Anti-Inflammatory and Gene Expression Studies of *Terminalia chebula* Extracts against Interleukins and TNF

Mustafa M. Farhan[1*], Saeed Arrak Turkey[2], Nadhim M.H[3]

Mustafa M. Farhan[1*], Saeed Arrak Turkey[2], Nadhim M.H[3]

[1,2]Al Mamoun University College, Department of Medical Laboratory technology, Baghdad, Iraq

[3]College of Biotechnology, Al-Qasim Green University, Iraq

Correspondence:
Mustafa M. Farhan
Al Mamoun University College, Department of Medical Laboratory technology, Baghdad, Iraq.
E-mail: muneerfarhan01@gmail.com

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ABSTRACT

Aims: Since ages, several plant extracts and Ayurvedic formulations were used to treat ailments and such studies were well documented in the recent decades. Even some of the plants were screened for their efficacy as immunomodulators to restore and rejuvenate the immune system. The present study deals in screening for the possible healing effects of *Terminalia chebula* on IL-2 and IFN-γ levels.

Methods: The raw and dried fruits of the sample were pulverized finely and extracted with methanol. Following which their aqueous solutions are reextracted with hexane, ethyl acetate and chloroform to study the possible cytotoxic effects. Lipopolysaccharide (LPS)-stimulated macrophage cells were used throughout the study to measure the effect of extracts on nitric oxide (NO) production using Griess method. Expression of Cyclooxygenase-2 (COX2) and tumor necrosis factor (TNF)-α were studied by real time PCR quantification along with estimation of IL-1β and IL-6 cytokine levels using the enzyme-linked immunosorbent assay (ELISA).

Results: The chloroform extract showed maximum NO inhibition at about 18.31± 1.6µmol/L. In accordance to the above result, COX2 and TNF-α were found to be downregulated by 12 and 7 fold respectively at 100µg/mL ($P < 0.005$). Chloroform extract significantly reduced IL-1β levels to 21.23 ± 0.21pg/ml (100µg/mL) and also lowered the levels of IL-6 to 45.67± 3.31pg/ml (100µg/ml)

Conclusion: The present study confirmed the positive effect of the chloroform extract in reducing the NO secretion and also by showing an inhibition in the expression of COX2, IL-1β, IL-6, and TNF-α. Thus to conclude *Terminalia chebula* could be used as the best anti-inflammatory candidate drug in addition to the many chemical compounds available in the medical markets.

Keywords: *Terminalia*, Antiinflammation, real time PCR, COX2, IL-6

INTRODUCTION

Immunomodulation is an adaptive terminology of the body to suppress its extra aggressive immune molecules. During the immune response, several immune molecules gets activated and this processes many free radicals like reactive oxygen and reactive nitrogen species are generated [1]. Nitric oxide (NO) which is a by product of reactive nitrogen species produced inducible nitric oxide synthase (iNOS) and superoxide (O_2^-) are released by pyrogen-activated macrophages and activated immune cells respectively increasing the cellular toxicity and oxidative stress within the body [2].

Such an imbalance between the free radical molecules and the anti-oxidative system leads to accumulation of free radicals which eventually leads to inflammation. Such a large number of free radicals inducing oxidative stress leads to structural changes within the biomolecules causing mutagenesis [3]. This stress was also found to aid in developing chronic diseases like pulmonary and cardiovascular diseases including cancer [4]. Thus studies are entirely focussed of how to immunosuppress the immune system and to increase the activity of the anti-oxidative systems to reduce the inflammatory activities.

Many natural compounds which are derived from traditional botanical plants and herbs are found to be rich in anti-oxidative substances which are studied and being studied to scavenge the free radicals. *Tithonia diversifolia* and *T. diversifolia*, members of Asteraceae which grows commonly in many parts of Central and South America and Asian countries [5] was used in healing dermatitis, fever, arthritis and many pathogen infections [6].

Chronic inflammation is considered a major threat which progresses into cancer as the inflammatory molecules generated during this process creates a hostile environment for the tumor and aids in metastasis. In many instances, such an environment is said to be the major predisposing factor for viral mediated tumorigenesis.

On entry of the pathogen into the body, the innate immune system gets activated recruiting granulocytes to the injured site, producing inflammatory mediators like TNF-α, IL-1β and IL-6 and many such lipid mediators like Prostaglandins (PGs) and leukotrienes (LTs). All these mediators invoke an acute inflammatory process within hours to clear off the infection from the damaged tissues [7]. In due course, such an acute inflammation gets resolved when all of the pathogens and damaged tissue debris are cleared off from the damaged site. But sometimes, these acute cases turn into chronic within weeks to months or sometimes years, leading to autoimmune, neurodegenerative and vascular diseases [7].

Interleukin-2 (IL-2) was considered as a cytokine responsible for the proliferation and differentiation of effector T cells and aids in cancer and several other infectious diseases. IL-2 is used in passive therapy on the patients with melanoma or renal cell carcinoma which has been approved by the US Food and Drug Administration

[8]. IL-2 is the first line of defensive molecule which aids in treating autoimmunity and as such considered as potent therapeutic agent [9]. It is primarily produced by the induced T cells and dendritic cells (DCs) [10] but sometimes differential expression of IL-2 receptors also leads to selective IL-2-driven expansion of Tregs which increases the affinity for IL-2 receptor alpha which pairs with the IL-2 receptor beta [11]. Current studies confirmed that under expression of IL-2 will resolve the inflammation by attracting and expanding the immune regulatory cells [12].

COX2 is one of such inflammatory mediators which is seen to be over expressed during an immune response against the pathogens. Cyclooxygenase (COX), also known as prostaglandin (PG) H synthase, catalyses the first committed step in the synthesis of prostanoids, a large family of arachidonic acid metabolites comprising PGs, prostacyclin, and thromboxanes [13]. COX-2 is rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules and has emerged as the isoform primarily responsible for prostanoid production in acute and chronic inflammatory conditions [14]. COX-2 is also a molecule which metabolizes the accumulated PGE_2.

This leads to up-regulation of several signaling pathways and down-regulation of apoptotic proteins which helps in physiological processes like proliferation, angiogenesis and metastasis [15]. When COX-2 is seen to get over expressed, inflammation increases within the body leading to reduction in apoptosis and metastasis which finally develops into cancer. COX-2 was said to create an immunosuppressive tumor environment where the pro-inflammatory eicosanoids, cytokines, chemokines and carcinoma cells all aids in forming an immunosuppressive environment. It also leads to down-regulation of TH_1 cytokines like TNFα, IFNγ, IL-2, IL-12, and also said to upregulate the TH_2 cytokines like IL-4, IL-10 which are proven immunosuppressive agents [16].

Many traditional herbs were screened for their anti inflammatory nature. *Terminalia chebula* (*T. chebula*) was found to exhibit a vast number of medicinal properties owing to their phytoconstituents. Its fruit is said to possess vast number of health benefits and is used since ages for several and human ailments. *T. chebula* was also quoted extensively in Ayurveda, Unani and Homoeopathic literatures [17]. Its aqueous extract was found to contain anti-inflammatory properties inhibiting the inducible nitric oxide synthesis [18]. Even studies done so far, proved that the Chebulagic acid present within this fruits could suppress the onset and progression of arthritis within mice. *T. chebula* was also found to be a potent anti-inflammatory agent and is used in Freund's adjuvant [19].

Thus, this study was designed to study the effect of the fruit extracts as antiinflammtory agents. *T.chebula* was studied for their anti-oxidative stress-related immunomodulation properties. But none of the studies were focussed towards the inflammatory mediators like TNF and IL-6. The study was planned to screen the No inhibition, and expression of the inflammatory mediators like TNF-, IL-6, COX2 by real time amplification and ELISA methods. LPS induced (J774.1A) macrophage cell lines were used in the study.

MATERIALS AND METHODS

Materials

ELISA kits for IL1β and IL6 were purchased from Everone Biosciences. All the reagents used in the study were of molecular grade and were purchased from HiMedia Deionized water was produced in-house using a Milli-Q System from Millipore. All the primers were ordered from Sigma Aldrich. Murine macrophage cell line (J774.1A) was donated from Stellixir Ltd, Bangalore, and was cultured in Dulbecco's modified eagle medium (DMEM) containing 2mM L-glutamine, 100U penicillin/mL, 0.1mg streptomycin/mL, and 10% heat-inactivated FBS (HiMedia) and incubated in a humidified CO_2 incubator. The cells were maintained until confluence and were passaged until further use.

Extract preparation

Raw fruits and dried ripened fruits of *Terminalia chebula* were collected from the nearby farms, Bangalore. The samples each of 500gm were extracted with 1000mL methanol for 3 days using soxhlet extraction (45^0C). The extract obtained was then concentrated in a rotary evaporator and lyophilized. About 5gm of this this extract was suspended in water and then partitioned with n-hexane, ethyl acetate and chloroform extracts. The extracts obtained were then concentrated in a rotary evaporator and the residues obtained were stored at -20 °C until further use. The yields obtained were 16.89, 18.21 and 19.26% (w/w) for n-hexane, ethyl acetate and chloroform extracts respectively. Each extract was then weighed and suspended in 5% sterile dimethyl sulfoxide (DMSO) to give a 20mg/mL solution.

Cell viability assay

The effect of the obtained extracts on the viability of the cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. About 7500-9000 cells per well were incubated with 1-200μg/mL of the extracts for 24hr at 37°C in a humidified CO2 incubator. All the tests were done in triplicates. Wells containing DMSO as the solvent was considered as negative control. 10μL of MTT (5mg/mL) was added to all the wells and the plate was incubated for 4hr at 37°C. Following incubation, the plates were removed and added with lysis buffer (0.1N HCl in isopropanol) to dissolve the formazan produced from the treated cells. Absorbance was measured with a microplate reader (Genetix, Germany) at 570nm. Absorbance of the negative control wells was considered as 100% viability. The Results were expressed as percentages and was calculated using the following equation: [%viability $=(OD_{sample}-OD_{Blank})/(OD_{control}-OD_{Blank})$ *100].

Quantification of nitric oxide production

Nitric oxide production was assayed according to the protocol prescribed by Najmeh Naseri *et al* (2018) [20]. In brief, the cell lines at a concentration of 1×10^6/100μl were seeded into 96-well plates. The seeded cells were then treated with 1μL lipopolysaccharide (LPS, HiMedia; 1μg/ml)

and extracts at varying concentrations of 6.25-200µg/ml for 24hr. All the tests were done in triplicates and the cells treated with LPS and 0.1% DMSO were considered as positive control and negative control respectively. NG-nitro-L-arginine-methyl ester (L-NAME, SIGMA; 1mM/mL) was used as an iNOS inhibitor in the study.

Following 24hr incubation, the supernatants were collected from the cultures and assayed for the production of NO with Griess reagent. The supernatant was incubated with the reagent for 15min at room temperature and the absorbance was read at 550nm in a plate reader (Genetix). The concentration of nitrate and the % of NO inhibition along with IC_{50} was calculated by sodium nitrite standard curve. Positive control was considered 100%.

Cytokine assay

The cytokines like IL1β and TNFα which would be released by the cell lines were measured using the ELISA kit (Everone Biosciences). The protocol was followed according to the manufacturers instructions. The supernatant of cells cultured (control and treatment) was spinned down at 8000rpm, 10min and stored at −20°C until further use. The cells without stimulation with LPS was used as negative control. The day before the assay, the 96well plate was impregnated with the capture antibody against the specific molecules. The samples were added in their respective wells and done in triplicates.

The plate was incubated for about 2hours and following washing with secondary antibody conjugated with HRP. H_2O_2-tetramethylbenzidine was sued as the substrate and after the incubation, the reaction was stopped using 2N H_2SO_4 and the absorbance was read at 405nm in a plate reader (Genetix). The concentration of the study proteins released into the media was estimated using the standard graph.

RNA extraction

Since the chloroform extract showed maximum rate of reduction, it was considered for the expression studies. The cells (treated and control) with confluency were used for the RNA extraction [21] using RNeasy Mini Kit (Qiagen 74104). The test was done according to the manual instructions. Following trypsinization, the cells were spinned down at 6000rpm for 5min and resuspended in about 560µl of AVL buffer and 560µl chilled ethanol. After thorough mixing, the contents were centrifuged at 8000rpm for about 1min and the column was then washed with 700µl of wash buffer and then incubated with 60µl of AVE buffer. The RNA was then eluted and stored at −20°C. The RNA was analyzed qualitatively using UV spectrophotometer (260/280) and used in the cDNA synthesis.

cDNA synthesis

cDNA synthesis was carried according to the manual instructions from RT PCR kit using SuperScript TMII Reverse Transcriptase, 200U/µl (HiMedia). About 1.22µl (concentration obtained was 1.97µg/µl) of the RNA was used in the reaction. Random primers and 1µl of RT enzyme was added and incubated at 25°C for 10min. Following incubation at 70°C for 45 min, the cDNA was then stored and used in the real-time PCR assay.

Real-time PCR

Primers used in the real time assay (Table 1) were designed using primer3 software and were purchased from Sigma-Aldrich. The real-time amplification was carried according to Salam Abbas *et al*. (2019) [21] using the iQTM SYBR Green Supermix (HiMedia). The primers (600nM) and 1µl of the RT products were used and the reaction was carried in a total volume of 12.5µl. All the reactions were done in duplicates along with their respective negative controls.

Table 1: List of the primers used in the real time PCR study. FW: forward primer; RV: reverse primer. Tm: melting temperature.

Gene		Template strand	Length	Tm	GC%	Product length
COX2	FW	GTACTCCCGATTGAAGCCCC	20	60.18	60	138
	RV	TCGTGTAGCGGTGAAAGTGG	20	60.32	55	
TNF-α	FW	GGGTGTGAGAAGAGAGATGGG	21	59.52	57.14	342
	RV	GGCCAGAGGGCTGATTAGAG	20	59.6	60	
GAPDH	FW	GCTGAGTACGTCGTGGAGTC	20	60.18	60	455
	RV	CCCATTCCCCAGCTCTCATA	20	58.56	55	

Expression of COX-2 and TNF- α members

Quantitative PCR was done on the samples (Control and treatment) in the Corbett Research cycler (Bio-Rad). The COX-2 (forward: 5' GTACTCCCGATTGAAGCCCC 3' and reverse: 5' TCGTGTAGCGGTGAAAGTGG 3'; 138bp) and TNF- α (forward: 5' GGGTGTGAGAAGAGAGATGGG 3' and reverse: 5' GGCCAGAGGGCTGATTAGAG 3'; 342bp) of about 600nM was used in the amplification study. 1.22µl of the RNA products were used, and the program was run for about 40 cycles at 93°C for 60s, 62°C for 40s, and with an elongation at 72°C for 60s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5' GCTGAGTACGTCGTGGAGTC 3' and reverse: 5' CCCATTCCCCAGCTCTCATA 3'; 455bp) was also amplified along with the gene members for a comparative analysis of the mRNA expression. The comparative analysis was done using $\Delta\Delta^{Ct}$ method and the Ct values obtained for each sample were normalized to the housekeeping gene (GAPDH).

Statistical analysis

Throughout the study the data was analyzed using a one-way ANOVA and using SPSS software (USA). All the values were expressed as means ± SD of at least three independent experiments and the significance level was maintained at $P < 0.05$.

RESULTS

Cell viability assay

MTT colorimetric assay was done to assay the possible cytotoxic effect of varying concentrations (6.25to 100.00µg/mL) of the extracts on the growth of J774.1A cells. Extracts of n-hexane, ethyl acetate and chloroform showed no significant cytotoxicity on the cell lines even at a concentration of 100µg/mL. None of the extracts showed any reducing effect on the cell lines.

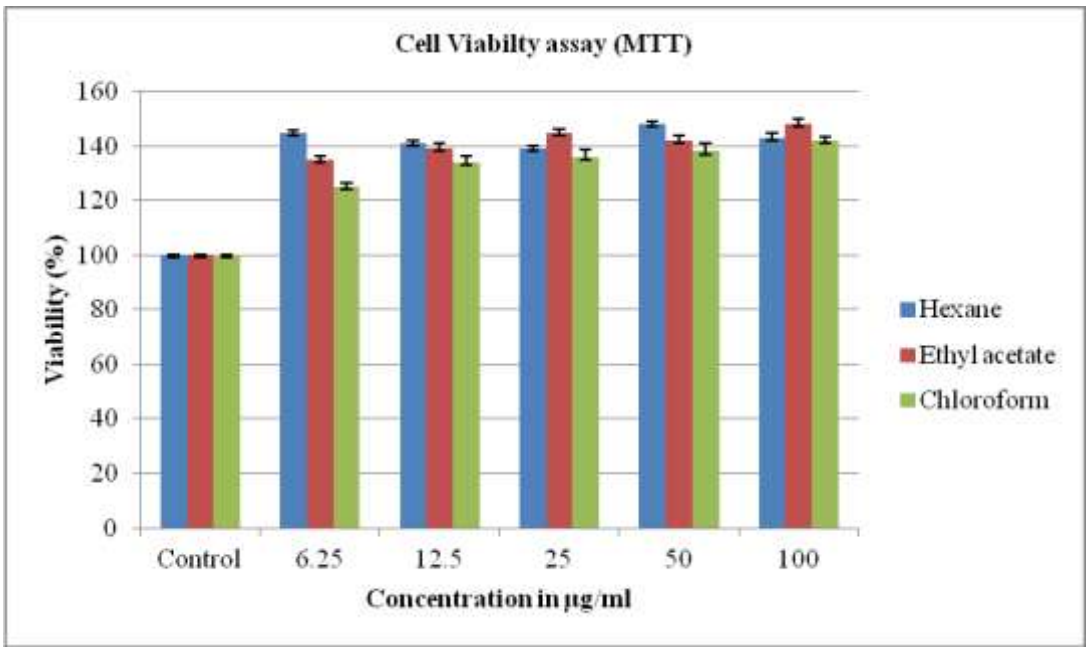
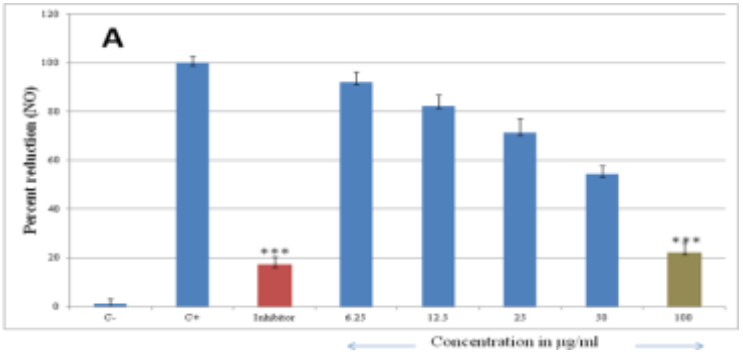


Figure1: Cytotoxicity assay of *T.chebula* extracts on J774.1A cells. Cell viability was determined by MTT method. Negative control was considered 100% viable. The values were expressed as mean ±S.D and were performed in triplicates (p<0.05).

Reduction in NO production

The cell lines were treated with LPS, extracts and inhibitors to study the effect on NO production. Cells added with LPS stimulant showed a significant increase in the NO production to 87.23 ± 2.1µmol/L ($p<0.005$). Negative control without LPS treatment showed 15.67± 1.1µmol/L. The cells treated with inhibitor obviously showed a reduction in the NO production to 16.94± 2.9µmol/L ($P < 0.005$).

Chloroform extract showed significant reduction when compared to ethyl acetate and n-hexane. Chloroform extract showed significant reduction from 89.83± 1.6 µmol/L to 18.31± 1.6µmol/L. Hexane extract showed a reduction from 92.16 ± 3.5% at 6.25µg/ml to 22.3 ± 10.1% at 100µg/ml (Fig. 2B). Ethyl acetate on the other hand also showed reduction but was significantly lower when compared to the other two extracts (32.4 ± 3.9% from 96.11± 2.3% at 100µg/ml, $p<0.005$).



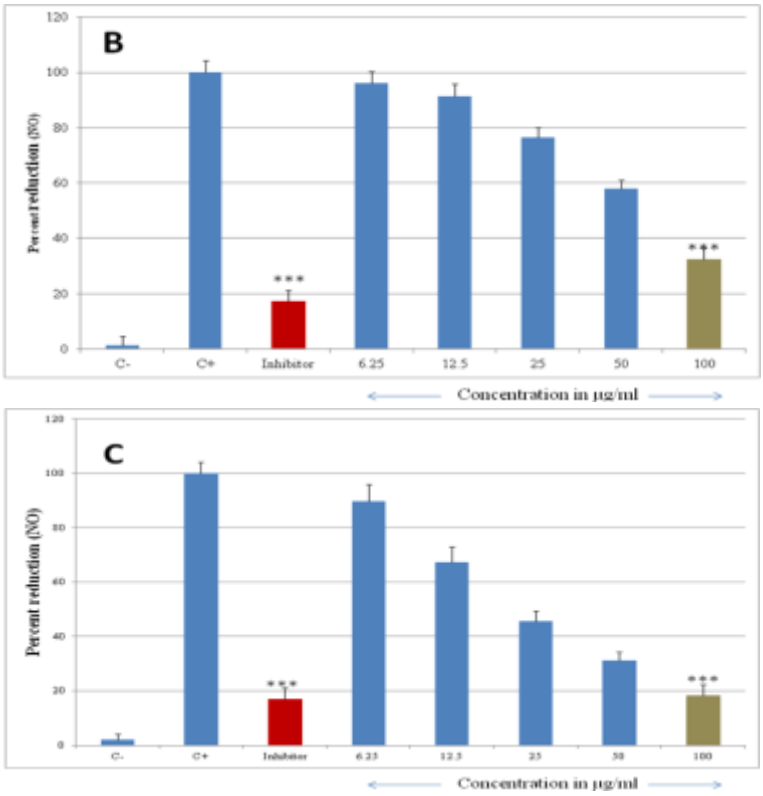


Figure 2: Effect of *T. chebula* extracts on LPS stimulated nitric oxide production from J774.1A cells. C-: Negative control (absence of LPS stimulus); C+: positive control (LPS treatment); inhibitor: L-NAME, NG-nitro-L-arginine-methyl ester; NO, nitric oxide. All the tests were done in triplicates and expressed as percent ± SD. (*$P < 0.05$). A: Hexane treatment; B: Ethylacetate; C: Chloroform.

Cytokine assay

This ELISA method was used in estimating the expression of the IL-1β and IL-6 cytokines on treatment with the hexane and chloroform extracts only (ethyl acetate was excluded from the study). From the figure it was found that LPS stimulation has significantly increased the levels of IL-1β levels (47.84 ± 1.53pg/ml) when compared to the negative control (21.54 ± 2.11pg/ml), and even IL-6 levels were also increased to 894±17.01pg/ml from 11.3 ± 2.54pg/ml (negative control). Chloroform extract showed significantly higher reduction of the IL-1β and IL-6 levels when compared to the hexane extract.

Chloroform extract with varying concentrations (6.25-100µg/ml) significantly reduced IL-1β levels to 44.22 ± 0.31pg/ml (6.25µg/ml), 40.31 ± 1.48pg/ml (12.5µg/ml), 32.9 ± 0.21pg/m (25µg/mL), 28.75 ± 1.48pg/ml (50µg/mL), and 21.23 ± 0.21pg/ml (100µg/mL); $P < 0.005$. Hexane extract also showed reduction in the levels with varying concentrations (6.25-100µg/ml) to 46.12 ± 0.31pg/ml (6.25µg/ml), 42.82 ± 2.48pg/ml (12.5µg/ml), 39.45 ± 1.21pg/ml (25µg/mL), 32.45 ± 1.48pg/ml (50µg/mL), and 30.83 ± 2.21pg/ml (100µg/mL); $P < 0.005$. Similar results were seen in IL-6 secretion also and chloroform extract showed significantly lower levels of 45.67± 3.31pg/ml (100µg/ml) and 789.67± 1.39pg/ml (6.25µg/ml). Hexane showed reduction in IL-6 levels to 234.54± 1.77pg/ml (100µg/ml) and 821.91± 3.12pg/ml (6.25µg/ml).

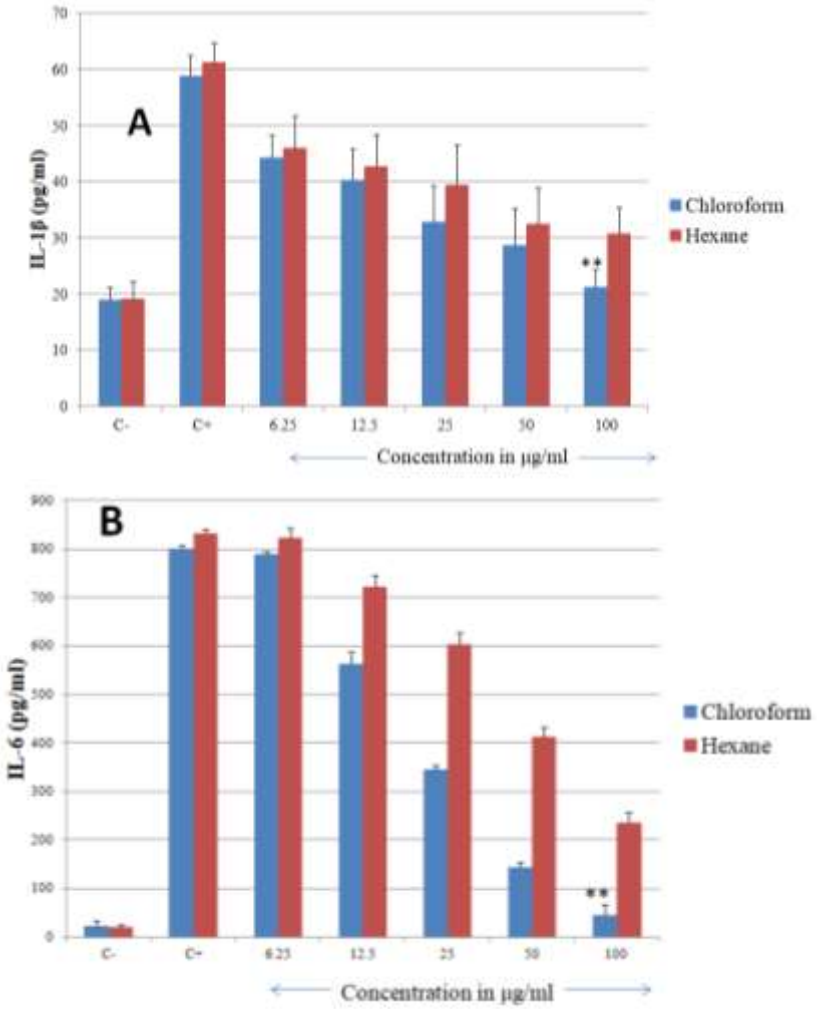


Figure 3: Graph showing the effects of the extracts on LPS stimulated cytokine levels. Cells are treated with 6.25-100µg/mL of the chloroform and hexane extracts on stimulus with LPS for 24hr. A: IL-1β; B: IL-6 cytokine levels. Values are expressed as pg/ml ± SD. All the values were average of triplicates ($P < 0.05$)

Expression of gene members

The results obtained were normalized to GAPDH for comparative expression study. The mRNA expression was studied separately for each gene member (GAPDH, TNF- α and COX-2) was studied separately. The sample with the lowest $\Delta\Delta^{Ct}$ value was used as calibrator, and the remaining members were compared in relation to calibrator.

The Ct values of the GAPDH, TNF- α and COX-2 were found to be 11, 21, and 22 respectively for the control. And the Ct values for the GAPDH, TNF- α and COX-2 after treatment was found to be 11, 30 and 33 respectively.

From the Ct values, (Figure 4) after normalizing with the GAPDH, it was observed that the relative expression of both the gene members were downregulated in treatment samples when compared to the control. The GAPDH gene expression was considered as 100%.

From the graph, (Figure 5) and the calculated $2^{-\Delta\Delta Ct}$ values, it was found that the relative expression of the COX-2 and TNF- α was donwregulated by 12 and 7 times respectively when compared to their respective controls.

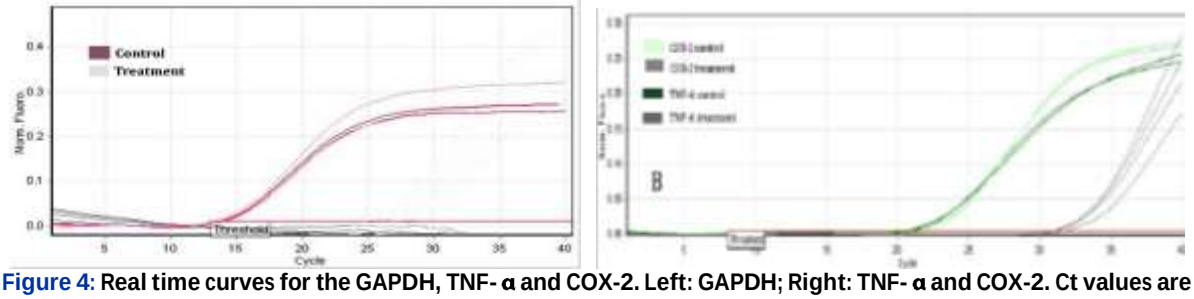


Figure 4: Real time curves for the GAPDH, TNF- α and COX-2. Left: GAPDH; Right: TNF- α and COX-2. Ct values are average of duplicates.

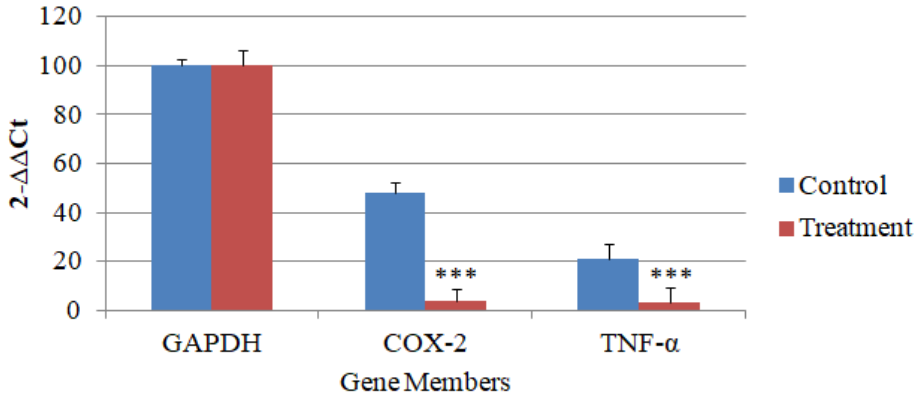


Figure 5: Graph showing the expression levels of GAPDH, TNF- α and COX-2 on treatment with the chloroform extracts. Negative control was without stimulus and not shown in the graph. GAPDH was used as calibrator. Its expression was considered as 100%. (*P*< 0.005).

DISCUSSION

Macrophages are considered very critical in case of inflammation due to their characteristic antigen presenting nature. Moreover, they are good at phagocytosis and other immunomodulation properties. The main role of these macrophages is to enhance the production of inflammatory molecules and mediators like cytokines [22]. During this crucial time, host tries to suppress the activity of these cytokines to minimise the damage caused to the tissues. Many previous studies reported that several medicinal plants are employed to heal the inflammatory process [23]. *E.amoenum* is studied for its anti-inflammatory and analgesic properties and as such commonly used as folk medicine in Iran to cure the common cold [24]. In the present study the effects of *T.chebula* as anti-inflammatory on stimulation with LPS was studied.

The extracts were first studied for their healing effects on J774.1A macrophage cell lines viability to nullify the cytotoxic effects, followed by estimating the inhibition of NO production. NO is said to be crucial mediator in regulating the inflammation process [25] as such its effects were studied. In the current study, the percent of cytotoxicity as shown by MTT assay determines the safety of using the sample material. None of the extracts (hexane, ethyl acetate and chloroform) showed any cytotoxicity on the cell lines. The cytotoxicity was significantly less even at the highest concentration of 100µg/mL.

In this study, *T. chebula* extract showed significant inhibition on the NO production in a dose dependent manner. Most of the studies reported so far suggests that the extracts which are significantly good at lowering the No production are good anti-inflammatory agents [26]. From the results, it was proved that chloroform extract (18.31± 1.6µmol/L) was significantly good at reducing maximum NO produced followed by hexane (22.3 ± 10.1µmol/L) at highest concentration of 100µg/ml. The inhibition activity was quite promising when compared to the inhibitor reduction activity (16.94± 2.9µmol/L). This reduction might be either due to the inhibition of iNOS activity or at the level of locking the transcription signalling methods. As such the study was planned in the method specified by Booke *et al* [27].

The study so far concluded the possible evaluation on the inhibition of NO by the xtracts. Additionally there are several studies reporting the same NO reducing activities. *Eucalyptus globules* and *Thymus vulgaris* [22] were studied for their capability in reducing the NO production among the LPS induced J774.1A cells. Even studies done on *Andrographis paniculata* [28] and *Echinacea* [25] also confirmed the possible inhibitory role of the extracts in NO production and iNOS activity among the LPS-stimulated macrophages. *Mentha longifolia* also was concluded that it is a good anti-inflammatory agent on regulating the production of NO among the LPS-stimulated J774.1A cells [23].

COX2 is also said to play a crucial role in inflammation [29] which converts arachidonic acid to prostaglandins. Moreover, NSAIDs are used to suppress the inflammatory process by inhibiting the COX enzyme [30]. Studies done on the hexane extract of *E. amoenum* proved that they are potent anti-inflammatory by inhibiting the COX2 gene expression among the J774.1A macrophage cell lines.

This study confirmed that the extracts strongly showed a reduction in the COX2 and TNF-α gene expressions. TNF-α and IL-1β were said to cytokines mostly involved in inflammation, where TNF-α produced by the macrophages takes part in initiating the acute phase of inflammation. This mode of action is done by attracting the neutrophils to the target site.

IL-1β also aids in bringing an inflammatory response on infection with a pathogen. Many chemical moieties were used as inhibitors to reduce the effect of IL-1β so as to relieve the inflammatory arthritis among the experimental organisms [31]. IL-6 is also said to be proinflammatory in nature and takes part in the generation of inflammation [32]. Our study confirmed that, chloroform extract showed a significantly higher downregulation of these two gene members (COX2 and TNF-α).

The real time expression studies done on the cell lines on induction with LPS confirmed the possible role of the extracts (chloroform) in downregulating the expression of

inflammatory chemokines. COX2 and TNF-α were downregulated by 12 and 7 times when compared to the control. Even studies done on several medicinal plants *Mentha longifolia* [23] and *Echinacea* [25] showed that they could reduce the expression of TNF-α, IL-6, and IL-1β proteins at the mRNA level among the LPS induced macrophages. As major reduction effects were observed with chloroform extracts on the cell lines, further studies need to be planned to isolate and purify the phytochemcials from the extract and study the same at pin point level.

CONCLUSION

The plant material *T.chebula* and its extracts were studied for the possible effects on the macrophages as anti-inflammatory agents. Among them chloroform extract showed significant reduction of NO production followed by the hexane extract. Chloroform extract was found to be more effective in reducing the levels of IL- and Il-6 gene members. Real time PCR analysis also confirmed that the chloroform extract was significantly down regulating the expression of COX2 and TNF- levels when compared to control.

The findings in the current study reveal that the plant material can act as strong anti-inflammatory agent as confirmed on the LPS induced macrophages. Further if the study could isolate and purify the extract into its constituent photochemical, then the mode of action could be traced to the pin point level. Hence the study confirms the possible potent role of *T.chebula* as strong anti-inflammatory agent.

CONFLICT OF INTEREST

None

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