**J Young Pharm, 2018; 10(4) : 481-483**
A multifaceted peer reviewed journal in the field of Pharmacy
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*Original Article*

# Inhibition of Nitric Oxide Production and Nitric Oxide Synthase Gene Expression in LPS Activated RAW 264 .7 Macrophages by Thyme Oleoresin from *Thymus vulgaris*

**Chidambaram Shunmugam Kumar Mangal[1], Roy Anitha[2*], Thangavelu Lakshmi[2]**
[1]Graduate Student, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, INDIA.
[2]Faculty of Pharmacology, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, INDIA.

## ABSTRACT

**Objective:** To evaluate the effect of Thyme oleoresin on nitric oxide production and nitric oxide synthase gene expression in RAW264.7 macrophage cell lines. **Methods:** The efficacy of Thyme oleoresin at different concentrations (3.175–150µg/ml) was determined on nitric oxide production in RAW264.7 cells macrophages. The optical density was measured at 540 nm with a microplate reader. RT - PCR was used to examine the expression of the iNOS gene in activated macrophages. The Statistical analysis of the data was carried out by Dunnett's following one way ANOVA and Posthoc comparisons in Graph pad Prism 5.0 software version. **Results:** Thyme oleoresin at its tested concentrations exhibited dose – dependent decrease in the production of NO. The $IC_{50}$ value was 24.24 µg/ml. LPS stimulated RAW macrophages strongly up regulated the iNOS gene expression levels. Thyme oleoresin at three different concentration, 12.5µg/ml, 25µg/ml and 50µg/ml, significantly suppressed the iNOS levels compared to that of LPS treatment only. **Conclusion:** Thyme oleoresin extract may be used for its anti-inflammatory effect as it could significantly suppress the iNOS gene expression as well as the production of NO.

**Key words:** Anti inflammatory, Macrophage cell line, Nitric oxide, Thyme oleoresin.

**Correspondence**

**Dr. Anitha Roy,** Associate Professor, Department of Pharmacology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, INDIA.

Phone: (Off) +91-44-26801580-87, + 91-9840787458

Email: anitharoy2015@gmail.com

**DOI:** 10.5530/jyp.2018.10.104

## INTRODUCTION

Nature has provided a complete store-house of remedies to cure all aliments of mankind.[1] Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects.[2-4] Thyme (*Thymus vulgaris,* family *Labiatae* ) is a medicinal plant which has been used as a folk medicine against asthma, arteriosclerosis, colic, bronchitis, coughs, diarrhea, rheumatism and cancer. Thyme has been used to promote perspiration.[5]

Nitric oxide is considered as a pro-inflammatory mediator that induces inflammation due to its over production in abnormal situations. In mammalian cells NO is synthesized during the conversion of L-arginine to L-citrulline which is catalyzed by the isoform of nitric oxide synthase (NOS).[6] The inducible NOS [iNOS]) is expressed by a variety of factors such as inflammatory cytokines, TNF-α and lipopolysaccharide which binds to the heme group of soluble guanylyl cyclase to generate cGMP. Activated cGMP then binds specifically to transcription factors, protein kinases and phosphodiesterases to elicit downstream effects. NO can also act by directly modifying proteins or contributing to the oxidation of proteins and lipids leading to normal as well as pathophysiologic functions.[7-10] NO inhibitors represent important therapeutic advance in the management of inflammatory diseases because NO is involved in the pathogenesis of inflammatory disorders of the joint, gut and lungs.[6] The aim of the present study was to evaluate the effect of thyme oleoresins on nitric oxide production and nitric oxide synthase gene expression in macrophage cell lines.

## MATERIALS AND METHODS

### Chemicals

Lipopolysaccharide (LPS), Phenol free Dulbecco's modified Eagle medium (DMEM), MTT, Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), and antibiotic-antimycotic solution (100U penicillin, 100µg streptomycin, and 0.25µg amphotericin B per ml) were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from GIBCO/BRL Invitrogen.

### Plant extract

Thyme oleoresin was obtained from Synthite Industries Limited, Kerala as gratis.

### Cell culture

Macrophage RAW 264.7 cells were obtained from the NCCS, Pune with Passage no 16. Cells were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37°C with 5% $CO_2$. Cells were washed with DMEM medium and detached with 0.25% trypsin-EDTA. The cells were seeded at a density of 5 x $10^5$cells/well in 24 well plate and incubated for 18h at 37°C and 5% $CO_2$. Then media of each well were aspirated and fresh FBS-free DMEM media were replaced. Different concentrations of Thyme extract (3.175–150µg/mL) were prepared in FBS-free DMEM to give a total volume of

500µl in each well of a microtiter plate. The cells were co - incubated with 1µg/ml of LPS for 24h.

### Estimation Nitric oxide (NO)

The presence of nitrite, a stable oxidized product of nitric oxide (NO), was determined in cell culture media using Griess reagent. 50 µl of supernatant from the test culture was mixed with 50µl of 1% (w/v) sulphanilic acid in 5% (v/v) phosphoric acid in a 96-well plate, followed by incubation for 10 min at room temperature. After that 50 µl 0.1% (w/v) N-1-naphthyl ethylenediamine HCl in distilled water was added and incubated for 10 min at room temperature. The optical density at 540 nm was measured with a microplate reader. The NO concentration was calculated by comparison with a $NaNO_2$ (0–100 µM) standard curve. The final concentration of DMSO was adjusted to less than 0.1% for all treatments. The results were expressed as inhibition of NO production compared to the control (LPS) using: $([nitrite]_c - [nitrite_t)/[nitrite]_c$, where $[nitrite]_c$ and $[nitrite]_t$ are the nitrite concentration in the control and test sample, respectively.[11,12]

### RNA Isolation and q - PCR Analysis

To determine whether thyme extract inhibits NO production at the level of transcription, RT - PCR was used to examine the expression of the iNOS gene in activated macrophages. RAW macrophages were treated with 12.5µg/ml, 25µg/ml and 50µg/ml of Thyme extract with 1µg/ml of LPS and incubated for 24h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and 2µg of RNA was used for complementary DNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time polymerase chain reaction (q-PCR) was performed in an ABI 7500 Real-Time System with SYBR Green PCR Master Mix (Takara). Reactions were initiated with an initial incubation at 50°C for 2 min and 94°C for 10 min, followed by 40 cycles of 94°C for 5s, 60°C for 15s, and 72°C for 10 s. The relative gene expression levels were calculated using the 2−ΔΔCt method. The specific primer sequences used were given below and β-actin was used as an internal reference gene between different samples.

INOS: Forward:5′-ATGTCCGAAGCAAACATCAC-3′

Reverse: 5′-TAATGTCCAGGAAGTAGGTG-3′

### Statistical analysis

Data obtained from the experiments were expressed as Mean ± SEM (n=3). The Statistical analysis of the difference between the groups was evaluated by Dunnett's following one way ANOVA Posthoc comparisons in Graph pad Prism 5.0 software version. $p<0.001$, $p<0.01$ and $p<0.05$ were considered to be statistically significant.

## RESULTS

### Effect of Thyme oleoresin on NO production

Nitrite production was dependent on the activating state of the cells. LPS unstimulated macrophages (Control) for 24h produced lowest levels of NO, whereas LPS stimulated group showed 89.61±0.47 % of NO. The effect of thyme extract on nitric oxide production in RAW macrophages was dose dependant. Thyme oleoresin at its tested concentrations exhibited dose – dependent decrease in the production of NO. At 3.175 µg/ml of thyme oleoresin showed 86.13±0.47 of 86.13±0.47 % where as at 150µg/ml it produced only 3.84±0.82% of nitric oxide. The $IC_{50}$ value was found to be 24.24 µg/ml (Figure 1).

### Gene Expression of iNOS

LPS stimulated RAW macrophages strongly up-regulated the iNOS gene expression levels. In the presence of thyme extract at three different doses of 12.5µg/ml, 25µg/ml and 50µg/ml, the iNOS level was significantly suppressed, compared to that of LPS treatment only (Figure 2).

## DISCUSSION

The present study results indicate that thyme oleoresin has good NO inhibitory activity. With increase in the concentration of the extract, the nitrous oxide level continues to decrease by inhibiting the effect of iNOS. Hence, thyme oleoresin may be used as an anti-inflammatory agent as it can inhibit one of the major pro-inflammatory constituent in certain disease conditions.

NO is known for its physiological role including immune defense against microorganisms but excess production of NO is associated with various diseases such arthritis, diabetes, stroke, septic shock, autoimmune, chronic inflammatory diseases and atherosclerosis. In the pathogenesis of several neurodegenerative diseases, excessive NO production has been identified as one of the major causative reasons.[13] Inducible nitric

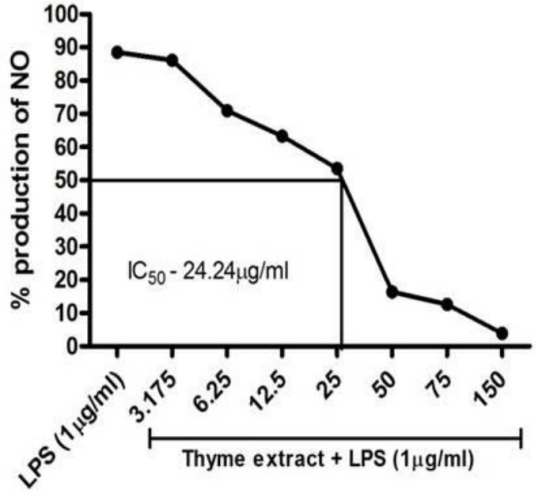


**Figure 1:** Graphical representation of effect of Thyme extract on production of NO production in LPS stimulated RAW 264.7 macrophages.

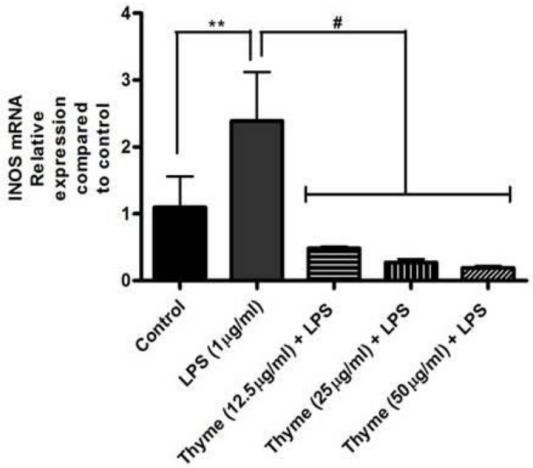


**Figure 2:** Effect of Thyme extracton LPS – stimulated iNOS expression in RAW 264.7 macrophages.

oxide synthase (iNOS) is responsible for the large production of nitric oxide which in turn is responsible for the vasodilation and hypotension observed during septic shock and inflammation. Hence, inhibitors of iNOS may be useful candidates for the treatment of inflammatory diseases accompanied by the overproduction of NO.[14]

Inflammation is actually a defense and protection to multiple harmful stimuli, however; when it is self-amplified and uncontrolled it leads to pathogenesis of a wide variety of inflammatory illness. It involves pro-inflammatory cytokines such as nitrous oxide and it acts as an important biological response toward injury. Several studies have demonstrated the properties of various compounds from plants which tend to possess rich pharmacological properties that play beneficial roles in many conditions including inflammation-related diseases.[15,16] NO has the property to modify or generate intercellular signals and thus it has an effect on immune cells, tumour cells and the cells of different tissues or organs. In the present study, the effect of thyme on NO production on LPS stimulated Raw 264.7 macrophage cell lines was carried out and has shown a dose dependent inhibitory effect on nitric oxide production which is indicative of its anti-inflammatory effect. Hence, this extract might be used in conditions where, excessive NO production may be a reason and can be treated in a natural way.

Natural plant compounds which are able to suppress the production of inflammatory mediators from activated macrophages can act as potential anti-inflammatory agents. Many researchers have explored different plants for their anti-inflammatory potential. *Solanum nigrum* Linn is reported to have anti-inflammatory activity on both acute and sub-acute stages of inflammation.[17] *Solanum melongena* and *Solanum macrocarpon* extracts have shown inhibitory effect on NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.[18] Phytoconstituents present in plants such as phenols, triterpenes, tannins, anthraquinones, and flavonoids may be responsible for these anti-inflammatory properties.[19] Similarly, *P.alliacea* could reduce inflammation in RAW264 macrophages. It suppressed the oxidative stress and the induction of various pro-inflammatory mediators in RAW264.7 macrophages through NF-B inactivation.[20]

## CONCLUSION

Thyme oleoresin showed a significant decrease in production of pro-inflammatory mediator NO and suppression iNOS gene expression even when enhanced with LPS. Hence, thyme may be used in optimum concentration in particular dosage form for inflammatory conditions after further validation.

## ACKNOWLEDGEMENT

The authors thank Synthite industries private Limited for providing Thyme oleoresin as a gift sample for carrying out this project. Also express gratitude to Ms Parameswari, Senior Research Fellow, Sri Ramachandra Medical College and Research Institute, Chennai for interpreting the results.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**q-PCR**: Quantitative real-time polymerase chain reaction; **iNOS:** inducible Nitric Oxide Synthase; **LPS:** Lipopolysaccharide; **DMEM:** Dulbecco's modified Eagle medium; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DMSO:** Dimethyl sulphoxide; **PBS:** Phosphate Buffer Saline; **FBS:** Fetal Bovine Serum.

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**Article History:** Submission Date : 18-04-2018 ; Revised Date : 02-07-2018; Acceptance Date : 27-07-2018.
**Cite this article:** Mangal CSK, Anitha R, Lakshmi T. Inhibition of Nitric oxide Production and Nitric oxide Synthase Gene Expression in LPS Activated RAW 264 .7 Macrophages by Thyme oleoresin from *Thymus vulgaris*. J Young Pharm. 2018;10(4):481-3.