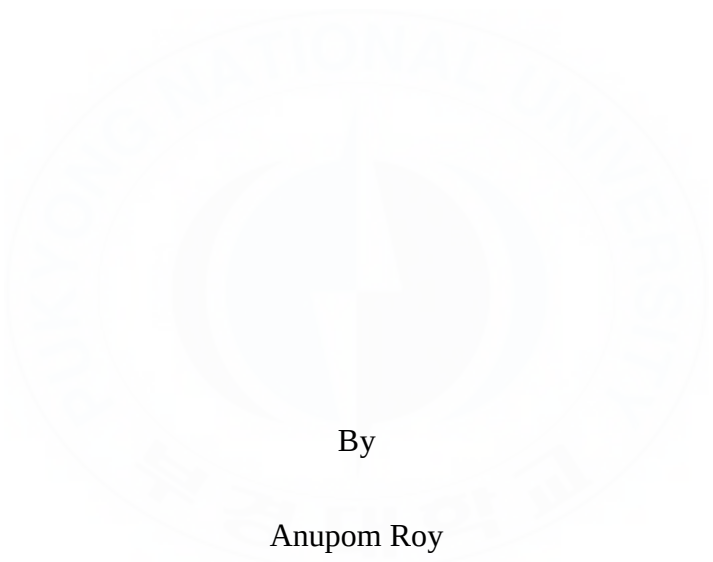
Thesis for the degree of Master of Science

The anti-inflammatory effects of eugenol, methyl eugenol, estragole, and pulegone through the regulation of NF-κB and Nrf2 signaling pathways in LPS-stimulated RAW 264.7 cells.



By

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Department of Food and Life Science

The Graduate School

Pukyong National University

August 2016

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LPS 유도 RAW 264.7 세포에서 eugenol, methyl eugenol, estragole, pulegone 의 NF-κB 와 Nrf2 신호 경로를 통한 항염증 효과

Advisor: Prof. Jae Sue Choi

By

Anupom Roy

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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A dissertation

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Abstract

Eugenol, methyl eugenol, estragole, and pulegone are naturally occurring organic compounds obtained from essential oils of a variety of plants. The aim of this study was to investigate the anti-inflammatory effects through the inhibitory mechanism of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK) pathways and the activation of nuclear factor erythroid 2-related factor 2 (Nrf2)/ heme oxygenase (HO)-1 pathways in Lipopolysaccharide (LPS) stimulated RAW 264.7 cells. The production of nitric oxide (NO) was detected by Griess reaction and ELISA. The expression of

iNOS and COX-2 was detected by Western blot. The expression of NF-κB and MAPK was also detected by Western blotting analysis. Furthermore, the intracellular ROS level was measured using fluorescent probe DCFH-DA. Then the expression of HO-1 and Nrf-2 was detected by western blotting analysis. Additionally, molecular docking studies were performed to evaluate binding affinities and binding sites of eugenol, methyl eugenol, estragole, and pulegone towards iNOS. Results revealed that those four compounds significantly inhibited NO production as well as iNOS and COX-2 expression. Meanwhile, western blot analysis showed that pulegone, estragole, methyl eugenol, and eugenol inhibited LPS-induced NF-κB and MAPK activation in RAW 264.7 cells. Furthermore, all those compounds suppressed LPS-induced intracellular ROS production in RAW 264.7 cells, while the expression of stress response gene HO-1 was up-regulated via the activation of transcription factor Nrf-2. In addition, molecular docking results showed that pulegone exhibits higher binding affinity in iNOS inhibitory complex compared to others tested compounds. Collectively, these findings provide that pulegone, estragole, methyl eugenol, and eugenol inhibit the LPS-induced expression of inflammatory mediators via the down-regulation of iNOS, COX-2, NF-κB, and MAPKs pathways as well as up-regulation of Nrf-2/HO-1 pathway indicating that all four compounds have a potential therapeutic and preventive application in various inflammatory diseases

1. Introduction

Inflammation is a complex physiological and pathological processes in response to infection and tissue injury. Inflammation is the result of various factors such as microbial infections, chemicals, and immunological reactions. It is a protective response that involves immune cells, blood vessels, and molecular mediators. The purpose of inflammation is to enclose injury, destroy invading microorganisms and inactivate toxins, and to restore the tissue or organ for recovery (Morson, 1970, Cline, 1970). Although, inflammation is a beneficial host-response upon tissue injury, but upon long persistence, it may result in chronic conditions such as cancer, cardiovascular disease, diabetes, pulmonary disorders, neurological disease, and arthritis (Choudhari et al., 2013). Inflammation can be acute or chronic. The initial response of the body to harmful stimuli is called acute inflammation and prolonged inflammation is known as chronic inflammation. The two main integral components of the host's defense system for inflammatory response are innate immunity and an adaptive immune response. The first line of defense against noxious material is provided by innate immunity and as well as after recognition of an appropriate stimulus, it provides the necessary signals to instruct the adaptive immune system to mount a response.

In turn, the adaptive response deals with the initiating stimulus as phagocytes and granulocytes after the innate immune system response (Lawrence et al., 2002).

The treatment of inflammatory diseases focuses on the suppression of few inflammatory mediators or signaling pathways including NO, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), prostaglandin E_2 (PGE_2), nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK), reactive oxygen species (ROS), and pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) (Joung et al., 2015). During inflammation, macrophages play essential roles. It produces inflammatory cytokines when activated by endotoxin. Nitric oxide (NO) is a vital cellular signaling molecule involved in many physiological and pathological processes. NO is produced as a metabolic by-product when L-arginine is converted into L-citrulline by the interfere of nitric oxide synthase (NOS). There are three isoforms of NOS including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Laroux et al., 2001). During the inflammatory disease condition, iNOS produces an excessive amount of NO. Eventually, NO and iNOS are the important targets for the treatment of inflammatory diseases.

Prostaglandin (PG) H synthase is known as cyclooxygenase (COX) which converts arachidonic acid to prostaglandins. COX plays a pivotal role in inflammatory responses as a mediator (Fu et al., 1990). Unlike isozymes COX-1 and COX-3, COX-2 is inducible in certain cells in response to inflammatory stimuli such as cytokines, endotoxin, mitogens, tumor promoters and growth factors (Fu et al., 1990, Chandrasekharan et al., 2002). It is well reported that COX-2 acts as a pathogen in chronic inflammatory diseases, and selective COX-2 inhibitors have been favorably reported in various experiments and clinical treatments (Suh et al., 1998, Patrignani et al., 2003).

Nuclear factor kappa B (NF-κB) is a transcription factor that plays a fundamental role in the inflammatory and acute response (Giuliani et al., 2001). Normally, NF-κB subunits are inactive and bound with IκB. Phosphorylation of IκB activates NF-κB to enter the nucleus and activate gene expression (May and Ghosh, 1998). NF-κB is one of the key regulators of proinflammatory gene expression which mediates the synthesis of cytokines such as TNF-α, IL-1β, IL-6 and IL-8 (Tak and Firestein, 2001). It also regulates the transcription of other inflammatory mediators such as COX-2 or iNOS (Tak and Firestein, 2001).

Therefore, NF-κB becomes a vital target for the treatment of inflammatory diseases.

Mitogen-activated protein kinases (MAPKs) are the chain of protein exist in the cell which communicates a signal from a cellular surface receptor to DNA in the nucleus of the cell. MAPKs are also known as protein Ser/Thr kinases that transform extracellular stimuli into a wide range of cellular response. This class includes the extracellular signal-regulated kinases 1/2 (ERK 1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK 1/2/3), and p38 isoforms (α, β, γ, and δ) (Carqnello and Roux, 2011). In contrast to JNK and p38, the ERK cascade shows relatively little information in inflammation where bacterial endotoxin (LPS) activates ERK in macrophages (Karin, 2001). In turn, JNK and p38 are more actively induced in response to proinflammatory stimuli by the induction inflammatory cytokines (Karin, 2001). So it is evident that the inhibition of MAPKs is the critical target for novel anti-inflammatory drugs.

Classically, ROS are defined as partially reduced metabolites of oxygen that have strong oxidizing capabilities. At high concentration, ROS are deleterious but at low concentration, they serve complex signaling functions. ROS produces injurious effect due to oxidizing protein and lipid cellular

constituents and damage DNA. The chronic or prolonged production of ROS contributes central role to the progression of inflammatory disease (Mittal et al., 2014).

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the degradation of heme and produces biliverdin, ferrous iron, and carbon monoxide. The degradation of heme and production of biliverdin, ferrous iron, and carbon monoxide contribute to the anti-inflammatory effects of HO-1 (Pae and Chung, 2009). Previous studies showed that HO-1 inhibits the excessive production of pro-inflammatory cytokines as well as reactive oxygen species (ROS) in LPS-stimulated RAW 264.7 cells (Chen et al., 2013). At the transcription level, HO-1 induction is regulated by a transcription factor called nuclear transcription factor-E2-related factor 2 (Nrf-2). Nrf-2 contributes to the anti-inflammatory process through the regulation of gene expression of HO-1 and eventually becomes the drug target for the treatment of inflammatory diseases (Taha and Blaise, 2014).

Essential oils are volatile, concentrated hydrophobic, natural, complex compound extracted from aromatic plants. They are characterized by strong odor. Essential oils were first developed in Middle Ages by Arabs and known for their antiseptic, i.e. bactericidal, fungicidal, and virucidal, and medicinal properties and

their smell (Bakkali et al., 2008). Essential oils were used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and anesthetic (Bakkali et al., 2008). Eugenol is a phenylpropene compound that is found in certain essential oils especially olive oil, nutmeg, cinnamon, lemon balm, and basil (Pavithra, 2014). Methyleugenol is an another natural constituent of the essential oils that found in a number of plants (Bakkali et al., 2008). Estragole is also a phenylpropene and a colorless natural primary constituent of essential oil of tarragon (Bakkali et al., 2008). On the other hand, pulegone is a monoterpene and a naturally occurring organic compound obtained from the essential oils of a variety of mint species (Bakkali et al., 2008).

The aim of this study is to focus on the comparative anti-inflammatory activities of eugenol, methyl eugenol, estragole and pulegone through the inhibition of iNOS, COX-2, NF-κB, and MAPKs expression and the induction of Nrf2/HO-1 expression in LPS-stimulated RAW 264.7 cells. In addition, molecular docking simulation is also performed to identify potential binding sites and the binding difference between selected compounds towards iNOS.

2. Materials and methods:

2.1. Chemicals and reagents:

LPS from *Escherichia coli,* Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2′ 7′-dichlorodihydrofluorescein diacetate (DCF-DA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), phenylmethylsulfonyl fluoride (PMSF), fetal bovine serum (FBS) and antibiotics were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and Dulbecco's modified Eagle's medium (DMEM) from Hyclone (Logan, UT, USA). Various primary antibodies (iNOS, COX-2, NF-κB (p65), p-ERK, ERK, p-JNK, JNK, p-p38, p38, HO-1, Nrf-2 and β-actin) and secondary antibodies were obtained from Cell signaling Technology Inc. (Beverly, MA, USA) and Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and diluted 1:1000. Polyvinylchloride fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billerica, MA, USA). Super-signal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other chemicals and solvents were purchased from Sigma-Aldrich Co., unless stated otherwise.

2.2. Preparation of compounds

For this *in vitro* study, eugenol, methyl eugenol, estragole, and pulegone were purchased from Sigma-Aldrich Co. (St. Louis, UT, USA).

2.3. Cell culture

Marine RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC Rockville, MD, USA). RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, and 1% penicillin-streptomycin and 0.1% amphotericin B. The cells were incubated in humidified atmosphere with 5% CO_2 at 37^{o}C.

2.4. Cell viability

Cell viability was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl tetrazolium bromide (MTT) assay. Briefly, RAW 264.7 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated at 37^{o}C for 24h. The cells were then treated with various sample concentration. After incubation for an additional 24h at 37^{o}C, 100 µl MTT (0.5 mg/ml in PBS) was added to each well and the incubation continued for another 2 hours. The

resulting color was assayed at 540 nm using a microplate spectrophotometer (Molecular Devices).

2.5. NO production

The nitrite concentration in the medium was measured using Griess reagent as an indicator of NO production. Briefly, RAW 264.7 cells (2×10^4 cells/well in a 24-well plate with 500 µl culture medium) were pretreated with samples various concentration for 2 hours and incubated for 18 hours with LPS (1 µg/ml). After incubation, the nitrate concentration of the supernatants (100 µl/well) was measured by adding 100 µl Griess reagent. The absorbance values of mixtures were determined using a micro-plate spectrophotometer (Molecular devices) at 540 nm. The iNOS inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) was used as a positive control.

2.6. Measurement of intracellular ROS

The intracellular ROS scavenging activity of eugenol, methyl eugenol, estragole, and pulegone was measured using fluorescent probe DCFH-DA. Cells plated in a black 96-well plate at a density of 1×10^4 cells/well were co-treated with various concentration of above four compounds and LPS (1 µg/ml) for 2 h.

Cells were treated with 20 µM DCFH-DA for 30 min at 37ºC. The fluorescence intensity was measured at excitation wavelength of 485 nm and an emission wavelength of 528 nm using of fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Co., Sunnyvale, CA, USA).

2.7. Western blot analysis

Western blotting was used to measure the protein expression of iNOS, COX-2, MAPKs, HO-1, and Nrf-2. Firstly, RAW 264.7 cells (5×10^4 cells/ml) were cultured in 100-mm culture dishes in the presence or absence of LPS (1.0 µg/ml) and with or without test samples for 18 h. Afterward, the cells were washed twice with ice-cold PBS and lysed with cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% Tween 20, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO^4, 10 µg/ml leupeptin, 50 mM NaF, and 1 mM PMSF, pH 7.5) on ice for 30 min. Cell extracts were obtained at 14, 000 × g at 4ºC for 20 min. Protein amount was determined by Protein Bradford assay. Cytosolic proteins were electrophoretically separated on SDS-PAGE and transferred to PVDF membranes. Membranes were immediately blocked with nonfat dry milk (50 g/l) in Tris-buffered saline containing 0.1% Tween-20 (Ph 7.4) (TBST) buffer at room temperature for 1 h. The membranes were then

washed three times (10min) in TBST buffer and incubate with primary antibody, diluted 1:1000 in nonfat dry milk (50 g/l) in TBST buffer, 4^{o}C overnight. After three times of washings in TBST buffer (10 min), the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:2000 in nonfat dry milk (50 g/l) in TBST buffer at room temperature for 2 h. After three times washings in TBST buffer, (10 min), the antibody labeling was visualized with the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and was exposed to X-ray film (GE Healthcare Ltd., Amersham, United Kingdom). Pre-stained blue markers were used for molecular weight determination. Bands were quantified by densitometry analysis using ATTO CS analyzer.

2.8. Molecular docking simulation

Molecular docking studies were performed with eugenol, methyl eugenol, estragole, and pulegone to identify the binding affinity and binding sites against human inducible nitric oxide synthase (iNOS). The crystal structure of human inducible nitric oxide synthase (PDB ID: 4nos) was obtained from RCSB Protein Data Bank with a resolution of 2.25 Å. For docking simulation, the reported iNOS inhibitor ITU (ethyl isothiourea) was removed and water molecules were

also removed from the protein structure using Accelrys Discovery Studio 4.1 (DS 4.1) (DS, http://www.accelrys.com; Acclrys, Inc. San Diego, CA, USA). All hydrogen atoms were added to the protein using automated docking tool Autodock 4.2.6 (Goodsell et al., 1996). During docking simulation, ITU binding areas of the protein were considered as the most likely region for the appropriate binding area to inhibit the enzyme. The 3D sdf structure of eugenol, methyl eugenol, estragole, and pulegone were obtained from PubChem compound (CID: 3314, 7127, 8815, and 442495, respectively) and converted into pdb and geometry optimized using DS 4.1. Automated docking simulation was performed using Autodock tools (ADT) to assess the appropriate binding orientations and conformations of the ligand molecule with protein inhibitor. A Lamarckian genetic algorithm method implemented in the program Autodock 4.2 was employed. For docking calculations, gasteiger charges were added by default and the rotatable bonds were set by the Autodock tools and all torsions were allowed to rotate. The grid maps were generated by Autogrid program where grid box size of 126 × 126 × 126 points with a default spacing of 0.375 Å between the grid points was executed covered almost the entire favorable protein binding site. The X, Y, Z centers were -2.046, 94.75, and 16.803, respectively. The docking

protocol for rigid and flexible ligand docking consisted of 10 independent Genetic Algorithm (GA) and other parameters are used by defaults of ADTs. Binding aspect of iNOS residues and their corresponding binding affinity score regarded as best molecular interaction. The results were visualized and analyzed using UCSF Chimera (http://www.cgl.ucsf.edu/chimera/) and Ligplot+.

2.9. Statistical analysis

Data were expressed as the means ± standard deviations (SDs) of at least three independent experiments unless otherwise indicated. Data were compared using one-way ANOVA. *P* values < 0.05, 0.01, and 0.001 were considered statistically significant. All analyzes were performed using SPSS for windows, version 23 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of eugenol, methyl eugenol, estragole, and pulegone on RAW 264.7 cell viability

To determine the effects of eugenol, methyl eugenol, estragole, and pulegone were tested in the MTT cell viability assay using RAW 264.7 cells. Cell viability was tested to fixed the appropriate concentration ranges of selected compounds. As shown in Fig.1, eugenol, methyl eugenol, estragole, and pulegone did not show cytotoxicity to RAW 264.7 cells at the dose of 3.12, 6.25, 12.5, and 25 μg/ml for eugenol, 12.5, 25, 50, and 100 μg/ml for methyl eugenol and estragole and 6.25, 12.5, 25, and 50 μg/ml for pulegone. These non-toxic concentrations were used for the experiments in the entire experimental model.

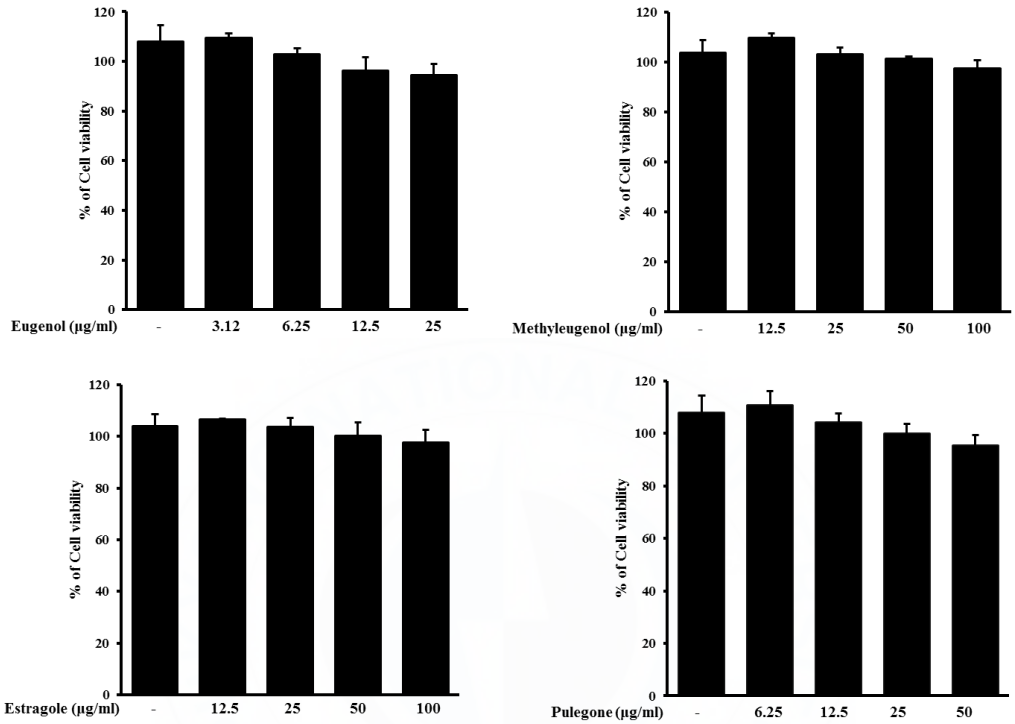
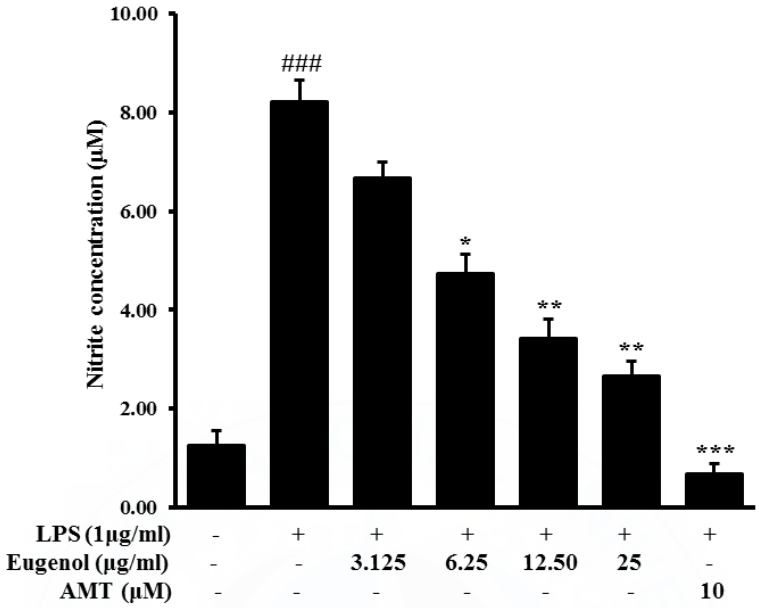


Fig. 1. Cell viability of eugenol, Methyleugenol, estragole, and pulegone. Cell viability was measured by MTT assay. Values represent the means ± SD of three independent experiments.

3.2. Effects on NO production

Nitrite concentration was determined in the culture media using Griess reagent to evaluate anti-inflammatory activities of eugenol, methyl eugenol, estragole, and pulegone on NO production in LPS-stimulated RAW 264.7 cells. Treatment of RAW 264.7 cells with selected compounds significantly suppressed LPS-induced NO production (Fig. 2). The results clearly indicated that eugenol, methyl eugenol, estragole, and pulegone dos-dependently inhibited LPS-induced NO production in RAW 264.7 cells. AMT, a positive iNOS inhibitor, significantly inhibited LPS-induced NO production (Fig. 2).


10.00
8.00
6.00
4.00
2.00
0.00
Nitrite concentration (µM)
###
*
**
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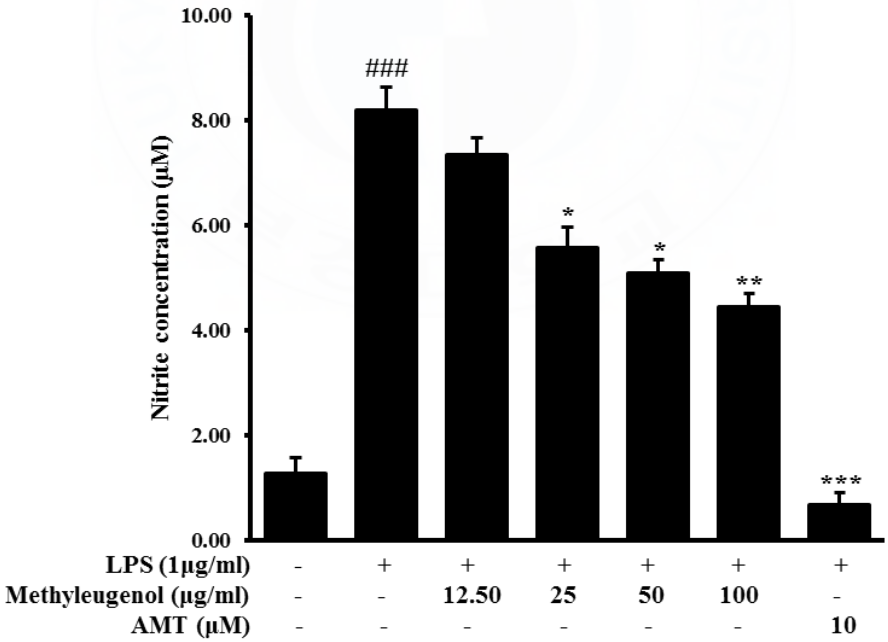
***
LPS (1µg/ml) - + + + + + +
Eugenol (µg/ml) - - 3.125 6.25 12.50 25 -
AMT (µM) - - - - - - 10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8.00
6.00
4.00
2.00
0.00
Nitrite concentration (µM)
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*
*
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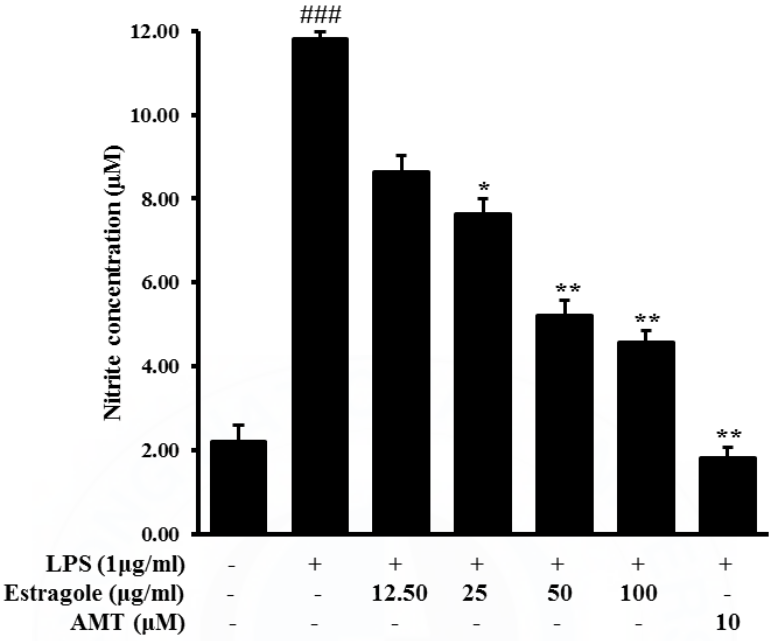
***
LPS (1µg/ml) - + + + + + +
Methyleugenol (µg/ml) - - 12.50 25 50 100 -
AMT (µM) - - - - - - 10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Nitrite concentration (µM)
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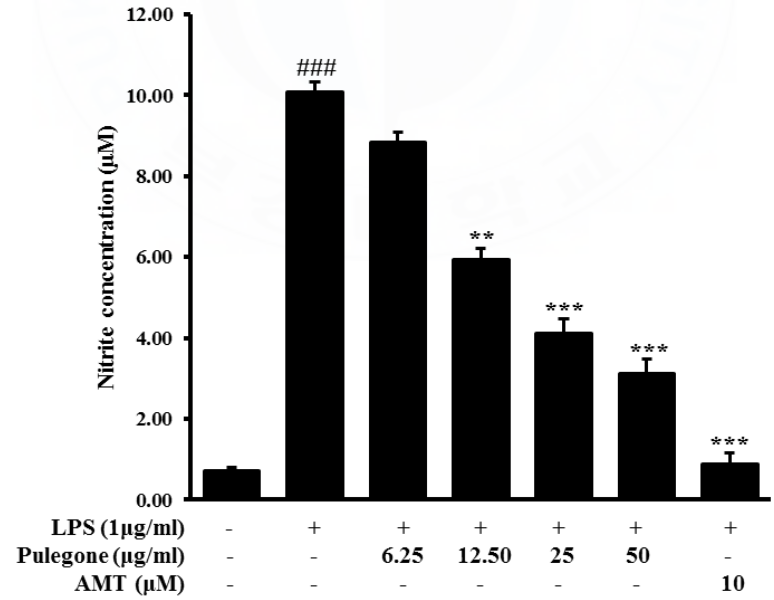
LPS (1µg/ml) - + + + + + +
Estragole (µg/ml) - - 12.50 25 50 100 -
AMT (µM) - - - - - - 10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12.00
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Nitrite concentration (µM)
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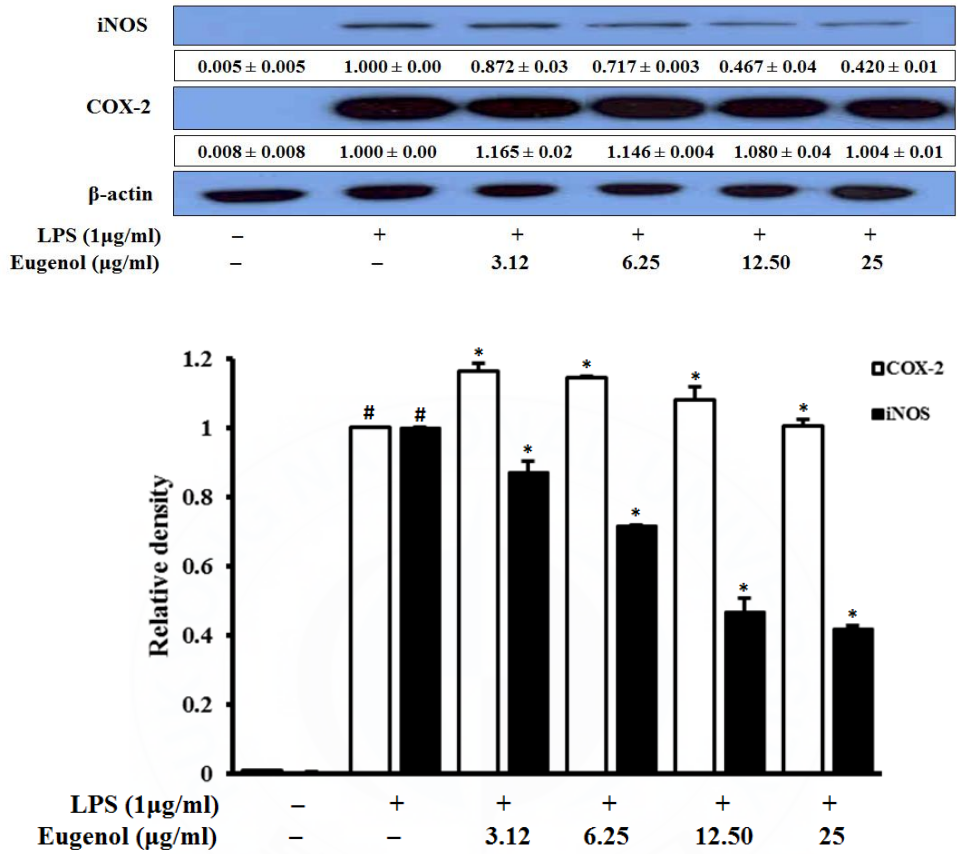
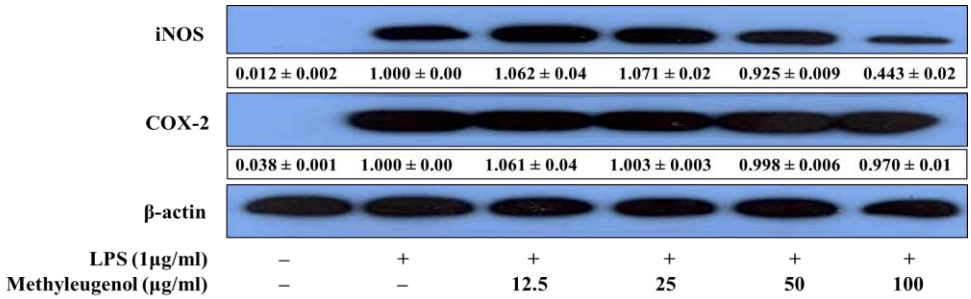
LPS (1µg/ml) - + + + + + +
Pulegone (µg/ml) - - 6.25 12.50 25 50 -
AMT (µM) - - - - - - 10

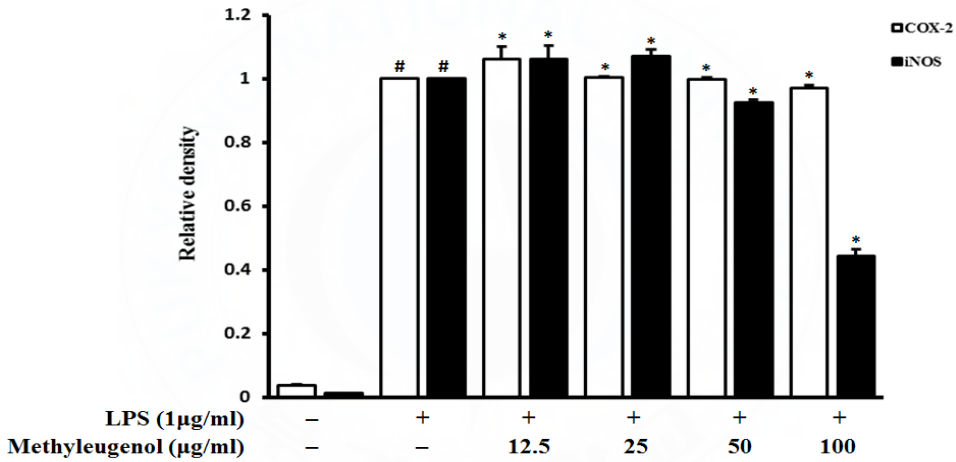
Fig. 2. Inhibitory effects of essential oils (eugenol, methyl eugenol, estragole, and pulegone) on the production of NO in LPS-stimulated RAW264.7 cells. Cells pretreated with different concentration of these essential oils for 2 h were stimulated with LPS (1 μg/ml) for 24 h. The cultured media were used to measure the amount of nitrite to determine NO production. Data are presented as means ± SDs of three independent experiments. ###$p < 0.001$ indicates significant differences from the control group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences from the LPS-treated group.

3.3. Effects on the production of iNOS and COX-2

The expression of iNOS was determined by western blot to evaluate the cause of decreased NO production. COX-2 expression was also determined to exhibit the inhibition of PGE2 production. RAW 264.7 cells were pretreated with the selected concentrations of eugenol, methyl eugenol, estragole, and pulegone for 2 h and stimulated with LPS (1μg/ml) for 18 h. Western blot analysis was used to measure iNOX and COX-2 protein levels. As expected, iNOS expression was significantly inhibited by eugenol, methyl eugenol, estragole, and pulegone after exposure to LPS for 18 h (Fig. 3). On the other hand, eugenol did not show any inhibitory activity against COX-2 (Fig. 3). Likewise, methyl eugenol and estragole showed significant inhibitory activity against COX-2 at 50 and 100 μg/ml. But pulegone exhibited strong COX-2 inhibitory activity (Fig. 3).


iNOS
0.005 ± 0.005
1.000 ± 0.00
0.872 ± 0.03
0.717 ± 0.003
0.467 ± 0.04
0.420 ± 0.01
COX-2
0.008 ± 0.008
1.000 ± 0.00
1.165 ± 0.02
1.146 ± 0.004
1.080 ± 0.04
1.004 ± 0.01
β-actin
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25
Relative density
1.2
1
0.8
0.6
0.4
0.2
0
COX-2
iNOS
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25


iNOS
0.012 ± 0.002
1.000 ± 0.00
1.062 ± 0.04
1.071 ± 0.02
0.925 ± 0.009
0.443 ± 0.02
COX-2
0.038 ± 0.001
1.000 ± 0.00
1.061 ± 0.04
1.003 ± 0.003
0.998 ± 0.006
0.970 ± 0.01
β-actin
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


Relative density
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1
0.8
0.6
0.4
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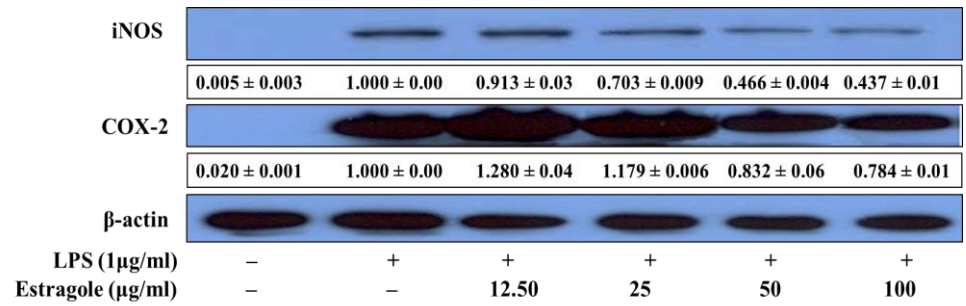
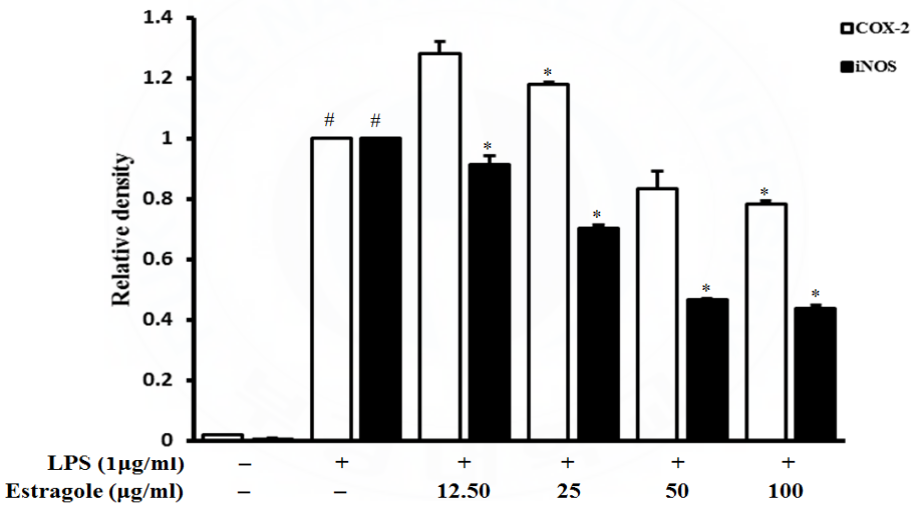
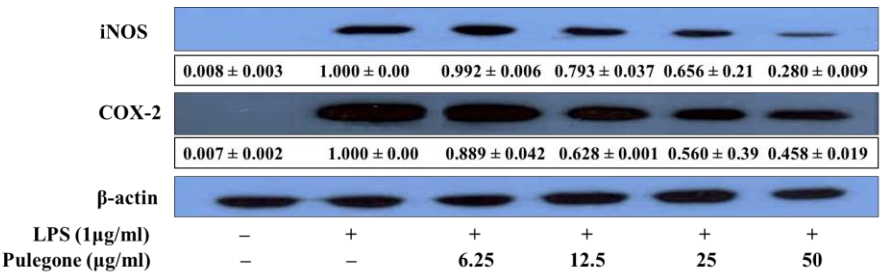
COX-2
iNOS
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


iNOS
0.005 ± 0.003
1.000 ± 0.00
0.913 ± 0.03
0.703 ± 0.009
0.466 ± 0.004
0.437 ± 0.01
COX-2
0.020 ± 0.001
1.000 ± 0.00
1.280 ± 0.04
1.179 ± 0.006
0.832 ± 0.06
0.784 ± 0.01
β-actin
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


COX-2
iNOS
Relative density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


iNOS
0.008 ± 0.003
1.000 ± 0.00
0.992 ± 0.006
0.793 ± 0.037
0.656 ± 0.21
0.280 ± 0.009
COX-2
0.007 ± 0.002
1.000 ± 0.00
0.889 ± 0.042
0.628 ± 0.001
0.560 ± 0.39
0.458 ± 0.019
β-actin
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

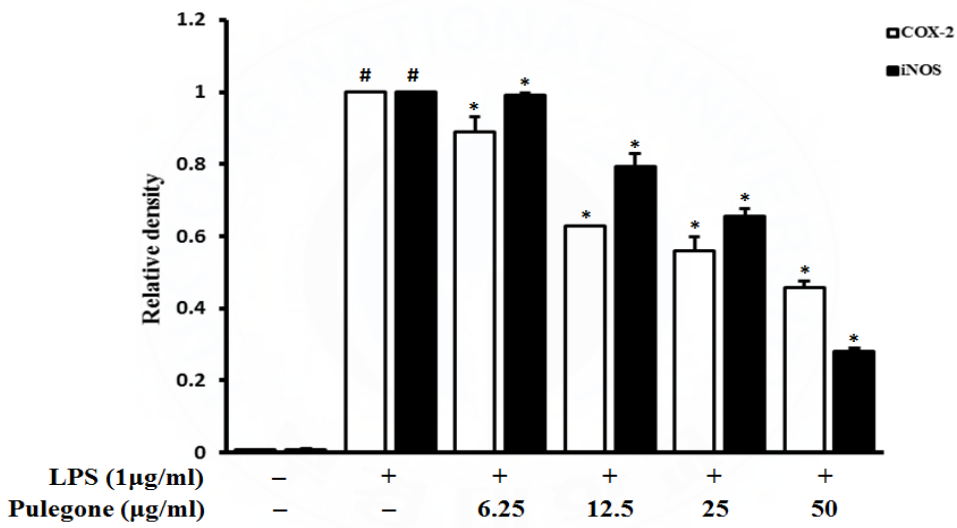
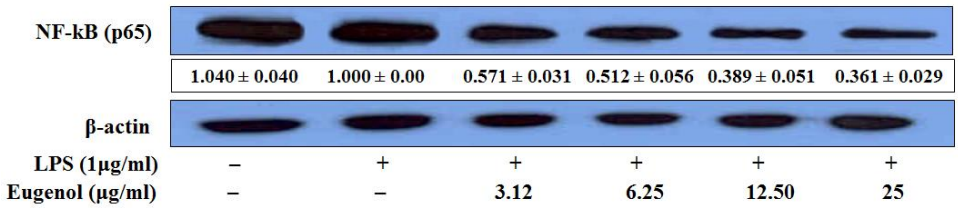
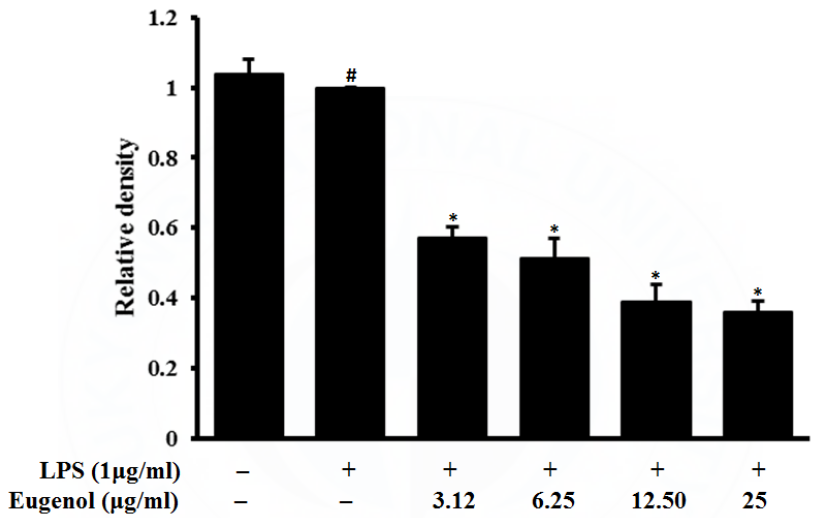

COX-2
iNOS
Relative density
1.2
1
0.8
0.6
0.4
0.2
0
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

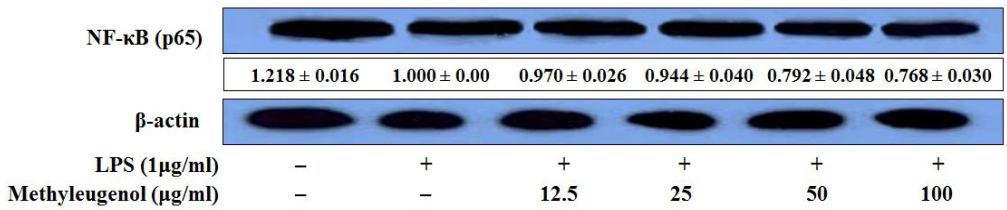
Fig. 3. Inhibitory effects of eugenol, methyl eugenol, estragole, and pulegone on the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells. Cells were pretreated with indicated concentration of these four compounds for 2 h and stimulated with LPS (1 μg/ml) for 18 h. The expression of iNOS, COX-2 and β-actin were detected by Western blot using corresponding antibodies. The results presented were representative of three independent experiments. #$p < 0.05$ indicates significant differences from the control group. *$p < 0.05$ indicates significant differences from the LPS-treated group.

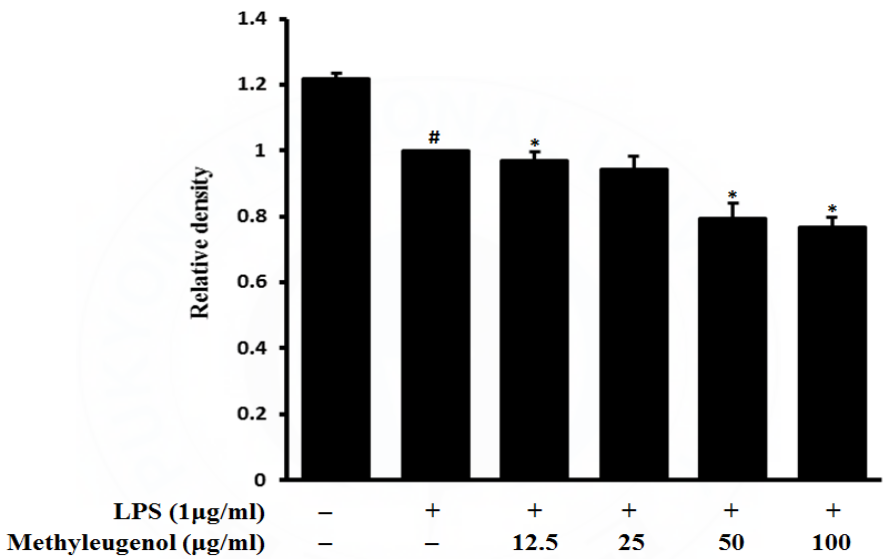
3.4. Effects on the activation of NF-κB

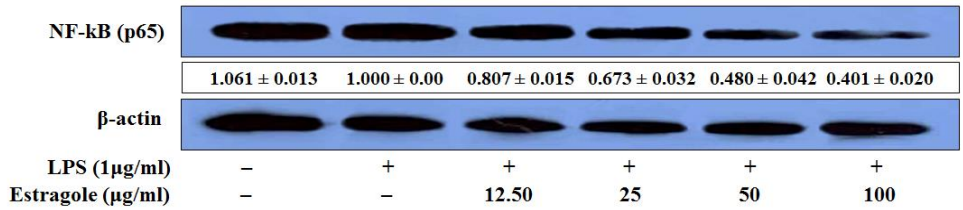
To evaluate the transcriptional controls of eugenol, methyl eugenol, estragole, and pulegone, it was measured that the effects of these four essential oils compounds on the transcriptional activation of NF-κB in LPS-induced RAW 264.7 cells. Fig. 4 clearly shown that the amount of p65 (NF-κB) subunits in the nucleus were rapidly increased after LPS treated in the control group and significantly inhibited in sample treated group. By the observation of these results, it was found that LPS evidently induced the translocation of NF-κB to the nucleus, and pretreatment with those four compounds significantly suppressed this process (Fig. 4).

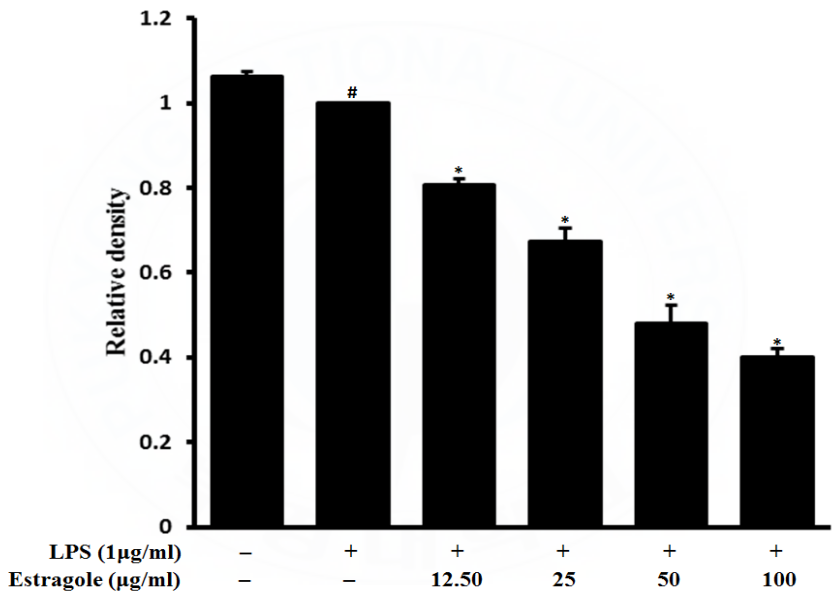

NF-kB (p65)
1.040 ± 0.040
1.000 ± 0.00
0.571 ± 0.031
0.512 ± 0.056
0.389 ± 0.051
0.361 ± 0.029
β-actin
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25

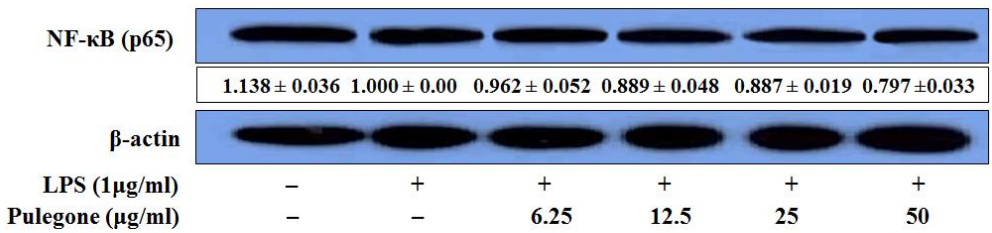

Relative density
1.2
1
0.8
0.6
0.4
0.2
0
#
*
*
*
*
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25


NF-κB (p65)
1.218 ± 0.016
1.000 ± 0.00
0.970 ± 0.026
0.944 ± 0.040
0.792 ± 0.048
0.768 ± 0.030
β-actin
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


Relative density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
*
*
*
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


NF-kB (p65)
1.061 ± 0.013
1.000 ± 0.00
0.807 ± 0.015
0.673 ± 0.032
0.480 ± 0.042
0.401 ± 0.020
β-actin
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


Relative density
1.2
1
0.8
0.6
0.4
0.2
0
#
*
*
*
*
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


NF-κB (p65)
1.138 ± 0.036
1.000 ± 0.00
0.962 ± 0.052
0.889 ± 0.048
0.887 ± 0.019
0.797 ±0.033
β-actin
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

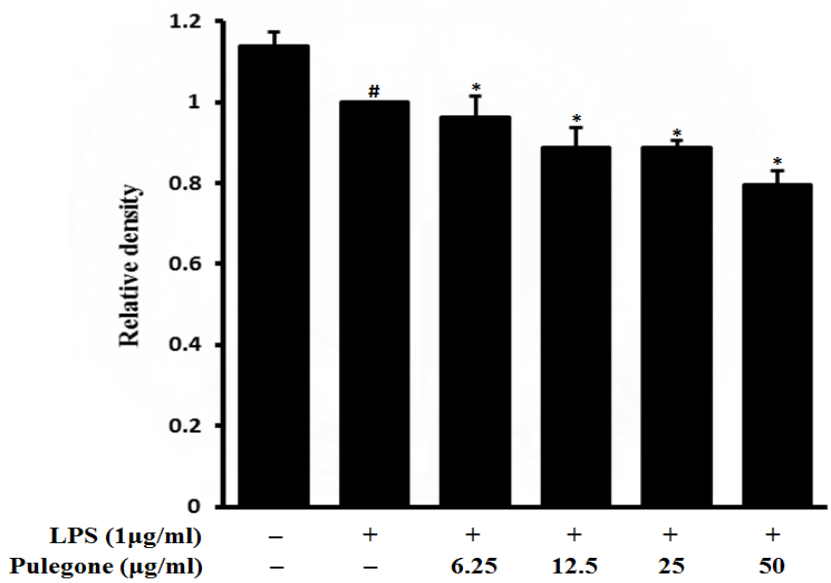
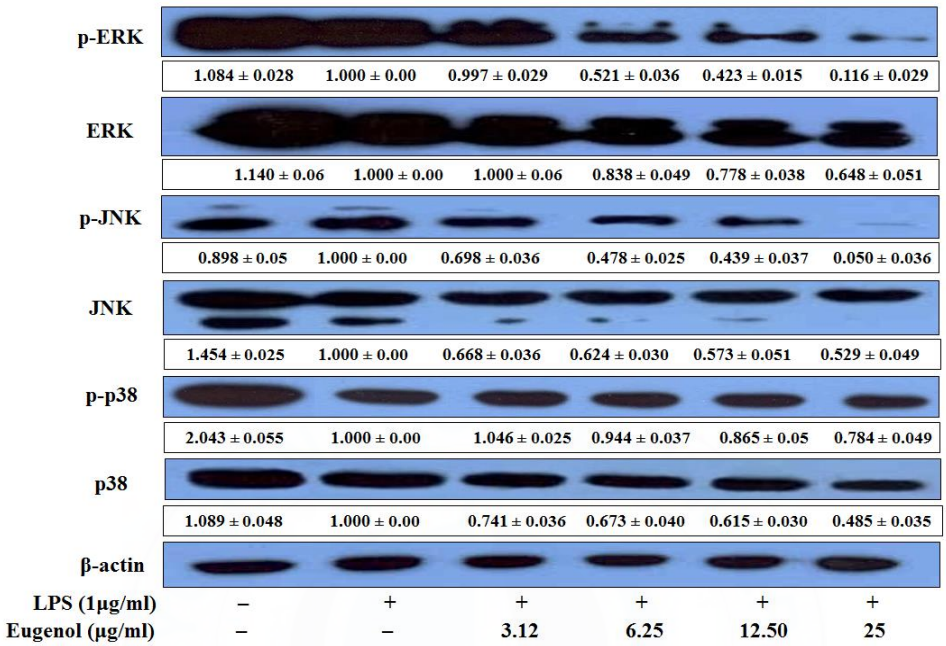
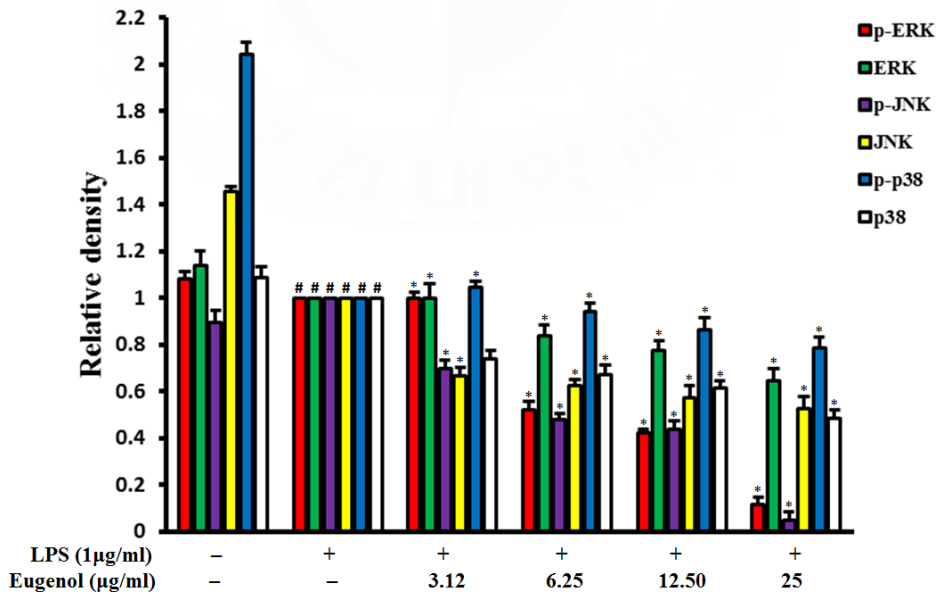

Relative density
1.2
1
0.8
0.6
0.4
0.2
0
#
*
*
*
*
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

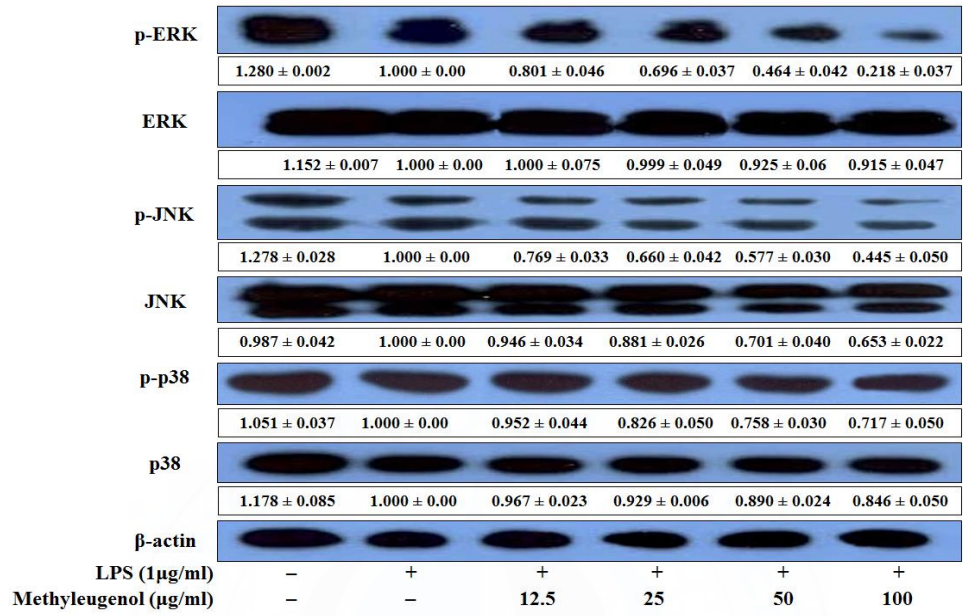
Fig. 4. Inhibitory effects of eugenol, methyl eugenol, estragole, and pulegone on the expression of NF-κB in LPS-stimulated RAW264.7 cells. Cells were pretreated with indicated concentration of these four compounds for 2 h and stimulated with LPS (1 μg/ml) for 18 h. The expression of NF-κB and β-actin was detected by Western blot using corresponding antibodies. The results presented were representative of three independent experiments. #$p < 0.05$ indicates significant differences from the control group. *$p < 0.05$ indicates significant differences from the LPS-treated group.

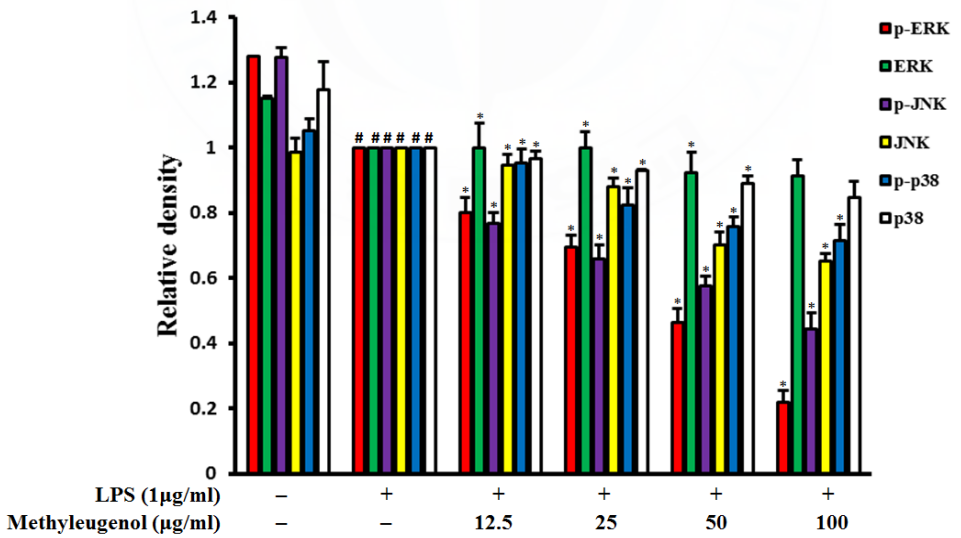
3.5. Effects on MAPK signaling pathways

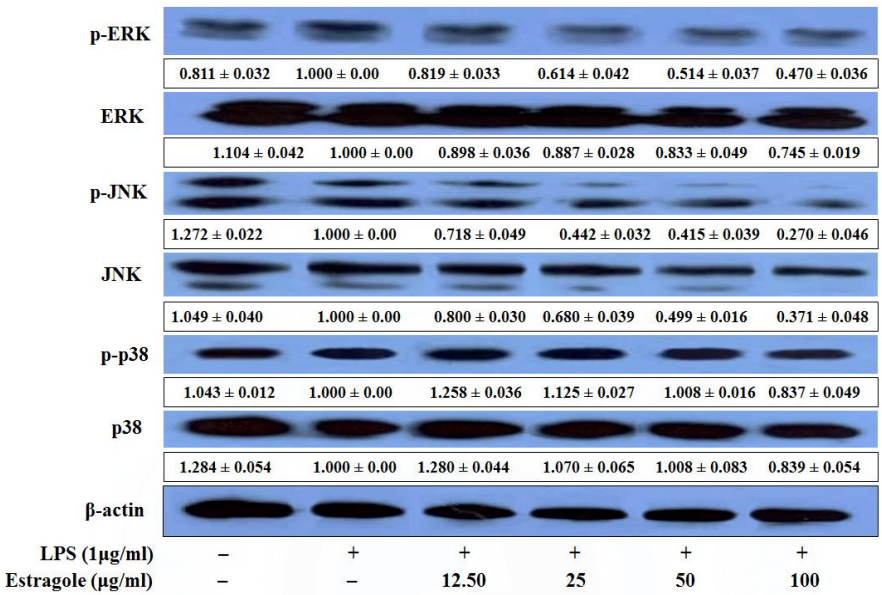
The phosphorylation levels of MAPKs were analyzed in LPS-treated RAW 264.7 cells by Western blotting because MAPK signaling molecules also play a critical role in regulating the LPS-induced inflammatory process. The phosphorylation of MAPKs is also closely related to the regulation of NF-κB activation. As Fig. 5 depicted that the inhibitory activities of eugenol, methyl eugenol, estragole, and pulegone on ERK, JNK, and p38 phosphorylation after 2 h of LPS stimulation in RAW 264.7 cells. These results demonstrated that tested four compounds significantly inhibit the phosphorylation of ERK, JNK, and p38 in LPS-treated RAW 264.7 cells. Exceptionally, phosphorylation of p38 for estragole and ERK for pulegone did not show significant inhibitory activity at lower concentration treated group.

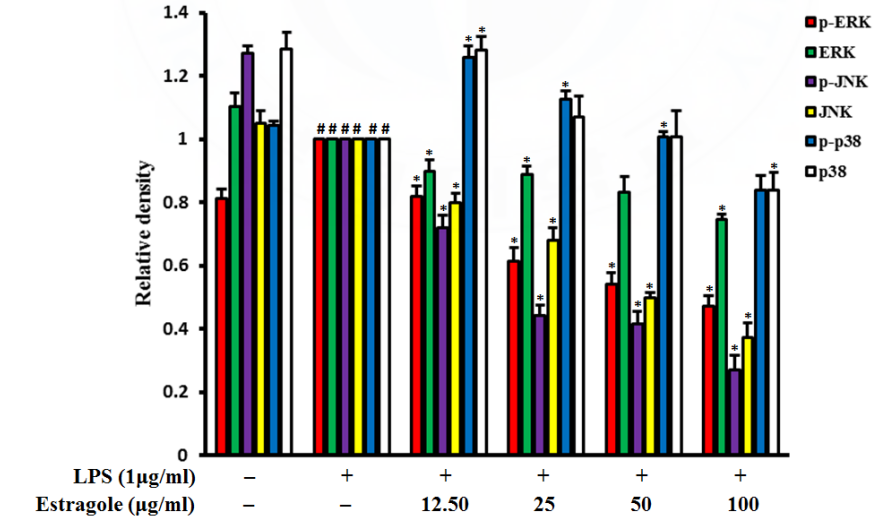

p-ERK
1.084 ± 0.028
1.000 ± 0.00
0.997 ± 0.029
0.521 ± 0.036
0.423 ± 0.015
0.116 ± 0.029
ERK
1.140 ± 0.06
1.000 ± 0.00
1.000 ± 0.06
0.838 ± 0.049
0.778 ± 0.038
0.648 ± 0.051
p-JNK
0.898 ± 0.05
1.000 ± 0.00
0.698 ± 0.036
0.478 ± 0.025
0.439 ± 0.037
0.050 ± 0.036
JNK
1.454 ± 0.025
1.000 ± 0.00
0.668 ± 0.036
0.624 ± 0.030
0.573 ± 0.051
0.529 ± 0.049
p-p38
2.043 ± 0.055
1.000 ± 0.00
1.046 ± 0.025
0.944 ± 0.037
0.865 ± 0.05
0.784 ± 0.049
p38
1.089 ± 0.048
1.000 ± 0.00
0.741 ± 0.036
0.673 ± 0.040
0.615 ± 0.030
0.485 ± 0.035
β-actin
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25

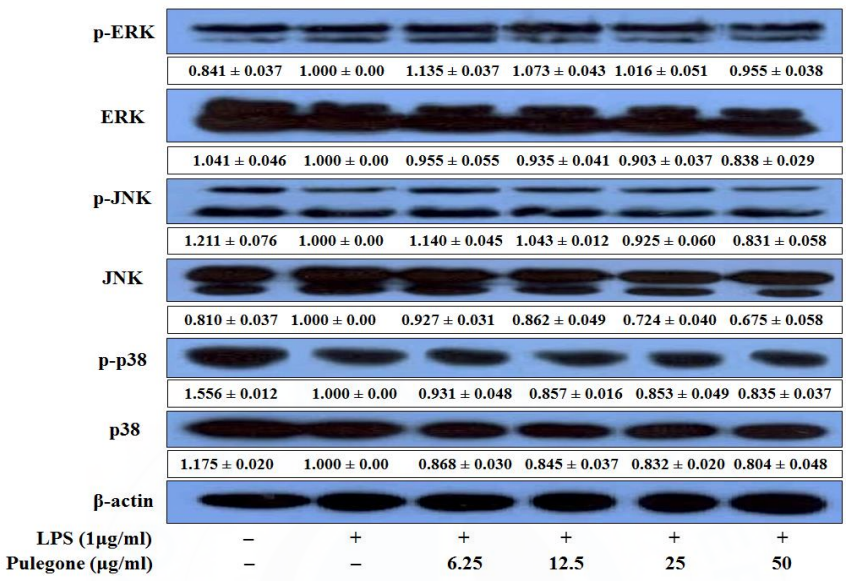

Relative density
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p-ERK
ERK
p-JNK
JNK
p-p38
p38
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25


p-ERK
1.280 ± 0.002
1.000 ± 0.00
0.801 ± 0.046
0.696 ± 0.037
0.464 ± 0.042
0.218 ± 0.037
ERK
1.152 ± 0.007
1.000 ± 0.00
1.000 ± 0.075
0.999 ± 0.049
0.925 ± 0.06
0.915 ± 0.047
p-JNK
1.278 ± 0.028
1.000 ± 0.00
0.769 ± 0.033
0.660 ± 0.042
0.577 ± 0.030
0.445 ± 0.050
JNK
0.987 ± 0.042
1.000 ± 0.00
0.946 ± 0.034
0.881 ± 0.026
0.701 ± 0.040
0.653 ± 0.022
p-p38
1.051 ± 0.037
1.000 ± 0.00
0.952 ± 0.044
0.826 ± 0.050
0.758 ± 0.030
0.717 ± 0.050
p38
1.178 ± 0.085
1.000 ± 0.00
0.967 ± 0.023
0.929 ± 0.006
0.890 ± 0.024
0.846 ± 0.050
β-actin
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


Relative density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p-ERK
ERK
p-JNK
JNK
p-p38
p38
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


p-ERK
0.811 ± 0.032
1.000 ± 0.00
0.819 ± 0.033
0.614 ± 0.042
0.514 ± 0.037
0.470 ± 0.036
ERK
1.104 ± 0.042
1.000 ± 0.00
0.898 ± 0.036
0.887 ± 0.028
0.833 ± 0.049
0.745 ± 0.019
p-JNK
1.272 ± 0.022
1.000 ± 0.00
0.718 ± 0.049
0.442 ± 0.032
0.415 ± 0.039
0.270 ± 0.046
JNK
1.049 ± 0.040
1.000 ± 0.00
0.800 ± 0.030
0.680 ± 0.039
0.499 ± 0.016
0.371 ± 0.048
p-p38
1.043 ± 0.012
1.000 ± 0.00
1.258 ± 0.036
1.125 ± 0.027
1.008 ± 0.016
0.837 ± 0.049
p38
1.284 ± 0.054
1.000 ± 0.00
1.280 ± 0.044
1.070 ± 0.065
1.008 ± 0.083
0.839 ± 0.054
β-actin
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


Relative density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
p-ERK
ERK
p-JNK
JNK
p-p38
p38
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


p-ERK
0.841 ± 0.037 1.000 ± 0.00 1.135 ± 0.037 1.073 ± 0.043 1.016 ± 0.051 0.955 ± 0.038
ERK
1.041 ± 0.046 1.000 ± 0.00 0.955 ± 0.055 0.935 ± 0.041 0.903 ± 0.037 0.838 ± 0.029
p-JNK
1.211 ± 0.076 1.000 ± 0.00 1.140 ± 0.045 1.043 ± 0.012 0.925 ± 0.060 0.831 ± 0.058
JNK
0.810 ± 0.037 1.000 ± 0.00 0.927 ± 0.031 0.862 ± 0.049 0.724 ± 0.040 0.675 ± 0.058
p-p38
1.556 ± 0.012 1.000 ± 0.00 0.931 ± 0.048 0.857 ± 0.016 0.853 ± 0.049 0.835 ± 0.037
p38
1.175 ± 0.020 1.000 ± 0.00 0.868 ± 0.030 0.845 ± 0.037 0.832 ± 0.020 0.804 ± 0.048
β-actin
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

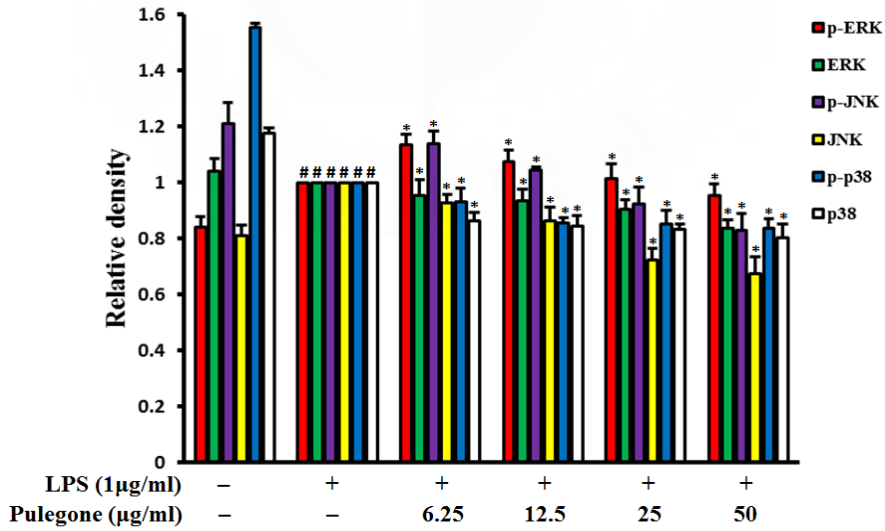
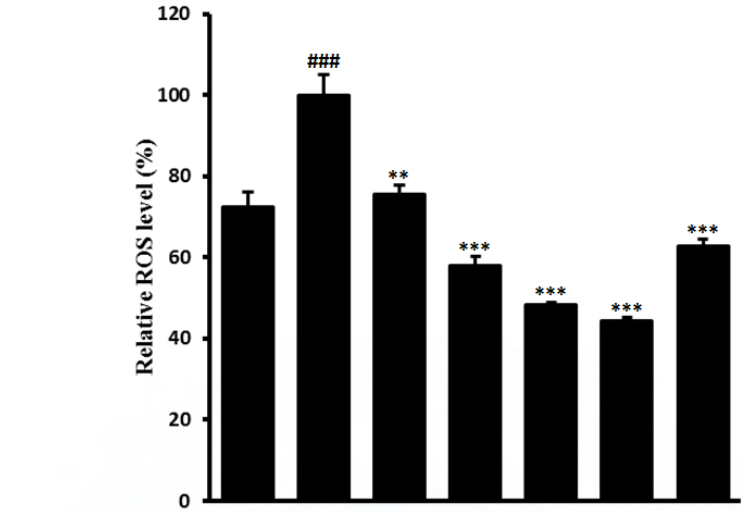
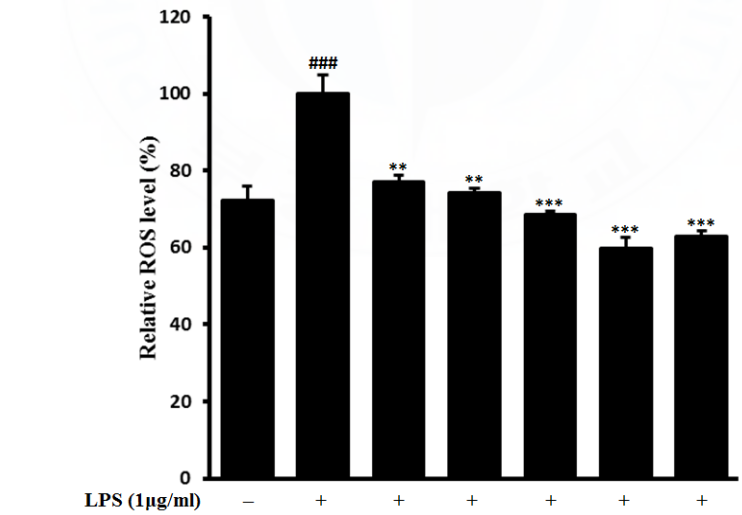

Relative density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
######
p-ERK
ERK
p-JNK
JNK
p-p38
p38
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

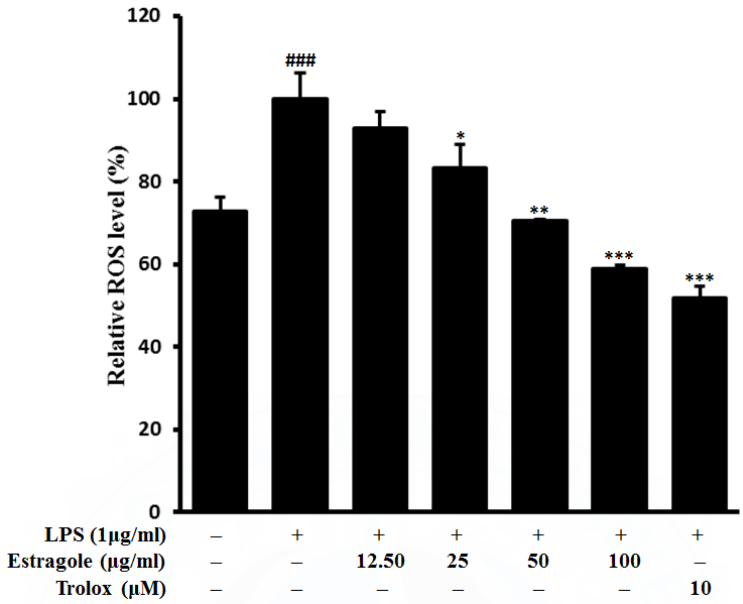
Fig. 5. Inhibitory effects of eugenol, methyl eugenol, estragole, and pulegone on the expression of MAPKs in LPS-stimulated RAW264.7 cells. Cells were pretreated with indicated concentration of these four compounds for 2 h and stimulated with LPS (1 μg/ml) for 18 h. The expression of MAPKs was detected by Western blot using corresponding antibodies. The results presented were representative of three independent experiments. #$p < 0.05$ indicates significant differences from the control group. *$p < 0.05$ indicates significant differences from the LPS-treated group.

3.6. Effects on intracellular ROS production

It has been suggested that inflammation is mediated by cellular oxidative stress. Therefore, it was investigated that the inhibitory effects of eugenol, methyl eugenol, estragole, and pulegone on LPS-induced ROS generation in RAW 264.7 cells. The cells were treated with LPS to generate ROS and DCFH-DA was used to detect ROS generation in a fluorescent microplate reader. As Fig. 6 shown that all four compounds exhibit significant inhibitory activities against ROS generation compared to positive control (Trolox). Among these four compounds eugenol showed higher inhibitory activity against ROS generation in LPS-induced RAW 264.7 cells.


120
100
80
60
40
20
0
Relative ROS level (%)
###
**
***
***
***
***
LPS (1μg/ml) – + + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25 –
Trolox (μM) – – – – – – 10


120
100
80
60
40
20
0
Relative ROS level (%)
###
**
**
***
***
***
LPS (1μg/ml) – + + + + + +
Methyleugenol (μg/ml) – – 12.50 25 50 100 –
Trolox (μM) – – – – – – 10


120
100
80
60
40
20
0
Relative ROS level (%)
###
*
**
***
***
LPS (1μg/ml) – + + + + + +
Estragole (μg/ml) – – 12.50 25 50 100 –
Trolox (μM) – – – – – – 10

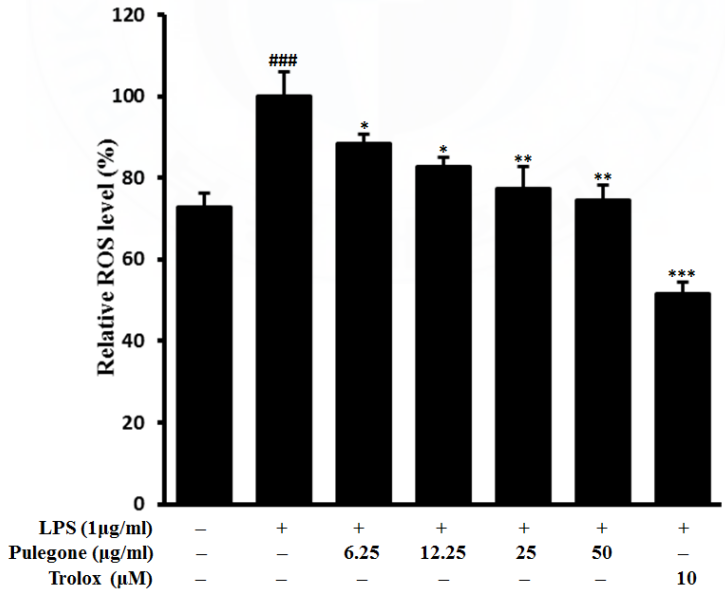
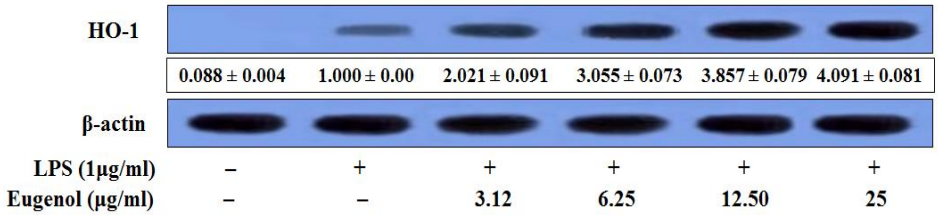
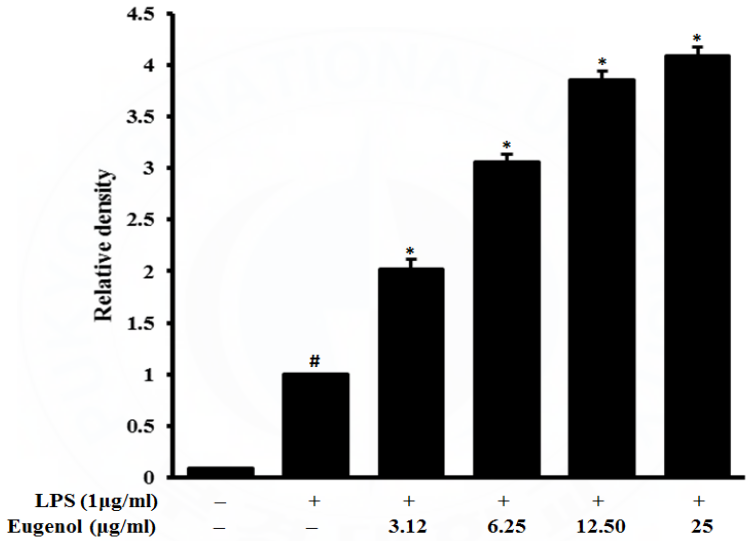

120
100
80
60
40
20
0
Relative ROS level (%)
###
*
*
**
**
***
LPS (1μg/ml) – + + + + + +
Pulegone (μg/ml) – – 6.25 12.25 25 50 –
Trolox (μM) – – – – – – 10

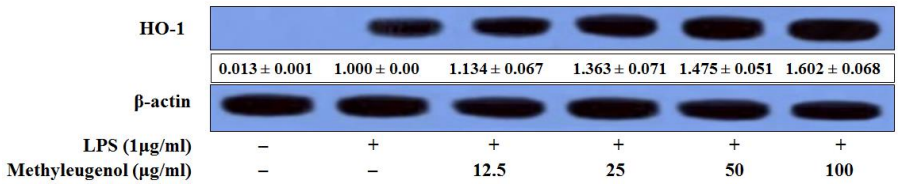
Fig. 6. Inhibitory effects of essential oils (eugenol, methyl eugenol, estragole, and pulegone) on the production of ROS in LPS-stimulated RAW264.7 cells. Cells pretreated with different concentration of these essential oils for 2 h were stimulated with LPS (1 μg/ml) for 24 h. ROS levels were measured by DCF-DA with fluorescent analysis. Data are presented as means ± SDs of three independent experiments. ###$p < 0.01$ indicates significant differences from the control group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences from the LPS-treated group.

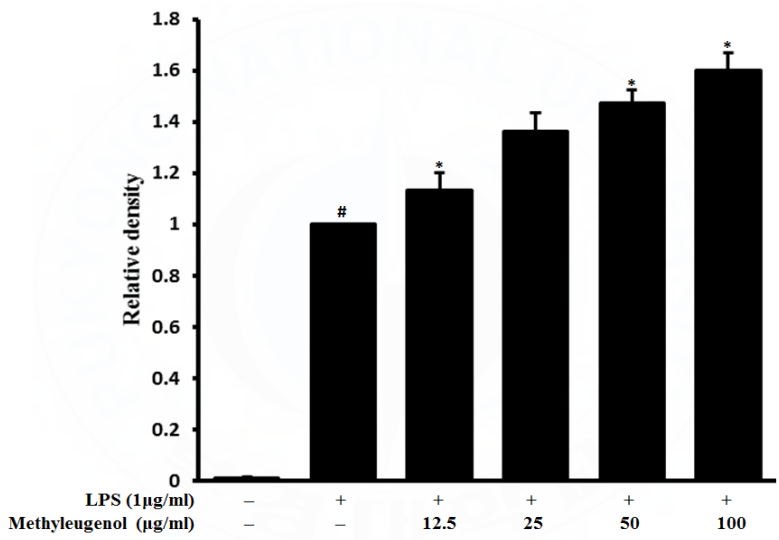
3.7. Effects on the regulation of HO-1

HO-1 is a cytoprotective enzyme which provides cellular protection against oxidative stress. Therefore, it was determined the effects of four selected compounds on the up-regulation of HO-1 protein expression by Western blot. As expected, selected four compounds treatment led to a significant increase in HO-1 protein expression in LPS-induced RAW 264.7 cells. Fig. 7 exhibits clearly the upregulation of HO-1 protein expression in LPS-treated RAW 264.7 cells.


HO-1
0.088 ± 0.004
1.000 ± 0.00
2.021 ± 0.091
3.055 ± 0.073
3.857 ± 0.079
4.091 ± 0.081
β-actin
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25


Relative density
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
#
*
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25


HO-1
0.013 ± 0.001
1.000 ± 0.00
1.134 ± 0.067
1.363 ± 0.071
1.475 ± 0.051
1.602 ± 0.068
β-actin
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100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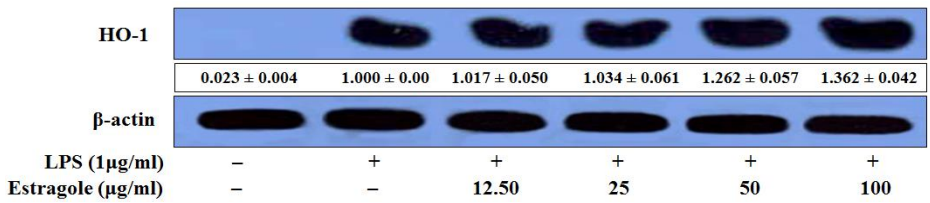
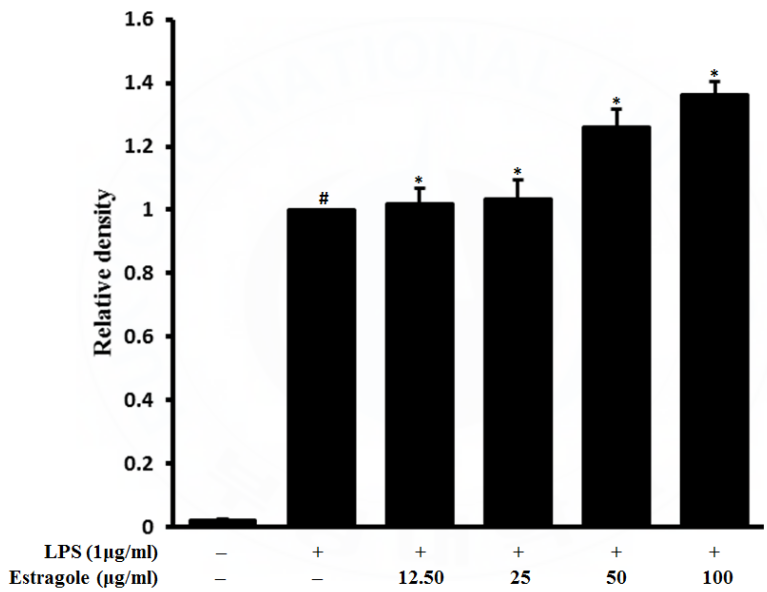
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


HO-1
0.023 ± 0.004
1.000 ± 0.00
1.017 ± 0.050
1.034 ± 0.061
1.262 ± 0.057
1.362 ± 0.042
β-actin
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


Relative density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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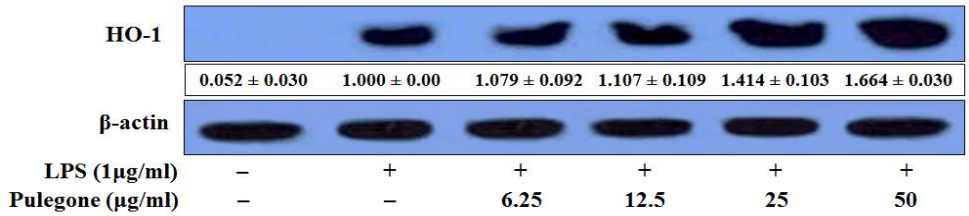
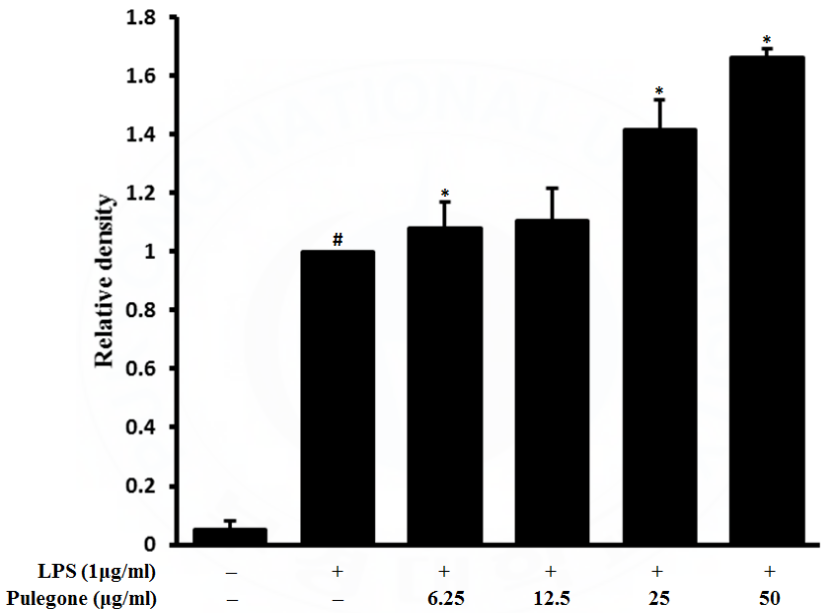
*
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


HO-1
0.052 ± 0.030
1.000 ± 0.00
1.079 ± 0.092
1.107 ± 0.109
1.414 ± 0.103
1.664 ± 0.030
β-actin
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50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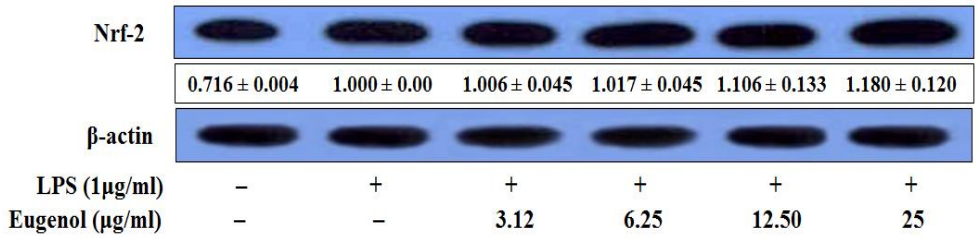
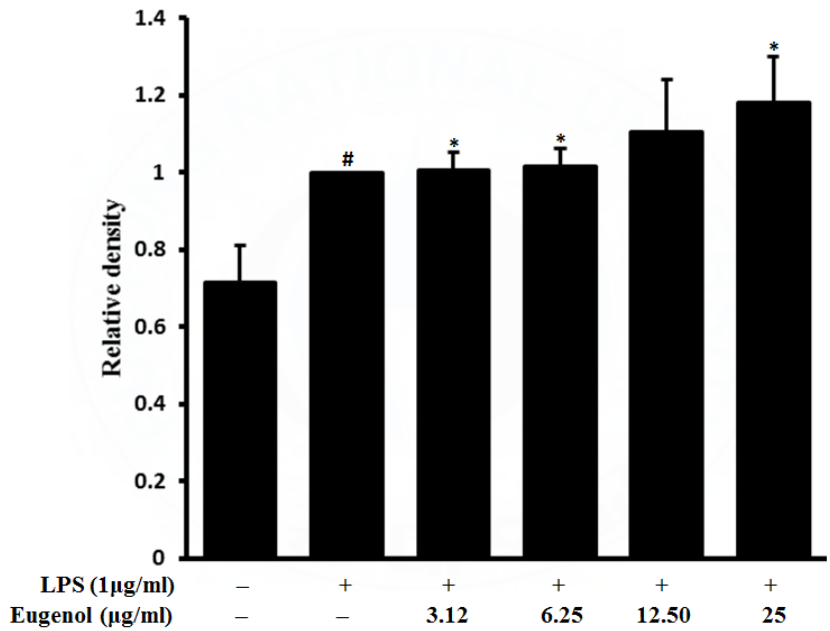
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
*
*
*
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

Fig. 7. Inhibitory effects of eugenol, methyl eugenol, estragole, and pulegone on the expression of HO-1 in LPS-stimulated RAW264.7 cells. Cells were pretreated with indicated concentration of these four compounds for 2 h and stimulated with LPS (1 μg/ml) for 18 h. The expression of HO-1 was detected by Western blot using corresponding antibodies. The results presented were representative of three independent experiments. #$p < 0.05$ indicates significant differences from the control group. *$p < 0.05$ indicates significant differences from the LPS-treated group.

3.8. Effects on the regulation of Nrf-2

Nrf-2 is a transcription factor which regulates the expression of HO-1 to protect against oxidative damage at the site of inflammation. Therefore, Nrf-2 protein expression was also investigated by Western blot in LPS-induced RAW 264.7 cells. As shown in results (Fig. 8), eugenol, methyl eugenol, estragole, and pulegone significantly up-regulate Nrf-2 expression compared to the non-treated group in LPS-treated RAW 264.7 cells. Pulegone showed higher up regulatory Nrf-2 protein expression among four tested compounds (Fig. 8).


Nrf-2
0.716 ± 0.004
1.000 ± 0.00
1.006 ± 0.045
1.017 ± 0.045
1.106 ± 0.133
1.180 ± 0.120
β-actin
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25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0.8
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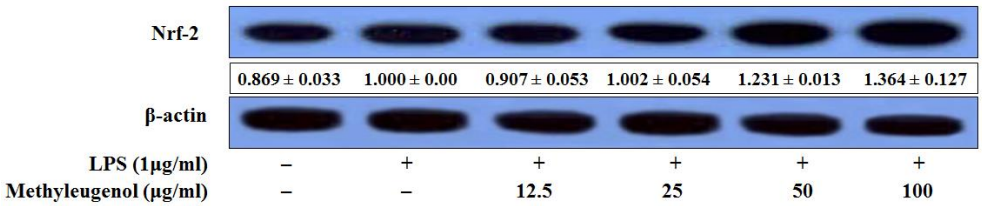
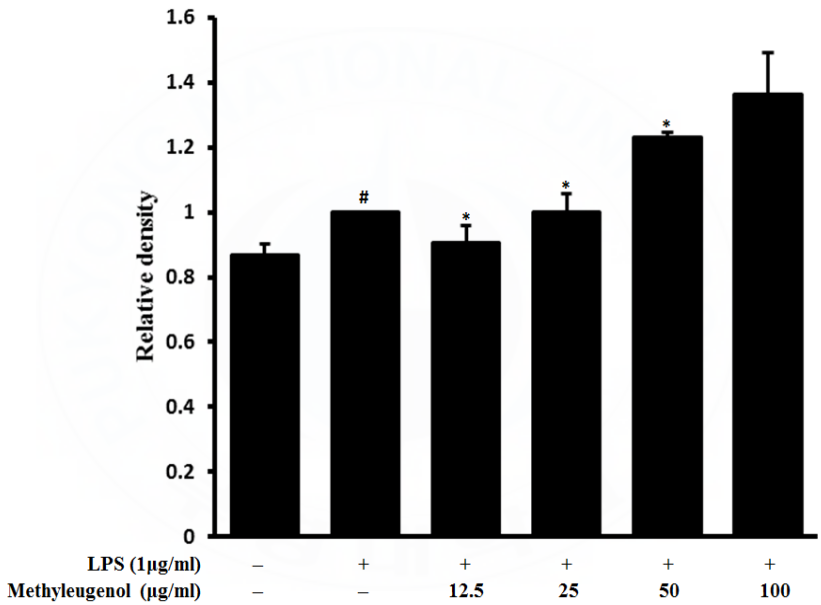
*
*
*
LPS (1μg/ml) – + + + + +
Eugenol (μg/ml) – – 3.12 6.25 12.50 25


Nrf-2
0.869 ± 0.033
1.000 ± 0.00
0.907 ± 0.053
1.002 ± 0.054
1.231 ± 0.013
1.364 ± 0.127
β-actin
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100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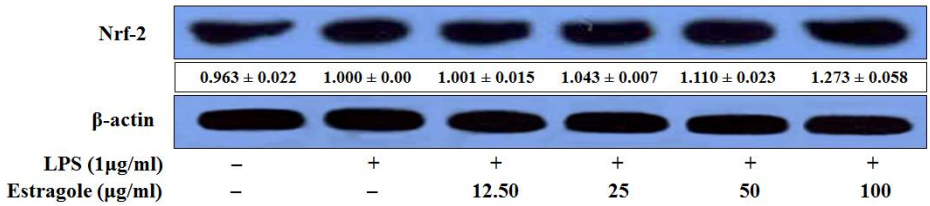
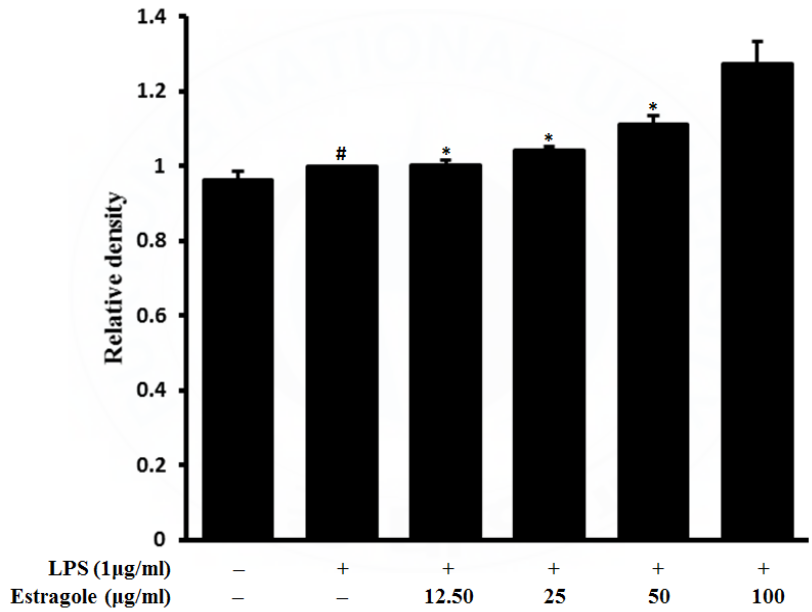
LPS (1μg/ml) – + + + + +
Methyleugenol (μg/ml) – – 12.5 25 50 100


Nrf-2
0.963 ± 0.022
1.000 ± 0.00
1.001 ± 0.015
1.043 ± 0.007
1.110 ± 0.023
1.273 ± 0.058
β-actin
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100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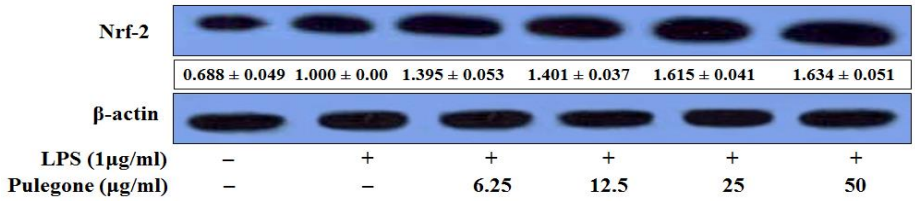
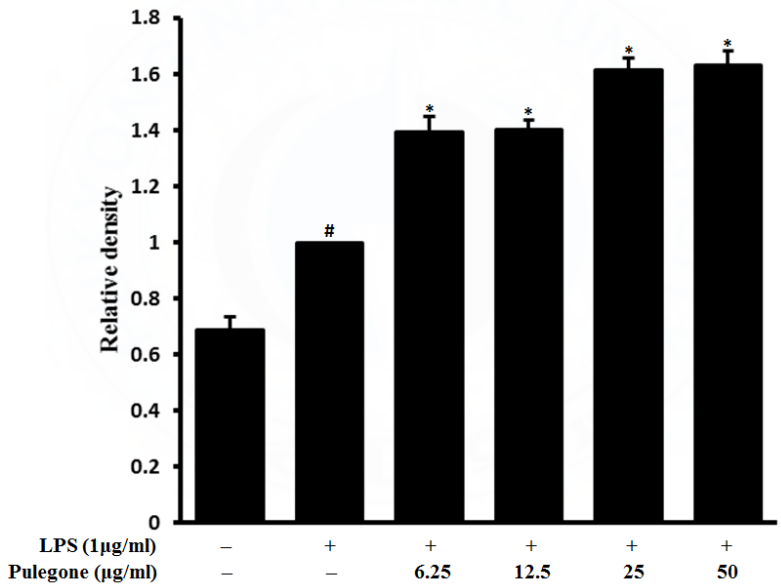
*
LPS (1μg/ml) – + + + + +
Estragole (μg/ml) – – 12.50 25 50 100


Nrf-2
0.688 ± 0.049
1.000 ± 0.00
1.395 ± 0.053
1.401 ± 0.037
1.615 ± 0.041
1.634 ± 0.051
β-actin
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
*
*
*
*
LPS (1μg/ml) – + + + + +
Pulegone (μg/ml) – – 6.25 12.5 25 50

Fig. 8. Inhibitory effect of eugenol, methyl eugenol, estragole, and pulegone on the expression of Nrf-2 in LPS-stimulated RAW264.7 cells. Cells were pretreated with indicated concentration of these four compounds for 2 h and stimulated with LPS (1 μg/ml) for 18 h. The expression of Nrf-2 was detected by Western blot using corresponding antibodies. The results presented were representative of three independent experiments. #$p < 0.05$ indicates significant differences from the control group. *$p < 0.05$ indicates significant differences from the LPS-treated group.

3.9. Molecular docking analysis

Molecular docking was performed and best poses were found to show the binding cavity of the enzyme exhibiting all the major interaction. Ten docking runs were performed using the Lamarckian Genetic Algorithm. Ligand-receptor complexes were analyzed for binding affinity. Hydrogen bonding and hydrophobic interactions from iNOS were visualized and analyzed by chimera and Ligplot+. Fig. 10 depicted molecular docking results of iNOS and ITU, eugenol, methyl eugenol, estragole, and pulegone. The iNOS-pulegone inhibitor complex lowest energy (- 6.97 kcal/mol) with highest binding affinity as compared with other compounds-iNOS complexes. Although iNOS-pulegone inhibitor complex did not show any hydrogen bond interaction but it showed hydrophobic interactions with active site residues such as Asn370, Gly371, Phe369, Glu377, Val352, Pro350, Trp372, and cofactor Hem510. Estragole, eugenol, and methyl eugenol exhibit binding affinities toward iNOS are as follows - 6.05, - 5.99, and – 5.94 kcal/mol, respectively. These three phenylpropenes have a slight structural difference which also may affect their binding affinity (Fig. 9). All inhibitors complexes were analyzed for a

geometrical parameter such as hydrogen bonding, hydrophobic interactions (Van der Waals bond) (Table 1).

Table 1: Molecular interaction results of the iNOS (4nos) active site with known inhibitor ethyl isothiourea (ITU), eugenol, methyl eugenol, estragole, and pulegone. The binding energy values of each compound with no. of H-bonds, H-bond interacting residues and Van der Waals bond interacting residues were described.

Compounds	Autodock 4.2 Score (kcal/mol)	No. of H-bonds	H-bonds interacting residues	Van der Waals bond interacting residues
Ethylisothiourea (ITU)	-4.81	2	Glu377, Trp372	Gly371, Phe369, Hem510, Val352, Pro350
Pulegone	-6.97	-	-	Asn370, Gly371, Phe369, Hem510, Glu377, Val352, Pro350, Trp372
Estragole	-6.05	-	-	Asn370, Gly371, Phe369, Hem510, Glue377, Val352, Pro350, Ala351
Eugenol	-5.99	1	Trp372	Gly371, Hem510, Glu377, Val352, Pro350, Tyr373
Mehtyleugenol	-5.94	-	-	Asn370, Gly371, Phe369, Hem510, Glu377, Val352, Pro350, Ala351

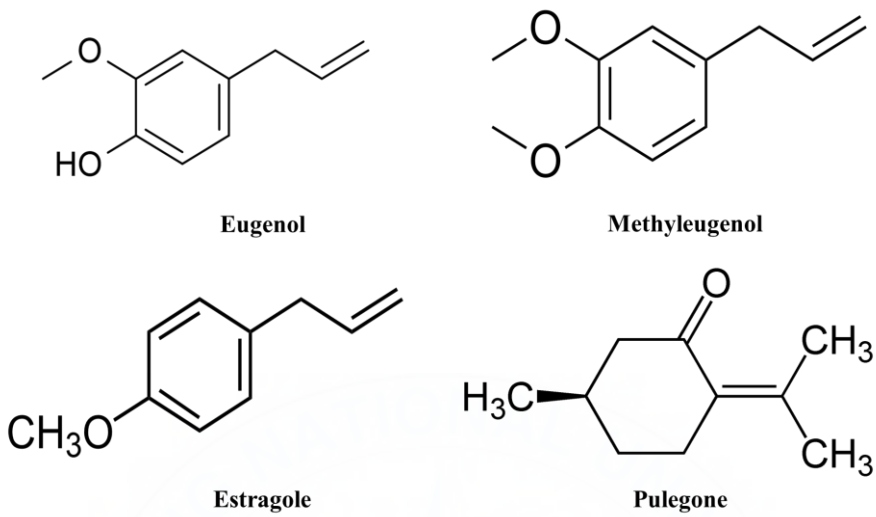
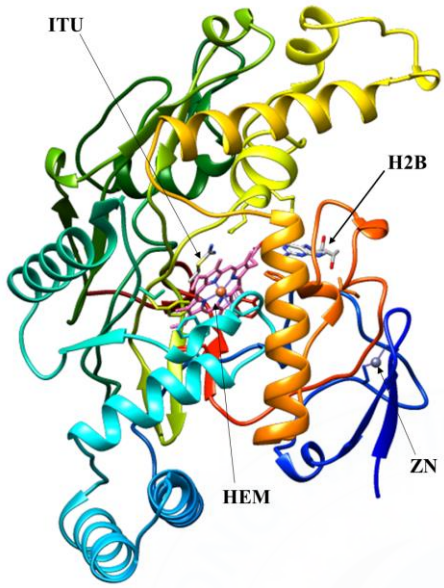
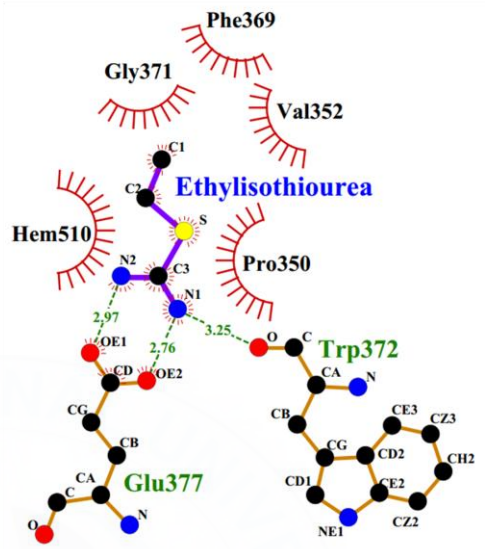
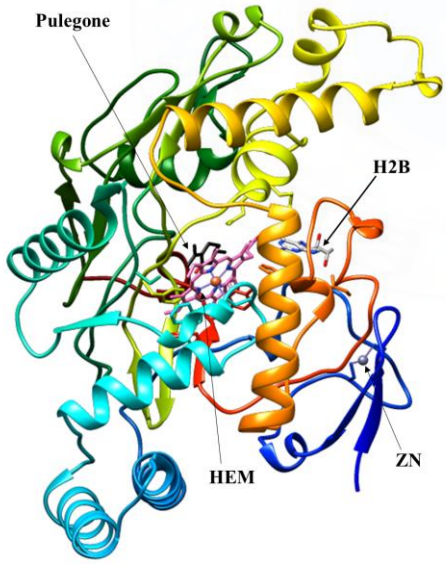
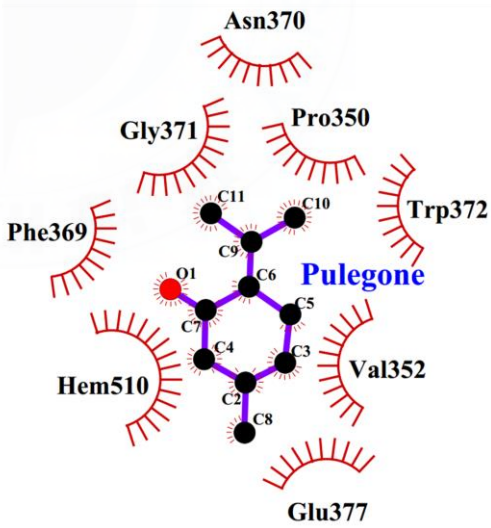


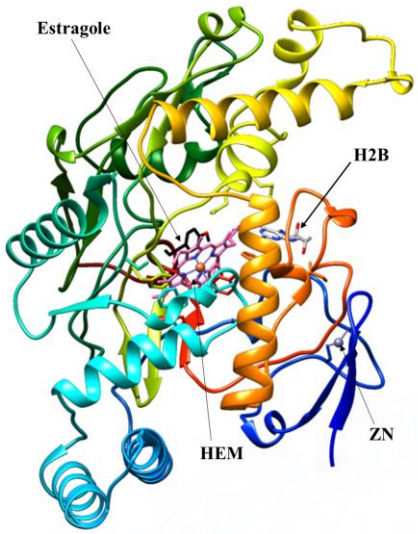
Fig. 9. The chemical structure of eugenol, methyl eugenol, estragole, and pulegone.

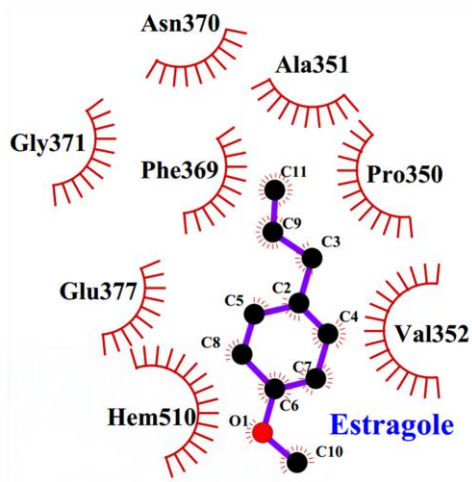

ITU
H2B
HEM
ZN

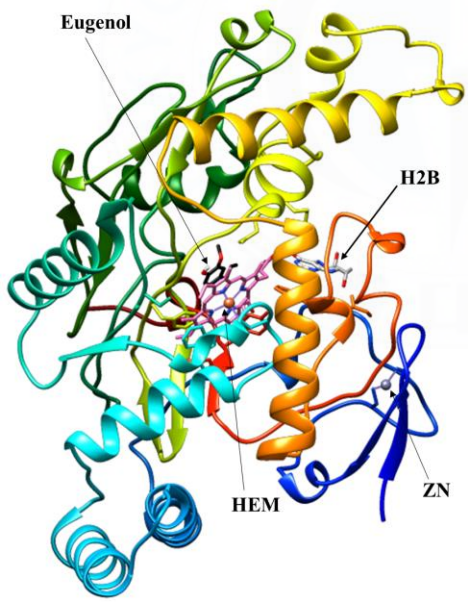

Phe369
Gly371
Val352
Ethylisothiourea
Hem510
Pro350
Trp372
Glu377
2.97
2.76
3.25

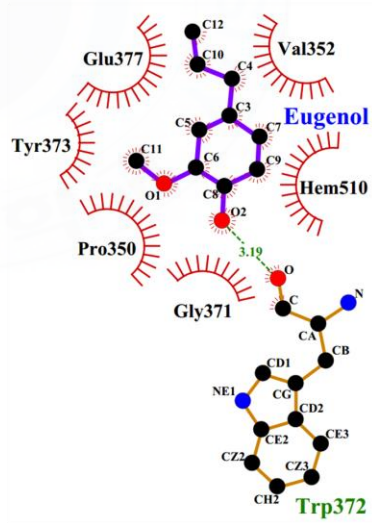

Pulegone
H2B
HEM
ZN


Asn370
Gly371
Pro350
Trp372
Phe369
Pulegone
Hem510
Val352
Glu377


Estragole
H2B
HEM
ZN


Asn370
Ala351
Gly371
Phe369
Pro350
C11
C9
C3
Glu377
C2
C5
C4
Val352
C8
C7
C6
Hem510
O1
Estragole
C10


Eugenol
H2B
HEM
ZN


C12
C10
Glu377
C4
Val352
C3
Eugenol
C5
C7
Tyr373
C11
C6
C9
Hem510
O1
C8
O2
3.19
Pro350
O
C
N
Gly371
CA
CB
CD1
NE1
CG
CD2
CE2
CE3
CZ2
CZ3
CH2
Trp372

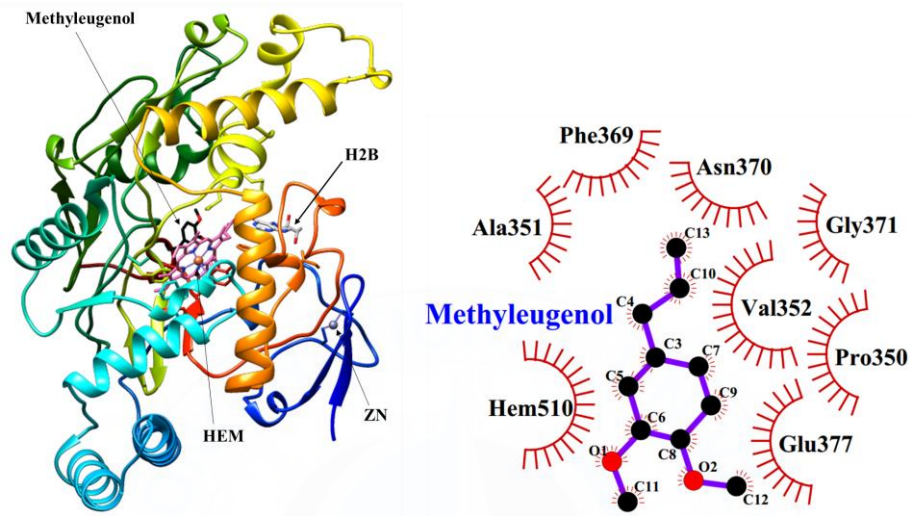


Fig. 10. The depiction of crystallographic docked conformation of ITU, eugenol, methyl eugenol, estragole, and pulegone with iNOS (4nos) as well as cofactor HEM, H2B, and Zn ion (left). Hydrogen bond and hydrophobic interactions from iNOS and ITU, eugenol, methyl eugenol, estragole, and pulegone inhibitor complex (right).

4. Discussion

Eugenol, methyl eugenol, estragole, and pulegone are essential oils compound obtained from various aromatic plants. Generally, essential oils containing plants located in warm countries like Mediterranean and tropical countries, which can play an important part of the traditional pharmacopeia (Bakkali et al., 2008). The density of essential oils is lower than water. Essentials oils are liquid, limpid, volatile, rarely colored, lipid soluble and soluble in organic solvents. Despite their wide use as fragrances, it is important to focus on their mode of biological action for the application in human health. Eugenol, methyl eugenol, and estragole are phenylpropene and the member of phenylpropanoids class of chemical compounds. On the other hand, pulegone is classified as a monoterpene. Although, it is already reported that eugenol exhibits anti-inflammatory activity in LPS-induced 264.7 cells (Li, et al., 2006) but in this study, it is used to compare the anti-inflammatory activities between eugenol, methyl eugenol, and estragole because all are structurally similar compounds.

MTT assay is a sensitive, accurate, and colorimetric assay for testing cell metabolic activity and widely used for the measurement of cytotoxicity (Keiser et al., 2000). In the present study, RAW 264.7 cells were treated with various

concentration of selected compounds (eugenol, methyl eugenol, estragole, and pulegone) for 24 h to test cell viability using MTT assay. The results clearly indicated that following compounds did not exhibit any cytotoxicity in the selected concentrations against RAW 264.7 cells. Therefore, these results strongly suggested that the concentrations of tested compounds are safe for the evaluation of anti-inflammatory activity in LPS-induced RAW 264.7 cells.

It is already proved that NO and PGE_2 are vital mediators of inflammation. NO contributes an important role in many body functions. Thus, the overproduction of NO can lead to inflammatory disorders (Meda et al., 1995, Dandona et al., 2010). iNOS regulates NO production from arginine in response to many inflammatory stimuli. The inducible enzyme COX-2 is responsible for the development of many chronic inflammatory diseases. Therefore, inhibition of NO and PGE_2 production and inhibition of iNOS and COX-2 protein expression might have crucial therapeutic value for the prevention of inflammatory diseases. This study revealed that eugenol, methyl eugenol, estragole, and pulegone significantly inhibit NO production compared to positive control. In the case of iNOS inhibition, eugenol, estragole, and pulegone significantly suppressed iNOS protein expression but methyl eugenol exhibited iNOS inhibitory activity at only

50 and 100 µg/ml concentration. Likewise, methyl eugenol and estragole exhibited COX-2 inhibitory activity at 50 and 100 µg/ml concentration where pulegone showed promising COX-2 inhibitory activity at all selected concentrations. The selected concentration used in this study for eugenol did not show any inhibitory activity against COX-2 but it was already reported that at higher concentration (up to 400 µM) eugenol shows COX-2 inhibitory activity (Li et al., 2006). Eugenol also showed the inhibitory activity against PGE_2 production (Tung et al., 2008). Unfortunately, the present study did not clarify the inhibition of PGE_2 production in LPS-induced RAW 264.7 cells. Further studies may require to evaluating the inhibition of PGE2 production for methyl eugenol, estragole, and pulegone in LPS-induced RAW 264.7 cells.

Recent studies have shown that LPS-induced inflammation is highly associated with different intracellular signaling pathways, such as NF-κB and MAPKs pathways. NF-κB is an important regulator for a number of proinflammatory genes such as iNOS, COX-2, TNF-α, IL-1β, and IL-6 in LPS-induced inflammation (Marks-Konczalik et al., 1998, Islam et al., 2012, Chen et al., 2005). Predominantly, the inactive NF-κB complex with IκB resides in the cytoplasm and IκB is rapidly phosphorylated in response to proinflammatory

stimuli (Rajapakse et al., 2008). Free NF-κB rapidly translocates to the nucleus and promotes the transcription process of target genes which are responsible for inflammation. So, NF-κB could be an effective therapeutic target for treating inflammatory diseases. It was observed that present data shows all four compounds significantly inhibit the NF-κB protein expression in LPS-induced RAW 264.7 cells. Eugenol and estragole showed higher inhibitory expression compared to others towards NF-κB. In addition, further studies may necessary to evaluate cellular cytokines inhibitory activities in LPS-induced RAW 264.7 cells.

The MAPK families consist of ERK1/2, JNK, and p38 that control cellular signal transduction in response to inflammation (Kaminska, 2005). LPS is a recognized activator of MAPK in macrophages. In inflammation, p38 and JNK pathways are known as a stress-activated protein kinase (Xia et al., 1995). These MAPK signaling pathways subsequently activate NF-κB. The mechanism of NF-κB inactivation is also related to the inhibition of phosphorylation of ERK1/2, JNK, and p38. Western blot analysis demonstrated that eugenol and methyl eugenol exhibit significant inhibitory activity towards the phosphorylation of ERK1/2, JNK, and p38. Estragole and pulegone showed significant down regulatory activities against the phosphorylation of JNK. Estragole has also

suppressed the phosphorylation of ERK1/2 significantly. But estragole for p38 and pulegone for ERK1/2 phosphorylation suppressed protein expression only at higher concentration. Interestingly, pulegone showed promising inhibitory activity against p38 phosphorylation in LPS-induced RAW 264.7 cells.

Uncontrolled production of free radicals in cellular systems can damage cellular components like cellular macromolecules, DNA, lipids and proteins in the cell. ROS generation is an important factor in the pathogenesis of inflammation (Hancock et al., 2001). LPS can facilitate the cellular ROS level which is associated with the expression of inflammatory signaling pathways mediated by NF-κB (Choi et al., 2007). Numerous studies revealed that oxidative stress strongly affects the activation of NF-κB (Siomek, 2012). The antioxidant can inhibit the production of proinflammatory cytokines including IL-8 (Ryan et al., 2004). In the present study, pretreatment of eugenol, methyl eugenol, estragole, and pulegone significantly reduced LPS-activated ROS production in RAW 264.7 cells. Among these four compounds, eugenol showed higher inhibitory activity against ROS production in LPS-induced RAW 264.7 cells.

The inducible HO-1 enzyme is a stress responsive protein acts against oxidative stress. It is currently believed that HO-1 has anti-inflammatory

activities (Kim et al., 2014). HO-1 degrades heme through catalyzation process and produces iron, carbon monoxide, and biliverdin. Later biliverdin is converted into bilirubin, which is a strong antioxidant. Several evidence indicates that the induction of HO-1 inhibits LPS-induced inflammatory response activated by Nrf-2 pathways (Lee et al., 2011, Paine et al., 2010). Transcription factor Nrf-2 activation is the primary defense against cellular oxidative stress. Nrf-2 translocates in the nucleus after the dissociation from keap1 and binds to antioxidant response element (ARE) and regulates the transcription of HO-1 protein in LPS-induced RAW 264.7 cells (Lee et al., 2011). HO-1 is known as an antioxidant protein. Inducible HO-1 inhibits the excessive production of TNF-α and IL-1β through Nrf-2 activation in response to LPS-induced RAW 264.7 cells (Tsoyi et al., 2009). This study revealed that all four compounds (eugenol, methyl eugenol, estragole, and pulegone) significantly up regulate HO-1 protein expression. Eugenol showed higher up regulatory activity among four tested compounds. Likewise, Nrf-2 protein expression also significantly up-regulated by these four selected compounds.

Additionally, in this study, molecular docking simulation was also performed to evaluate the binding affinity and binding difference among four

selected compounds towards iNOS. Computational docking studies exhibited important information to understand the ligand-protein interaction and active site residues (Mane et al., 2011, Pawar et al., 2010). Autodock 4.2 docking program used to predict the binding sites and energy after in vitro experiment of iNOS. The catalytic activity of an enzyme molecule can be assessed by their hydrogen bonds and hydrophobic interactions in a docking (Robert, 1998). These docking studies exhibit that monoterpene pulegone showed lowest binding energy (-6.97 kcal/mol) with highest binding affinity compared to other three phenylpropene regarding iNOS inhibition. Reported inhibitor ethyl isothiourea (ITU) showed -4.81 kcal/mol with 2 hydrogen bond interactions. Although pulegone did not show any hydrogen bond interaction toward iNOS active sites but several hydrophobic interactions are appeared in the active site. On the other hand, among eugenol, methyl eugenol, and estragole to iNOS binding complex, the only eugenol showed one hydrogen bond interaction with Trp372 residue. These three compounds showed almost similar binding affinity towards iNOS. Hence, hydrophobic interaction plays an important role which may differentiate binding affinity into iNOS active site residues. Interestingly, all four tested compounds

with reported inhibitor ITU showed hydrophobic interaction with cofactor heme (Hem510).

Taken together, the *in vitro* cell line assay for MTT, NO production, ROS generation, the Western blot analysis for the inhibition of iNOS, COX-2, NF-κB, MAPKs protein expression and the up-regulation of HO-1/Nrf-2 signaling pathways along with molecular docking studies for iNOS inhibition revealed the selected phenylpropene namely, eugenol, methyl eugenol, and estragole, and selected monoterpene called pulegone may be useful for the development of anti-inflammatory therapeutics.

5. Conclusion

In conclusion, these findings demonstrated that eugenol, methyl eugenol, estragole, and pulegone significantly inhibit the production of inflammatory mediators and up-regulate antioxidant protein expression in LPS-stimulated RAW 264.7 cells. Moreover, the inhibitory actions of eugenol, methyl eugenol, estragole, and pulegone are likely associated with the regulation of NF-κB, MAPKs, and HO-1/Nrf-2 signaling pathways. In addition, monoterpene pulegone showed higher overall down regulatory and up regulatory activity compared to rest of three phenylpropenes, as well as molecular docking confirmed that pulegone also showed a strong binding affinity towards iNOS. Verification of anti-inflammatory action of eugenol, methyl eugenol, estragole, and pulegone and justifying relative mechanisms in *in vivo* models will be beneficial for application of eugenol, methyl eugenol, estragole, and pulegone as therapeutic agents for inflammatory diseases.

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