulation of anti-inflammatory cytokine (IL-10), LPS induced inflammatory THP-1 cell based model.

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