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ORIGINAL ARTICLE

The Effect of Capsicum Oleoresin on Nitric Oxide Production and Nitric Oxide Synthase Gene Expression in Macrophage Cell Line

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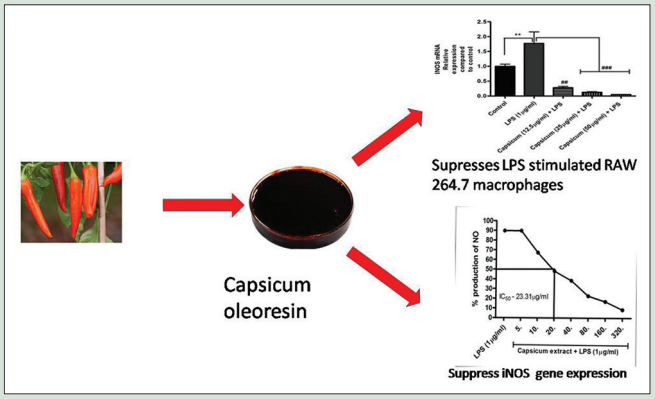
ABSTRACT

Background: Nitric oxide (NO) is an inflammatory agent produced by macrophages. It also acts as a neurotransmitter. However, overproduction of NO results in acute or chronic inflammation. Capsicum is well known for its anti-oxidant, anti–inflammatory, and anticancer properties. **Objective:** The objective of this study was to evaluate the effect of capsicum oleoresin on NO production and NO synthase gene expression in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophage cell line. **Materials and Methods:** Macrophage RAW 264.7 cells were obtained from the National Centre for Cell Science, Pune with Passage no 16. RAW macrophages were treated with 12.5 μg/ml, 25 μg/ml, and 50 μg/ml of Capsicum extract with 1 μg/ml of LPS and incubated for 24 h. **Results:** When capsicum was added at three different doses of 12.5 μg/ml, 25 μg/ml, and 50 μg/ml, the inducible NO synthase (iNOS) levels was significantly suppressed, compared to that of LPS treatment only. The level of NO increased by LPS induction was significantly decreased in a dose-dependent manner when treated with different concentrations of capsicum extract and capsicum had a suppressing effect on iNOS gene expression in LPS – stimulated RAW 264.7 macrophage. **Conclusion:** This study concludes that capsicum oleoresin is good enough to suppress iNOS gene expression and NO production. Hence, it may be used in inflammatory conditions with excessive NO production.

Key words: Anti-inflammatory, anti-oxidant, capsicum oleoresin, macrophages, nitric oxide

SUMMARY

- Capsicum oleoresin used was isolated from *Capsicum annuum* L.
- It showed dose-dependent inhibitory effect on nitric oxide production
- Different concentration of capsicum oleoresin suppressed lipopolysaccharide-induced inducible nitric oxide synthase gene expression in lipopolysaccharide-stimulated RAW 264.7 macrophages
- The ability of capsicum oleoresin extract in inhibiting nitric oxide production and suppressing inducible nitric oxide synthase gene expression may be used in future for the management of acute and chronic inflammatory conditions associated with high level of nitric oxide



Abbreviations Used: NO: Nitric oxide, LPS: Lipopolysaccharide, iNOS: Inducible nitric oxide synthase, DMEM: Dulbecco's modified Eagle medium, MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide for, DMSO: Dimethyl sulfoxide, PBS: Phosphate buffer saline, FBS: Fetal bovine serum.

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INTRODUCTION

Capsicum annuum Linn. (Family *Solanaceae*) is a medicinal herb. It has analgesic, antiangiogenic, antiparasitic, antiplatelet, anti-arthritic, antioxidant, antiviral, antifungal, antineoplastic, hypoglycemic, gastroprotective, and larvicidal effects.[1,2] Oleoresin from *Capsicum annunm* L is reported to have hypocholesterolemic and antidiabetic activity.[3] Oleoresins are formed of resins and essential oils with characteristic flavor and aroma of the spice from which it is obtained.[4] Capsicum oleoresin contains capsaicin. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is one of the most common capsaicinoids.[5] Capsaicinoids are responsible for antioxidant activity.[6] The presence of phenolic ring in them may be responsible for their antioxidant property.[7] Capsaicin is a therapeutic agent used to treat fevers, nausea, vomiting, malaria, and different pain conditions.[8] Topically, it is used for the management of pain in arthritis, postoperative neuralgia, diabetic neuropathy, and psoriasis.[9]

Oxidative stress due to increase of reactive oxygen and nitrogen species can initiate tissue damages by damaging carbohydrates, proteins, lipids, and DNA, ultimately causing cell death.[10] Nitric oxide (NO) and inducible NO synthase (iNOS) can cause production of reactive nitrogen species.[11] Hence, the oxidative stress, inflammation and associated disease can be better managed by inhibiting iNOs.[12] Macrophage activation and NO production are necessary for proper defense mechanism. The key enzyme for the production of NO is iNOS. However, hyperactivation of macrophages can end up in pathophysiological condition by the release of cytokines and other mediators.

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Many plants such as *Atractylodis rhizoma alba*, *Etlingera pavieana*, *Zingiber cassumunar, Achillea millefolium L.* and *Dioscorea villosa*.[13-17] were explored for their inhibitory effect on NO production and inducible nitric oxidase synthase so that they could be effectively used for controlling the oxidative stress caused by the production of reactive nitrogen species through NO and for the management of cancer, atherosclerosis, diabetes, vascular disease, Alzheimer's, Parkinson's, and periodontal diseases. Researchers could prove that by inhibiting NO and guanylate cyclase, herbal agents such as (−)-epigallocat-echin-3-gallate, inhibited migration of mammary cancer cell, and proanthocyanidins from grape seed inhibited migration of non-small cell lung cancer cell.[18] Since, inhibitors of NO production can have a diverse and versatile use, in this study, the effect of capsicum oleoresin on NO production and inducible nitric oxidase synthase was explored.

MATERIALS AND METHODS

Chemicals and extract

Lipopolysaccharide (LPS), Phenol-free Dulbecco's modified Eagle medium (DMEM), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide for, dimethyl sulfoxide (DMSO), phosphate buffer saline, and antibiotic-antimycotic solution (100U penicillin, 100 µg streptomycin, and 0.25 µg amphotericin B per ml) were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from GIBCO/BRL Invitrogen. The Capsicum oleoresin extract was provided by Synthite Industries Ltd., Kerala, as gratis.

Cell culture

Macrophage RAW 264.7 cells were obtained from the National Centre for Cell Science, Pune with Passage no 16. Cells were cultured in phenol red-free DMEM supplemented with 100units/ml penicillin, 100 µg/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-ethylenediaminetetraacetic acid. The cells were seeded at a density of 5×10^5 cells/well in 24 well plate and incubated for 18 h at 37°C and 5% CO_2. Then, media of each well were aspirated and fresh fetal bovine serum (FBS)-free DMEM media were replaced. Different concentrations of Capsicum extract (5–320 µg/mL) were prepared in FBS-free DMEM to give a total volume of 500 µl in each well of a microtiter plate. The cells were co-incubated with 1 µg/ml of LPS for 24 h.

Estimation of nitric oxide

The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media using Griess reagent.[19] Briefly, after treatment protocol, 50 µl of supernatant from the test culture was mixed with 50 µl of 1% (w/v) sulfanilic 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 micro plate reader. The NO concentration was calculated by comparison with a NaNO2 (0–100 µM) standard curve. The final concentration of DMSO was adjusted to <0.1% for all treatments. The results were expressed as inhibition of NO production compared to the control LPS using: $([\text{nitrite}]_c\text{-}[\text{nitrite}]_t)/[\text{nitrite}]_c$, where $[\text{nitrite}]_c$ and $[\text{nitrite}]_t$ is the nitrite concentration in the control and test sample, respectively.

RNA isolation and quantitative real-time polymerase chain reaction analysis

RAW macrophages were treated with 12.5 µg/ml, 25 µg/ml, and 50 µg/ml of Capsicum extract with 1 µg/ml of LPS and incubated for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and 2 µg of RNA was used for complementary DNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time polymerase chain reaction 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 by40 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′-ATGTCCGAAGCAAACATCAC-3′

Reverse: 5′-TAATGTCCAGGAAGTAGGTG-3′

Statistical analysis

Data obtained from the experiments were expressed as mean ± standard error of mean. The Statistical analysis of the difference between the groups was evaluated by Dunnett's following one-way ANOVA *post hoc* comparisons in Graph Pad Prism 5.0 software version (San Diego, USA). $P < 0.001$, $P < 0.01$, and $P < 0.05$ were statistically significant.

RESULTS

Effect of capsicum extract on nitric oxide production

NO is a pluripotent signaling molecule produced by different isoforms of enzyme, nitric enzyme synthase (NOS). Though NO is having many beneficial effects, overproduction of NO can lead to various inflammatory diseases. LPS significantly increased NO production in RAW macrophages. The level of NO increased by LPS induction was significantly decreased in a dose-dependent manner when treated with different concentrations of Capsicum extract [Table 1 and Figure 1].

Gene expression of inducible nitric oxide synthase

LPS stimulation of RAW macrophages strongly up regulated the iNOS gene expression levels. However, when Capsicum was added at three different doses of 12.5 µg/ml, 25 µg/ml, and 50 µg/ml, the iNOS levels was significantly suppressed, compared to that of LPS treatment only [Figure 2].

DISCUSSION

Many studies have proven that capsaicin has anti-inflammatory activity in different models.[20-22] The NO inhibition at various concentrations of capsicum oleoresin was compared in this study. As shown in Table 1, it is inferred that NO production was less, when higher concentrations of capsicum oleoresin extract was added to LPS stimulated RAW

Table 1: Effect of Capsicum extract on no production in LPS stimulated RAW 264.7 macrophages

Concentration (µg/ml)	No production in LPS stimulated RAW 264.7 macrophages (%)
LPS (1µg/ml)	89.61±0.47
5	89.41±0.59
10	66.75±0.63
20	47.72±0.52
40	37.97±0.35
80	22.26±0.42
160	16.55±0.55
320	8.27±0.68

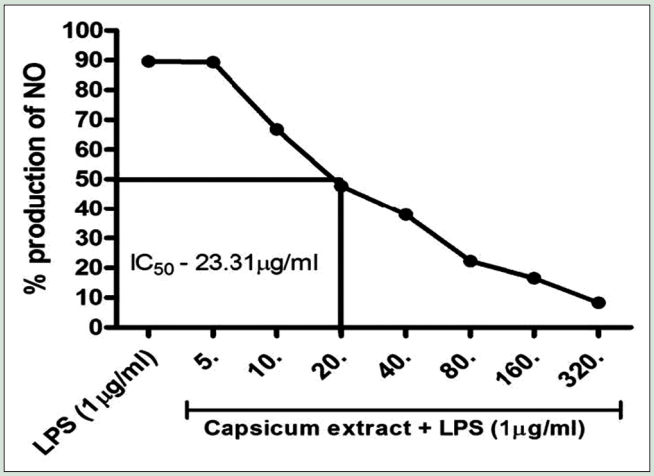

Figure 1: Graphical representation of effect of capsicum extract on production of nitric oxide production in lipopolysaccharide stimulated RAW264.7 macrophages

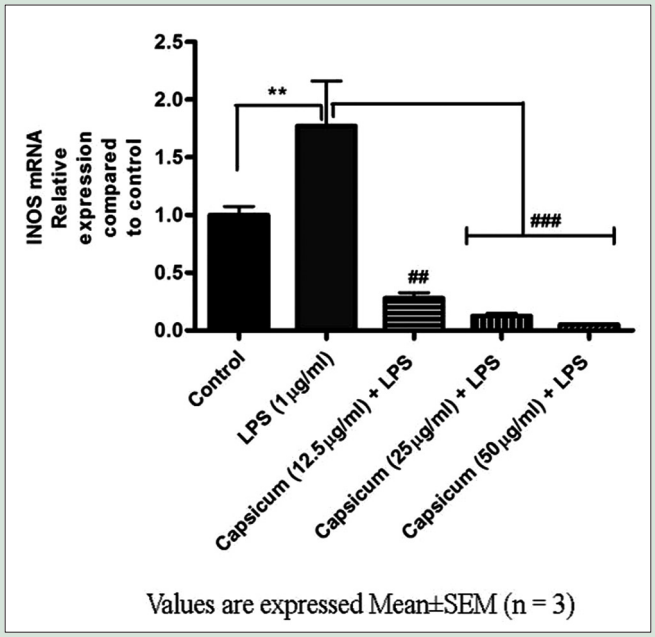

Figure 2: Effect of Capsicum extract on lipopolysaccharide-stimulated inducible nitric oxide synthase expression in RAW264.7 macrophages

264.7 macrophage cell line. Results revealed that capsaicin inhibits LPS-induced NO production.

The inflammatory response is a process involving complex interactions among inflammatory molecules that leads to tissue to respond to traumatic, infectious, postischemic, toxic, or autoimmune injury. Many plants have shown good NO inhibitory effect. In a study done by Makchuchit *et al.*, ethanolic extracts *Mesua ferrea* showed potent inhibitory activity, with IC_{50} value of 26.23 µg/ml.[23] The ethanolic extract of *Atractylodeslancea* exhibited the most potent NO production inhibitory activity (IC_{50} = 9.70 µg/ml) which is higher than that of indomethacin (IC_{50} = 20.32 µg/ml). This result is comparable to the study by Wang *et al.*[24] In study by Huang *et al.*, water and ethanolic extracts of root of *Angelica sinensis* exhibited inhibitory effect on NO production in LPS activated RAW 264.7 macrophage in the concentration range of 20–200 µg/ml.[25]

This study can be further expanded for its *in vivo* evaluation so that this extract may be used as a cure for inflammatory conditions. Many plant extracts such as *Prunus cerasus, Eugenia jambolana*, and *Punica granatum* were studied for their healing effects on diabetes.[26] Some plant extracts were used for aromatherapy which has a soothing effect on patients with anxiety.[27] Hence, there is an upward trend in exploring plants for their versatile potential in healthcare.

CONCLUSION

This study proves the ability of capsicum oleoresin extract in inhibiting NO production and suppressing iNOS gene expression in LPS – stimulated RAW 264.7 macrophage. Hence, this extract may be used in future for the management of acute and chronic inflammatory diseases such as ischaemia, rheumatic arthritis where NO production is responsible for the inflammation.

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Conflicts of interest

There are no conflicts of interest.

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