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Antioxidant and nitric oxide inhibition activities of myristica fragrans essential oil in RAW 264.7 Cells

Aktivitas antioksidan dan penghambatan nitric oxide dari minyak atsiri Pala (Myristica fragrans) pada sel RAW 264.7

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Abstract

The excessive production of free radicals, such as Nitric Oxide (NO), initiates several diseases. Compounds of nutmeg oil (MFEO) have been reported to exhibit antioxidant activity. This study aimed to evaluate the antioxidant activity, cytotoxicity, and NO inhibitory potential of MFEO. This experimental study was conducted using the MFEO obtained from plantations in South Aceh Regency, Indonesia. The antioxidant activities were tested using the ABTS and FRAP tests, cytotoxicity test used micro-tetrazolium solution and NO production inhibition were carried out on RAW 264.7 cells used Griess Reagent. The IC50 values were determined using linear regression statistical analysis ($p<0,05$). Results, the IC50 values of MFEO using the ABTS method were 8,400 ppm and 24,949 ppm, respectively, using the FRAP method. These results indicate that MFEO has a very weak antioxidant activity compared to Trolox and ascorbic acid. An MFEO concentration of 250 ppm showed a cell viability of more than 50% (75,57%), and the ability to inhibit NO production began to be shown at an MFEO concentration of 50 ppm (7,5%). The antioxidant activities of MFEO were very weak and MFEO concentrations starting from 50 ppm inhibited NO production, and up to a concentration of 250 ppm, more than 50% viability of RAW 264.7 cell. In conclusion, MFEO needs higher concentrations than Trolox and ascorbic acid for strong antioxidant effects. At lower doses, it remains safe for RAW 264.7 cells and effectively reduces NO production.

Keywords: Myristica fragrans, nitric oxide production, RAW 264.7 cells, cytotoxicity

Abstrak

Produksi radikal bebas seperti Nitric Oxide (NO) yang berlebihan menginisiasi beberapa penyakit. Senyawa dalam minyak pala (MFEO) dilaporkan memiliki aktivitas antioksidan. Penelitian bertujuan mengevaluasi aktivitas antioksidan, sitotoksisitas, dan potensi penghambatan produksi oksida nitrat dari MFEO. Penelitian ini menggunakan minyak pala (MFEO) dari Kabupaten Aceh Selatan. Aktivitas antioksidan MFEO diuji dengan metode ABTS dan FRAP, selanjutnya dilakukan uji sitotoksisitas dan uji inhibisi produksi NO pada sel RAW 264.7. Uji sitotoksisitas menggunakan larutan mikrotetrazolium dan uji inhibisi produksi NO menggunakan Reagen Griess. Selanjutnya dinilai IC_{50} dengan uji statistik regresi linier (nilai $p<0,05$). Hasil, nilai IC_{50} MFEO dengan metode ABTS adalah 8,400 ppm dan 24,949 ppm dengan metode FRAP. Hasil ini menunjukkan MFEO memiliki aktivitas antioksidan sangat lemah dibandingkan dengan *trolox* dan asam askorbat. Pada konsentrasi MFEO 250 ppm, viabilitas sel >50% (75,57%) dan kemampuan menginhibisi produksi NO mulai ditunjukkan pada konsentrasi MFEO 50 ppm (7,5%). Aktivitas antioksidan MFEO sangat lemah dan konsentrasi MFEO mulai 50 ppm mampu menginhibisi produksi NO dan hingga konsentrasi 250 ppm viabilitas sel >50%. Kesimpulan, MFEO

membutuhkan konsentrasi yang lebih tinggi daripada Trolox dan asam askorbat untuk efek antioksidan yang kuat. Pada dosis yang lebih rendah, tetap aman untuk sel RAW 264.7 dan secara efektif mengurangi produksi NO.

Kata Kunci: Myristica fragrans, produksi nitrit oksida, sel RAW 264.7, sitotoksisitas

Introduction

Free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), are by-products of normal metabolic processes, but their overproduction can lead to oxidative stress, contributing to cellular damage and disease (Pizzino et al., 2017). Nitric oxide (NO) is a free radical in the form of a gas molecule that acts as a non-specific immune response to eliminate pathogens (Lundberg & Weitzberg, 2022). Nitric oxide is produced by inducible nitric oxide synthase (iNOS), which is influenced by inflammatory reactions (Cinelli et al., 2020). Stimulation of macrophages by lipopolysaccharide (LPS) stimulates gene transcription, resulting in increased secretion of nitric oxide synthase (NOS) and NO (Jung et al., 2015). NO is a free radical that acts as a vital signalling molecule and regulates processes such as vasodilation and immune responses. While excessive NO production can contribute to oxidative stress and inflammation, its controlled release plays an essential role in cellular communication and defence (Piacenza et al., 2022).

These conditions that have been described require antioxidant compounds that can remove free radicals and inhibit NO production(Kang et al., 2022). Antioxidants break the chain reactions of free radicals, which are formed by providing electrons without becoming free radicals (Munteanu & Apetrei, 2021). Antioxidants can inhibit the formation of free radicals in cellular sources, and repair and modify damage (Chatterjee, 2016). Antioxidants can be produced by the body (endogenous) and are found in various sources of biological wealth, such as spice plants (Abeyrathne et al., 2022).

Nutmeg (*M. fragrans*) is a tropical plant native to Southeast Asia, particularly in Indonesia, containing bioactive compounds such as myristicin and eugenol, methyl eugenol, and other monoterpene and sesquiterpene compounds (Ashokkumar et al., 2022). The diversity of MFEO depends on soil type, location, plant origin, environmental conditions, and extraction methods (Nikolic et al., 2021).

Various in vitro testing methods have been developed to measure the cellular antioxidant capacities of medicinal plants (Munteanu & Apetrei, 2021). Inhibitors of NO production and cytotoxicity tests can be carried out on cell lines stimulated by LPS in culture media (Merly & Smith, 2017). RAW 264.7, a type of LPS-stimulated macrophage cell line from male Mus musculus ascites tumor tissue, was induced with murine Abelson leukemia virus in immunological studies to imitate the inflammatory process (Dong et al., 2017).

The progress of medicine in Indonesia depends on the utilization of biological riches, such as nutmeg, which are often used in medicine (Wongrakpanich et al., 2018). Free radicals are more interesting for the prevention and management of diseases at the molecular level (Di Meo & Venditti, 2020). A scientific study reported that the secondary phytochemical compound MFEO has antioxidant activity and can inhibit free radicals (Zhang et al., 2016). This study is important because it explores the possible therapeutic application of Myristica fragrans essential oil (MFEO) to reduce inflammation and oxidative stress, which are two factors linked to a number of chronic diseases. Considering an alternative option for synthetic medicines, MFEO shows promise for future pharmacological use. Therefore, we aimed to prove the effect of MFEO as a free radical scavenger and inhibitor of NO production in RAW 264.7, to evaluate the antioxidant activity, cytotoxicity, and NO inhibition potential of MFEO. This study presents an innovative approach to assess how MFEO affects NO production and cytotoxicity in RAW 264.7 cells. By investigating the ways in which MFEO may reduce inflammation and oxidative stress, we offer more evidence of its potential as a therapy.

Methods

This study is an experimental laboratory study using MFEO samples to assess antioxidant activity through ABTS, FRAP, cytotoxicity tests, and inhibition of NO production in RAW 264.7

cells. M. fragrans seeds were obtained from plantations in Air Berudang village, Tapak Tuan District, South Aceh, Aceh Province. The nutmeg plantation in the South Aceh Regency is situated between 02° 23' 24″–03° 44' 24″ N and 96° 57' 36″–97° 56' 24″ E. The MFEO distillation was conducted at the Atsiri Research Center, Syiah Kuala University. Antioxidant ABTS and FRAP tests, cytotoxicity tests, and NO production inhibitor tests were performed in Invilab Bogor, West Java Province, Indonesia. This study was conducted from September 2023 to December 2023.

Chemical and Reagents

The majority of chemicals used for extraction and characterization were of analytical grade and procured from Sigma-Aldrich (Castle Hill, NSW, Australia). ABTS and potassium persulfate were purchased from Sigma-Aldrich (St.Louis, MO, USA). Louis, MO, USA). Louis, MO, USA). Ethanol, sodium acetate, ferric chloride, and acetic acid were acquired from Thermo Fisher Scientific (Waltham, MA).

Plant Extract Prosedure

The extraction technique was conducted using the steam distillation method with 2000 g of nutmeg seeds that had been cleaned with distilled water. The extraction procedure was performed as previously described, with some modifications (Angilia et al., 2024).

Cell Culture Materi*al*

RAW 264.7 cells were acquired from the European Collection of Authenticated Cell Culture (EAACC) and were obtained from tumor ascites fluid in male mice that had been injected intraperitoneally with Abelson's Murin leukemia.

Cell Culture Condition

RAW 264.7 cells were grown in DMEM and maintained at 37°C in a 5% CO_2 environment. The living cells were diluted to a concentration of 1×10^5 cells/mL. The cells were cultured in a 96-well microplate and allowed to adhere for 1 h until the supernatant was obtained. The supernatant containing live cells was collected, washed with PBS, and added to each well of a microplate. The wells were exposed to MFEO samples, positive controls, and LPS, and incubated in a 5% CO_2 environment at 37°C for 24 h. Nitric oxide (NO) production was measured.

Antioxidant Radical Scavenging Activity (ABTS) Test

Antioxidant activity was assessed using the ABTS test with some modifications(Tang et al., 2020). Briefly, 5 mL of a 7 mmol ABTS solution was mixed with 90 µL of 140 mM potassium persulfate, creating a bluish-green radical cation (ABTS+) when left in the dark for 16 h at 25° °C. This solution reached maximum absorption at a wavelength of 734 nm. The ABTS solution was subsequently diluted with analytical-grade ethanol to reach an initial absorbance of 0.7 at 737 nm. Next, 10 µL of the extract or Trolox antioxidant was combined with 300 µL of the diluted ABTS solution, added to a 96-well plate, and incubated in the dark for 6 min. The antioxidant capacity was represented as mg of Trolox equivalents per gram (mg TE/g dw) of the sample using the calibration curve that was plotted over a concentration range of 0-125 µg/L.

Antioxidant Ferric Ion Reducing Power (FRAP) Test

Antioxidant activity was evaluated using a ferric ion reduction capability assay in accordance with the method outlined by, Tang et al (2020) with slight modifications. The iron-binding reaction produced a deep-sea blue color at an absorbance wavelength of 593 nm and a low pH of 3,6. The procedure started with the preparation of sodium acetate (450 mg, pH 3,6) and was homogenized in 50 ml of distilled water, following adding 4,0 ml of glacial acetic acid and diluting of the solution to a final volume of 250 ml. This produced a solution with acetic acid concentration of 0,30 mol/L. Subsequently, 50 mg of TPTZ was weighed and dissolved in distilled water, followed by the addition 0,17 ml of concentrated hydrochloric acid. The solution was diluted to a final volume of 100 ml, resulting in a TPTZ concentration of 10 mM.

In addition, 270 mg of $FeCl_3$ was dissolved in distilled water and diluted to a final volume of 50 ml, resulting in a concentration of 20 mmol/L. Acetic acid buffer, TPTZ solution, and $FeCl_3$ solution were mixed at a ratio of 10:1:1. This mixture was incubated at 37°C and was required to be used within 1-2 hours. For the testing procedure, 180 µL of FRAP working solution and 5 µl of the sample were added to a 96-well plate, thoroughly mixed, and incubated at 37°C in the dark for an hour. Absorbance was determined at a wavelength of 593 nm, and

FRAP findings were expressed as mg of ascorbic acid equivalents per gram (mg AAE/g dw) of MFEO.

Cytotoxic Activity Test

The viability of RAW cell 264.7 was assessed using the micro-tetrazolium (MTT) technique following a method based on the modified protocol(Kumar et al., 2018). This technique involves incubation of the sample at -20°C, and the cup is prepared to make a dilution solution until the total volume of the MFEO sample is 20 mL for the round-bottom plate and 30 mL for the flat-bottom plate before the given cells. The total volumes of the cell suspension and sample were 100 mL and 150 mL for the round-and flat-bottom plates, respectively. After adjusting the incubation time, the MTT solution was added at a ratio of 1:10 (5 mg/mL). The plate was shaken for 5 min on a plate shaker at a maximum rate of 900 shakes/min. The plates were incubated at 37°C for 4–6 h in a CO_2 incubator.

Cells were exposed to increasing concentrations of the MFEO extract, ranging from 0 to 200 µg/ml, and their viability was assessed after 24 h using a microplate reader set to 570 nanometers (nm). Dimethyl sulfoxide (DMSO) at a concentration of 0,75% was used as the negative control. The optical density was measured within a wavelength range of 540–720 nm, with excitation wavelengths of 540-570 nm and emission wavelengths–580-610 nm. Cells were counted and diluted to a density of 1×10 cells/ml in 96-well plates to evaluate cell viability.

The percentage of viable cells was calculated using the following equation:

$$\text{Cell Viability}(\%) = \frac{\text{OD of treated samples}}{\text{OD of untreated samples}} \text{ x } 100$$

Information:
Cell Viability = Percentage of alive samples (%)
OD = Optical density

NO Production Inhibitor Test

This will be carried out according to the protocol described with some modifications (Divate & Chung (2017). MFEO was administered at a certain concentration in a volume of 50 µL/well and reacted with Griess reagent, which consists of 1% solution, sulphanilamide, 2% phosphoric acid, and 0,1% naphthyl-ethylenediamine-dihydrochloride (NED) (50 µL), which was also added to the 96 well-microplate and incubate at room temperature for 5-10 minutes. Absorbance readings were recorded at 570 nm. The percentage inhibition of NO production was calculated using the following equation and compared with the positive control:

$$\text{NO Production Inhibition } (\%) = \frac{\mathbf{(Ac-Ae)}}{\mathbf{Ac}} \text{ x } 100$$

Information:
Ac = Absorbance of control reaction
Ae = Absorbance in the presence of the sample

A Diagram of the research is shown in Figure 1 below.

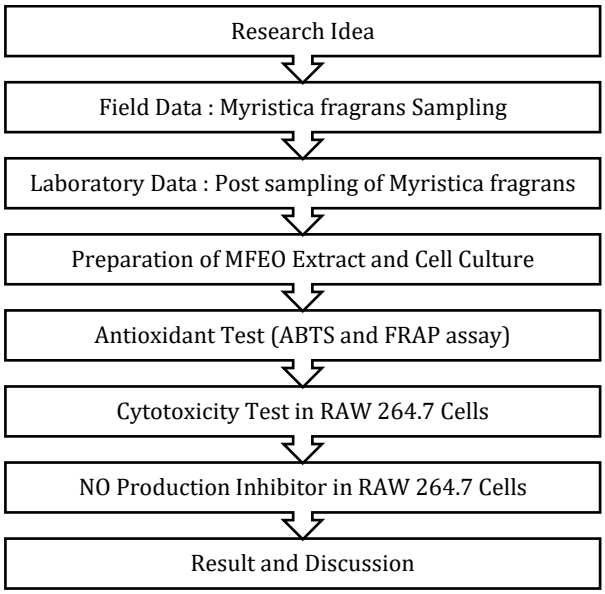

Figure 1. Flowchart Diagram of Research

Data analysis

Statistical data analysis was performed using linear regression with a 95% confidence interval (CI) and significance level of $p<0,05$.

Result and Discussion

Antioxidant ABTS Test

The results of MFEO Antioxidant tests using ABTS are shown in Table 1. This study showed that the IC50 MFEO value was 8,400 ppm, whereas the IC50 Trolox value was smaller at 87,8 ppm. This shows that the antioxidant ability of Trolox is stronger than that of MFEO. The IC50 value is the concentration of an antioxidant substance that causes a 50% loss of its radical character.

Table 1. MFEO antioxidant test using ABTS test

MFEO Concentration (ppm)	% inhibition	IC_{50} (ppm)
5,000	34,45±1,98	
10,000	60,33±1,10	
15,000	67,45±0,38	8,400
20,000	87,86±0,73	
25,000	91,98±0,27	

The results of the MFEO study above are in contrast to previous bionanocomposite studies by Amina et al. (2021) using MFEO extracted with ZnONP and polyutherane (PU) media through the ABTS test, which showed very good antioxidant potential with an IC_{50} value (0,49 of 0,36). This superior antioxidant activity was attributed to the presence of major phytochemicals including myristicin (33,25%), terpene hydrocarbons (40,58%), and terpene derivatives (8,45%) (Amina et al. 2021).

The results of the Trolox antioxidant assay using ABTS are shown in Table 2**.**

Table 2. Trolox antioxidant test using ABTS test

Trolox Concentration (ppm)	% inhibition	IC_{50} (ppm)
0	5,36±1,48	
1,953125	4,44±4,24	
3,90625	6,39±1,65	
7,8125	8,24±1,32	87,8
15,625	12,90±3,55	
31,25	20,90±2,64	
62,5	38,54±0,59	
125	72,45±2,35	

Another study Antasionasti et al (2021) using M. fragrans fruit flesh extract reported the ability to capture ABTS radicals at 89,98 ppm which is categorized as a strong antioxidant (Antasionasti et al., 2021). A Research by Li et al. (2020) also explained that in the ABTS antioxidant test using different solvents, the ethanol solvent had an IC_{50} value of 27,68 ppm, followed by methanol solvent IC_{50} 117,66 ppm, acetone solvent IC_{50} 64,35 ppm, dichloromethane IC_{50} 82,31 ppm and ethyl acetate solvent IC_{50} 91,19 ppm(Li et al., 2020). Research has shown that methanol, ethanol, and acetone exhibit relatively high antioxidant activity. These results highlight that solvents with higher polarities, such as methanol and ethanol, tend to exhibit greater antioxidant activity than solvents with lower polarities, such as n-hexane, chloroform, and dichloromethane (Li et al., 2020).

A study by Ibrahim et al. (2020), using distillation time (DT) to produce MFEO fractions with different chemical compositions and bioactivities, found that the phytochemical variations at the desired concentrations, such as the percentage concentration of α-thujene, α-pinene, camphene, sabinene, α-phellandrene, 3-carene, p-cymene, limonene, β-pinene, and myrcene compounds, decreased. The percentage concentrations of myristicin, α-terpinene, terpinolene, and γ-terpinene increased with subsequent distillation times. These findings can be utilized by industries that use MFEO to sell products (Ibrahim et al. 2020).

The ABTS antioxidant test uses a radical scavenging mechanism in an intense green reaction with ABTS radicals. A decrease in the green color indicates the effectiveness of the antioxidant (Suthisamphat et al., 2020). However, antioxidant activity is also influenced by other factors, such as the extraction method, solvent extraction, and environmental conditions (Sultan et al., 2023).

Environmental conditions in which M. fragrans affects the distribution of plant vegetation and the content of active compounds in herbal plants. More than 80% of phytochemical compounds can be used as medicines for the active compounds in plants (Almarfadi et al., 2022).

Antioxidant FRAP Test

The results of MFEO Antioxidant tests using FRAP are shown in Table 3. The IC_{50} value of MFEO was 24,949 ppm, while the IC_{50} value of ascorbic acid was 92 ppm. The IC_{50} value showed that MFEO required a relatively high concentration to produce a significant antioxidant effect compared to ascorbic acid. A lower IC_{50} value indicates stronger antioxidant activity. The IC_{50} value represents the concentration of antioxidant compounds required to reduce the free radical activity by 50%, serving as a key parameter for assessing antioxidant potency.

Research Imran et al (2024) using the FRAP test revealed an MFEO of 14,11 µg compared with seed flowers 10,71 µg and ascorbic acid 253,5 µg. Another research by Trifan et al. (2023) also explained the

antioxidant ability of M. fragrans found that the FRAP reduction potential of nutmeg seeds was 108,11 TE/g followed by nutmeg extract 105,28 mg TE/g and nutmeg essential oil 86,52 TE/g.

Table 3. MFEO antioxidant test using FRAP test

MFEO Concentration (ppm)	Absorbance	FRAP (Ascorbic Acid eq.)	IC_{50} (ppm)
1000	0,12±0,00	3,25±0,4	24,95
750	0,11±0,00	2,52±0,2	
500	0,10±0,00	1,66±0,1	
250	0,08±0,00	0,53±0,1	
0	0,07±0,00	-0,34±0,0	

The antioxidant activity of ascorbic acid, as evaluated through the Ferric Reducing Antioxidant Power (FRAP) assay, is comprehensively presented in Table 4, illustrating its reducing capacity under the specified experimental conditions.

Table 4. Ascorbic acid antioxidant test using FRAP method

Ascorbic Acid Concentration (ppm)	Absorbance	IC_{50} (ppm)
0	0,07 ± 0,00	92
15,625	0,30 ± 0,00	
31,25	0,53 ± 0,00	
62,5	0,98 ± 0,02	
125	1,87 ± 0,04	
250	2,80 ± 0,02	
500	2,74 ± 0,03	
1000	2,712 ± 0,02	

The FRAP test evaluates the ability of a substance to reduce Fe_{3+} to Fe_{2+}. The color changes that occur during this reaction can be measured using spectrophotometry at certain wavelengths in relation to the amount of Fe_{2+} and antioxidant capacity in the sample (Rumpf et al., 2023). The composition of essential oils is influenced by both biotic and abiotic factors, such as agronomic factors including climate, soil type, soil moisture, cultivation practices, insect and pathogen interactions, harvest timing, storage conditions, and drying of plant material prior to extraction (Trifan et al., 2023).

GC-MS analysis identified myristicin (13,06%) as the primary active compound, with potential antioxidant effects against oxidative stress. Similarly, another study Assa et al. (2014) found that M. fragrans extracts prepared using acetone and ethanol demonstrated antioxidant activities in the FRAP assay, attributed to the presence of myristicin, eugenol, and sabinene. The composition of essential oils can vary significantly, often consisting of more than 100 distinct compounds (Ivanović et al., 2021).

M. fragrans contains secondary metabolite compounds, such as phenolics, terpenoids, and alkaloids, that interact with each other between covalent and non-covalent bonds with molecular targets (Do Nascimento et al., 2020). The simplification of complex compounds from plant essential oils into single compounds through chromatographic techniques can produce semi-pure compounds that are more complementary (Abeyrathne et al., 2022). However, in traditional medicine, more complex essential oils are used than purified compound molecules (Suthisamphat et al., 2020).

Cytotoxic Activity Test

The cytotoxicity test results of MFEO on RAW 264.7 the micro-tetrazolium method are presented in Table 5.

Table 5. Cytotoxicity test of MFEO on RAW 264.7 cells using micro-tetrazolium method.

MFEO Concentration (ppm)	% viability
31,25	94,41±3,9
62,5	93,8±4,7
125	81,11±0,8
250	75,57±2,0
500	18,1±1,4
1000	17,26±6,1

The table indicates a significant reduction in the number of viable RAW 264.7 cells with more than 50% decrease observed at an MFEO concentration of 500 ppm (18,1%). However, MFEO concentrations below 250 ppm (75,57%) did not negatively affect cell viability, as determined by the micro-tetrazolium staining test. Suthisamphat et al. (2020) which looked at the effect of administering M. fragrans aryl extract using ethanol and water solvents and showed cell viabilities of 103,66% and 113,61%, respectively. This study showed that ethanol has higher anti-inflammatory and cytotoxic activities than those of water. In another cytotoxicity

study of M. fragrans using myristicin compounds as anti-inflammatory standards, the methanol extract of M. fragrans 12,5-200 µg/mL for 24 h showed that the toxicity of the methanol extract was stronger than that of MFEO (100 µg/mL) and myristicin compounds (200 µg/L), which could affect the viability of RAW 264.7 cells (Khamnuan et al., 2023). This suggests that myristicin is not the main anti-inflammatory bioactive compound present in nutmeg. Testing of isolated substances from fractionated extracts is still required to determine the main active compounds in nutmeg that have anti-inflammatory properties (Sultan et al., 2023).

Administration of MFEO at high concentrations causes poisoning with symptoms such as intense flushing, visual disturbances, dry mouth, elevated blood pressure, rapid heart rate, nausea, vomiting, euphoria, confusion, restlessness, abdominal discomfort, and sensory experiences without external stimuli (Yang et al., 2018). Volatile oils are considered to have low toxicity, although the use of essential oils and their co-constituents in pregnant women and during fetal development is still questionable. This is associated with potential risks, such as miscarriage, hormonal disruptions, maternal toxicity, teratogenic effects, and fetotoxicity in developing embryos (Dosoky & Setzer, 2021).

NO Production Inhibitor Test

The inhibitory effect of MFEO on nitric oxide (NO) production in RAW 264.7 macrophage cells was evaluated and is systematically presented in Table 6, demonstrating its potential anti-inflammatory properties under experimental condition.

Table 6. NO production inhibitor test

MFEO Concentration (ppm)	NO Production (µM)	% Inhibition
200	8,5 ± 0,3	29,4
100	10,2 ± 0,8	15,7
50	11,2 ± 0,1	7,5
25	12,4 ± 0,4	0,0
12,5	12,4 ± 0,4	0,0
6,25	12,5 ± 0,3	0,0
Control Media	8,3 ± 0,2	0,0
Media +LPS	12,1 ± 0,8	0,0

The research finding indicated that the administering of MFEO began to inhibit production of NO in RAW 264.7 cell at concentrations of 50 ppm (11,2 µM), 100 ppm (10,2 µM) and 200 ppm (8,5 µM), achieving inhibition rates of 7,5%, 15.7% and 29,4% respectively. In comparison, the control medium produced NO at a concentration of 8,3 µM, whereas LPS induced NO production without MFEO treatment at 12,1 µM. Previous studies demonstrated that myrisligan compounds isolated from MFEO can significantly inhibit NO production in RAW 264.7 cells. At doses of 6,25, 12,5, 25, and 50 mg/mL, myrislignans showed inhibition rates of 4,9%, 13,6%, 45,3%, and 64,3%, respectively. Myrislignan, a phytochemical found in M. fragrans, has been shown to inhibit the protein and mRNA expression of cyclooxygenase-2 and inducible iNOS in LPS-stimulated macrophages, indicating strong anti-inflammatory properties through the suppression of the NF-kB signaling pathway (Sultan et al., 2023).

NO is a free radical that is involved in various biological processes, including the regulation of vascular inflammation. When produced in excess, NO can damage nucleic acids, proteins, and lipids, leading to changes in membranes and plasma lipoproteins. This triggers the occurrence of arteriosclerosis plaque, dyslipidemia, and other diseases related to radical damage, such as rheumatoid arthritis (Lundberg & Weitzberg, 2022). Another study using Imran et al. (2024) in vivo inflammation studies in rat models for the treatment of rheumatoid arthritis (RA) using mace essential oil and M. fragrans seeds showed a significant decrease in inflammation ($p < 0,005$), which could potentially reduce inflammation and the recommendations for RA management. Another study using MFEO has the potential to be a chronic pain reliever because it can alleviate allodynia, joint swelling, and pain in mice with the injection of complete Freund 's adjuvant (CFA). Administration of 20 mg/kg BW MFEO significantly reduced swelling and decreased pain scores compared to the group receiving 30 mg/kg BW diclofenac (Zhanga et al., 2015).

Additionally, Vangoori et al (2018) we conducted an anti-dyslipidemia study using M. fragrans extract on normal and severely obese albino Wistar rats over 35 days. They found that the administration of 400 mg/kg M. fragrans

extract significantly reduced dietary intake and body mass. In the severely obese group, which was induced to obesity for the first five weeks and then treated with 200 and 400 mg/kg doses for the last five weeks, there was a notable decrease in body mass and dietary intake in the last five weeks. These results support the clinical value of M. fragrans extract in obesity treatment owing to its ability to inhibit hunger and body mass through the inhibition of pancreatic lipase (Vangoori et al., 2018).

Essential oils can act by adding hydrogen atoms and inhibiting free-radical chain reactions through their antioxidant properties(Imran et al. 2024). They can be used as complementary treatments through massage, inhalation, topical application, or oral ingestion (Thangaleela et al., 2022). The anti-inflammatory effects of essential oils are largely attributed to their bioactive compounds, such as monoterpenes and phenolic groups, which exhibit significant antioxidant activity (Gunathilake et al., 2018). Previous studies have consistently supported the potent antioxidant potential of MFEO (Matulyte et al. 2020).

Given their low molecular weight and high skin penetration, essential oils can enhance topical treatments by accelerating healing and reducing oxidative stress, which serves as an anti-inflammatory marker (Imran et al., 2024). However, the complex nature of the crude M. fragrans seed extract and its diverse active compounds means that high concentrations are required to achieve substantial antioxidant effects.

This study has various limitations, including results from in vitro studies that should be interpreted carefully because they might not accurately represent the effects that occur in living organisms (in vivo), and MFEO constituents may vary based on extraction techniques and the environment, which might influence the reproducibility of the results.

Conclusion

The ABTS and FRAP assay results indicated that MFEO requires relatively higher concentrations than Trolox and ascorbic acid antioxidants to achieve significant antioxidant effects. At lower concentrations, MFEO did not compromise the viability of RAW 264.7, and could effectively inhibit NO production.

We propose further studies to enhance the antioxidant and anti-inflammatory properties of MFEO by optimizing the extraction techniques and isolating its active ingredients. Furthermore, in vivo studies may represent the focus of future research to confirm the therapeutic effectiveness and safety of MFEO at various dosages.

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