Effect of lutein from marigold on nitric oxide production and nitric oxide synthase enzyme – An in vitro study

Dominic Pereira Westeous, Roy Anitha*, Thangavelu Lakshmi

Faculty of Pharmacology, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Tamil Nadu, India

ABSTRACT

Plants are well known for their versatile pharmacological activity because of the presence of various phytochemicals. Marigold plant is a well-known plant with many traditional uses including anti-inflammatory activity. Plants can produce an anti-inflammatory effect by inhibiting excessive nitric oxide production. The present study aimed to evaluate the effect of lutein extract on nitric oxide production and nitric oxide synthase gene expression in LPS stimulated RAW 264.7 macrophage cell line. Macrophage RAW 264.7 cells were obtained from the NCCS, Pune with Passage no 16. The presence of nitrite, a stable oxidised product of nitric oxide (NO), was determined in cell culture media using Griess reagent. RT - PCR was used to examine the expression of the iNOS gene in activated macrophages. LPS stimulated RAW macrophages strongly upregulated the iNOS gene expression levels. In the presence of lutein at three different doses of 17.5µg/ml, 35µg/ml and 70µg/ml, the iNOS levels were significantly suppressed, compared to that of LPS treatment only. The different concentrations used in the study have significantly suppressed the iNOS levels, compared to that of LPS treatment alone. 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 statistically significant. This study has concluded that lutein has a significant inhibitory effect on iNOS gene expression and nitric oxide production. Hence, it may be used in inflammatory conditions with excessive nitric oxide production.

* Corresponding Author

Name: Dr. Anitha Roy
Phone: +91-9840787458
Email: anitharoy2015@gmail.com

ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v9i4.1642

INTRODUCTION

In health and diseases, the inflammatory response plays a significant role (Ran and Montgomery, 2012). Macrophages have a significant impact on immune response and inflammation. The cells inducing inflammation, not only initiate and maintain specific immune responses but also secrete various types of cytokines (Heo et al., 2012). Activation of macrophages releases numerous inflammatory mediators including nitric oxide (NO) (Rossol et al., 2011). Overproduction of these inflammatory mediators is associated with many disorders. Hence, many inflammatory diseases can be managed by inhibiting the production of such mediators. (Ayroldi et al., 2011).

Marigold, *Tegetts erecta* (family *Compositae*) is a well-known plant with many medicinal properties. Marigold flower is a rich source of lutein which is a yellow plant pigment that belongs to the carotenoid family having good antioxidant
property (Sivel et al., 2014). Many studies have carried out for the nitric oxide inhibitory effect on LPS-stimulated RAW264 and have potential as therapeutic agents for inflammatory-related diseases (Rebecca Fang Ling et al., 2015). This study was carried out to examine the effect of lutein from marigold, on nitric oxide production and nitric oxide synthase gene expression in LPS stimulated RAW 264.7 macrophage cell line.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS), Phenol-free Dulbecco’s modified Eagle medium (DMEM), MTT, Dimethyl sulfoxide (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. Lutein extract from marigold flowers was provided by Synthite Industries Pvt Ltd, Kerala as gratis.

Cell culture

The efficacy of Lutein on nitric oxide production in RAW macrophages was determined. 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 100units/ml penicillin, 100µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum at 37°C with 5% CO2. 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 plates and incubated for 18h at 37°C and 5% CO2. Then media of each well were aspirated, and fresh FBS-free DMEM media were replaced. Different concentrations of lutein extract (5–320µg/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 of Nitric oxide (NO)

The presence of nitrite, a stable oxidised product of nitric oxide (NO), was determined in cell culture media using Griess reagent (Green et al., 1982). 50µl of supernatant from the test culture was mixed with 50 µl 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-naphthylethylenediamineHCl 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 NaNO2 (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. Values are expressed Mean ± SEM (n = 3).

RNA Isolation and q-PCR Analysis

RAW macrophages were treated with 17.5µg/ml,35µg/ml and 70µg/ml of lutein 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 two minutes 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. β-actin was used as an internal reference gene between different samples.

INOS: Forward: 5’-ATGTCCGAGAACATCATC-3’
Reverse: 5’-TAATGTCCAGGAAGTGGTG-3’

Statistical analysis

Data obtained from the experiments were expressed as Mean ± SEM. 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.

RESULT

Effect of Lutein 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 89.6± 0.47% of NO. Lutein at its tested concentrations exhibited a dose-dependent decrease in the production of NO. At 10µg/ml produced 79.14± 0.45% inhibition and 70 µg/ml showed 3.52 ± 0.49 % inhibition. The IC 50 Value was 36.55 µg/ml (Table 1).
inflammatory processes follow diseases such as atherosclerosis, Alzheimer's disease, cancer, asthma, periodontitis and infections such as tuberculosis with high production of chemical mediators, (Gaestel et al., 2009, Parwani et al., 2012). It is evidenced that nitric oxide is critically involved in obesity and thereby in conditions such as cardiovascular disease, hypertension and diabetes. (Dhorte et al., 2016). Inducible nitric oxide synthase is responsible for the large production of NO (Liew et al., 2011).

Plants are used in traditional and folk medicine from time immemorial for different health conditions such as diabetes (Ashwini and Anitha, 2017) cancer (Ashwini et al., 2012), anxiety (Sarah et al., 2012) and lipid-lowering property (Anitha et al., 2012). Many plants are known for their anti-inflammatory activity such as Crataeva Magna lour (Meera et al., 2017), Achillea millefolium L (Chou et al., 2013). Alkaloids, flavonoid, triterpenoid, phenols, saponins and tannins may be responsible for the anti-inflammatory activity. (Meera et al.; 2017; Coutinho et al., 2009). The anti-inflammatory activity of the methanolic extract of Schinus terebinthifolius was proved by its ability to inhibit nitric oxide production by LPS-stimulated macrophages (Bernardes et al., 2014). Lutein is a carotenoid obtained from marigold flowers. Carotenoids are known for their antioxidant and anti-inflammatory activity. (Rosanna et al., 2017).

The antioxidant property of lutein may be responsible for its nitric oxide inhibitory effect.

**CONCLUSION**

This study has concluded that lutein has a significant inhibitory effect on iNOS gene expression and nitric oxide production. Hence, it may be used in inflammatory conditions with excessive nitric oxide production such as atherosclerosis, Alzheimer's disease and cancer.

**Acknowledgement**

The authors thank Synthite Industries Ltd, Kerala for providing the lutein extract as a gift sample and also express their gratitude to Ms Parameshwarari P.R Senior Research fellow, SRMC, Chennai for interpreting the results.

**Conflict of interest:** Nil

**REFERENCES**


