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Anti-oxidative and anti-hyperglycemic properties of *Agastache foeniculum* essential oil and oily fraction in hyperglycemia-stimulated and lipopolysaccharide-stimulated macrophage cells: In vitro and in silico studies

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ABSTRACT

Ethnopharmacological relevance: Hyperglycemia (HG) and lipopolysaccharide (LPS) often promote superoxide accumulation, which may increase oxidative stress. Reducing superoxide production in hyperglycemia and the inflammatory condition is an emerging way to reduce protein and lipid oxidation and diabetes complication.
Aim of study: To examine the effect of *Agastache foeniculum* essential oil (AFEO) and oil fraction (AFoil) on HG- and LPS-stimulated oxidative stress, the pathogenicity of AFEO and AFoil on oxidative stress was assessed.
Methods: The stimulatory effects of AFEO and AFoil on the activity and expression of NADH oxide (NOX), catalase (CAT), superoxide dismutase (SOD), and the expression of nuclear respiratory factor 2 (NRF2) and nuclear factor-kappa B (NF-kB) in the stimulated macrophage cell line, J774.A1, was studied. The interaction patterns of AFEO and AFoil components with NOX, SOD, CAT, NRF2, and NF-kB proteins were also deduced using molecular docking.
Results: Estragole was the main ingredient in AFEO (97%). Linolenic acid (32.10%), estragole (16.22%), palmitic acid (12.62%), linoleic acid (12.04%), and oleic acid (8.73%) were the major chemical components of the AFoil. NOX activation was stimulated in macrophage cells by HG and LPS. At 20 μg/mL, AFEO and AFoil decreased NOX activity while increased SOD and CAT activities in stimulated macrophages. AFoil with estragole and omega-3 fatty acids was better than AFEO with estragole in anti-hyperglycemic and anti-oxidative activity. According to molecular docking research, estragole, linoleic acid, and linolenic acid bind to different hydrophobic pockets of NOX, SOD, CAT, NFR2, and NF-kB using hydrogen bonds, van der Waals bonds, pi-alkyl, and pi-anion interactions, with different binding energies.
Conclusion: AFEO and AFoil showed antioxidant and anti-diabetic activity. The mechanisms in lowering oxidative stress markers depended on down-regulating superoxide-producing enzymes and up-regulating superoxide-removing enzymes at gene and protein levels. The AFoil emulsion can be used to reduce the detrimental impacts of hyperglycemia and oxidative stress.

1. Introduction

Obesity, diabetes, cancer, respiratory illness, and cardiovascular disease are all common chronic diseases in today's world, and metabolic dysregulation plays a key role in their pathogenesis. Diabetes mellitus is one of the most severe multifactorial diseases, with serious symptoms such as hyperglycemia, hyperlipidemia, oxidative stress, and protein glycation (Oguntibeju, 2019). Increased glucose flux via hexosamine path, increased glucose flux via polyol path, increased protein kinase C (PKC) activation, increased protein glycation, increased protein oxidation, and increased lipid oxidation are all areas in that hyperglycemia facilitates superoxide accumulation (Zhang et al., 2019). Superoxide is generated by healthy cellular metabolisms, such as respiration and macrophage stimulation. In animal cells, the superoxide generating enzyme (NADPH oxidase, NOX) is the most important contributor to superoxide production, particularly in some pathological conditions (Moldogazieva et al., 2020). Superoxide oxidizes a variety of

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Abbreviations

HG	Hyperglycemia
LPS	Lipopolysaccharide
AFEO	*Agastache foeniculum* essential oil
AFoil	*Agastache foeniculum* oil
NOX	NADH oxide
CAT	Catalase
SOD	Superoxide dismutase
NRF2	Nuclear respiratory factor-2
NF-kB	nuclear factor-kappa B
PKC	Protein kinase C
PUFA	Polyunsaturated fatty acid
GC-MS	Gas chromatography-mass spectrometry
DMEM	Dulbecco modified eagle's medium (DMEM)
FOX	ferrous oxidation-xylenol orange
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
EDTA	Ethylenediaminetetraacetic acid
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GMS	Glycerol monostearate
MAPK	Mitogen-activated protein kinases
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
IkB	Inhibitor of kappa B
Keap1	Kelch-like ECH-associated protein 1

biomolecules, causing an increase in cellular oxidative stress and, ultimately, cellular death. Superoxide-induced cell death and disruption of the oxidants/antioxidants balance seem significant in pathological degenerative diseases such as diabetes and metabolic syndrome (Chukwuma et al., 2019).

To counterattack oxidative stress, the healthy body relies on the endogenous non-enzymatic (ascorbic acid, tocopherol, glutathione, carotenoid, and lipoic acid) and enzymatic (superoxide dismutase, peroxidase, catalase, peroxidases, and S-nitrosoglutathione reductase) antioxidant network (Viana da Silva et al., 2021). In some pathological disorders, such as metabolic syndrome, an endogenous antioxidant mechanism is overloaded, so the human body needs exogenous and natural antioxidants from edible plants and diet to keep free radicals at low levels (Seidkhani Nahal et al., 2019). Polyphenol compounds from medicinal plants are attracting growing scientific attention due to their potential beneficial effects on human health. Essential oils and phenolic compounds are inversely linked to hyperglycemia cases, according to the findings of epidemiological and experimental research (Lee et al., 2020). Polyphenols, flavonoids, alkaloids, and condensed tannins have been linked to various biological functions, including antioxidant potential against glucose, lipid, and protein oxidation (Koch, 2019). Polyphenol compounds in food slow down the progression of coronary, diabetes, and neurodegenerative diseases (Di et al., 2021).

However, only a few studies have looked into the roles of omega-3 and omega-6 polyunsaturated fatty acid (PUFA) in oxidative stress and diabetes pathogenesis. The essential fatty acids, linolenic acid, and linoleic acid are the precursor of omega-3 and omega-6 fatty acids, respectively. Via suppressing nuclear factor-kappa B (NF-kB) and enhancing nuclear respiratory factor-2 (NRF2) signaling pathways, omega-3 PUFA has been exhibited anti-inflammatory activities in the presence of various inflammatory conditions (Shi et al., 2020). Anti-inflammatory eicosanoids, neuroprotectant eicosanoids, and resolvins are all generated by omega-3 fatty acids (Bi et al., 2019). Proinflammatory mediators such as prostaglandins, leukotrienes, and thromboxane are formed from omega-6 fatty acids (Koletzko et al., 2020).

Agastache foeniculum (giant hyssop) is a Lamiaceae perennial plant that has long been used traditionally by Native American and Indian peoples as a medicinal plant for gastrointestinal, cardiovascular, and nervous treatments, as well as pain, cold, and fever (Ivanov et al., 2019; Matei et al., 2010). *Agastache* leaves, and flowers were used as poultice and infusions to treat blood cough, fever, heart conditions, and externally in treating burns (Najar et al., 2019). The essential oil of this plant is high in methyl chavicol (estragole), which gives it antioxidant, anti-inflammatory, antifungal, and antibacterial properties (Zielińska and Matkowski, 2014). Traditionally, in Mexico, the infusion obtained from leaves and flowers of this plant or even its essential oil has been shown to have the hypoglycemic capacity and used for diabetes treatment (Andrade-Cetto and Heinrich, 2005). *A. foeniculum* has been the subject of a great deal of research. Despite this, no research on the chemical composition of the oily extract or its biological activities has been released. The effect of *A. foeniculum* essential oil (AFEO) and *A. foeniculum* oil (AFoil) on diabetic oxidative stress pathogenicity has yet to be investigated.

As a result, this research aims to see how AFEO and AFoil affect the expression and function of NADH oxide (NOX), catalase (CAT), and superoxide dismutase (SOD) in hyperglycemia (HG) and lipopolysaccharide (LPS)-induced macrophages. In the treated macrophages, the capacity of AFEO and AFoil to modulate NRF2 and NF-kB expression was investigated. The authors discovered that AFEO and AFoil could alleviate oxidative stress in HG and LPS-stimulated cells and that they could be used as an antioxidant therapy in oxidative stress associated with diabetic and inflammatory responses. The observational results of this research add to our understanding of the possible utility of AFoil containing omega-3 fatty acids in avoiding oxidative stress in LPS- and HG-induced status.

2. Materials and methods

2.1. Preparation and characterization of essential oil

Zardband Pharmaceuticals Company (Tehran, Iran) provided the *Agastache foeniculum* (Kuntze) herbal ingredient. All details on the source, batch number, and purity confirmed by Zardband Pharmaceuticals Company. Experiments were carried out according to professor Reza Omidbaigi at Tarbiat Modares University (Omidbaigi and Mahmoodi, 2010). Professor Mohammad Jamal Saharkhiz from Shiraz University confirmed the plant species. His herbarium received the voucher sample (65110), and he maintains the collection. The AFEO was collected for 3 h using the hydrodistillation method from dry plant materials (100 g). Gas chromatography-mass spectrometry (GC-MS) was used to examine the composition of AFEO. A gas chromatograph system (Agilent 7890B GC 7955AMSD) with HP5MS column (30 m × 0.25 mm × 0.25 μm) and a quadrupole mass spectrometer were used for GC-MC research. The ion source temperature was 220 °C, and the interface temperature was 280 °C. The oven temperature plan was as follows: 4 min at 60 °C, rise to 140 °C at 20 °C/min, rise to 220 °C at 10 °C/min, then hold standby at 220 °C for 10 min. Basic essential oil components were identified using the NIST05 and Wiley 7n mass spectra libraries (Aminizadeh et al., 2020).

2.2. Preparation and characterization of the oil fraction

Plant powders (100 g) are suspended in 1000 mL hydrolysis

containing hydrochloric acid: methanol: normal saline (2:1:1). The samples were carefully combined and incubated for 48 h at 50 °C. After which, 500 mL hexane was added to the hydrolysate mixture and then incubated for 24 h at room temperature to separate fatty acid in the hexane phase. Gas chromatography-mass spectrometry (GC-MS) was used to analyze the fatty acid. An Agilent system (7890B GC 7955AMSD) with an HP5MS column and a quadrupole mass spectrometer is used for GC-MS analysis. The temperature of the ion source and interface was set at 230 °C and 290 °C, respectively. The heat setting for the oven temperature was as follows: 80 °C for 4 min, increasing ratio of 20 °C/min to 140 °C, increasing rate of 10 °C/min to 250 °C, then maintaining the temperature for 10 min. The fatty acid components were identified using the NIST05 and Wiley 7n mass spectra libraries (Yi et al., 2014).

2.3. Preparation of AFEO emulsion

First, to make an AFEO emulsion, combined 1.0 mL (1000 mg) of AFEO with 90 mL purified water. Glycerol monostearate (GMS) (10 mg/mL) was applied to this mixture, then incubated for 2 day at 35 °C before a milky solution (emulsion) was obtained. The AFEO emulsion was calibrated to a final volume of 100 mL. The volatile oil concentration was taken at 10 mg/mL in this state. The essential oil was diluted to 1.0 mg/mL in a culture medium for *ex vivo* antioxidant or anti-diabetic function screening (Siahbalaei and Kavoosi, 2021).

2.4. Preparation of oil (fatty acid) emulsion

10 mL (10 g) of oil fraction was mixed with 80 mL of 10 mM NaOH solution to make an oily fraction emulsion. Glycerol monostearate (10 mg/mL) was applied to this mixture, then incubated at 40 °C for two days before a milky solution (emulsion) was obtained. The oil emulsion was calibrated to a final volume of 100 mL. Under these conditions, the oil concentration was 100 mg/mL. Oil fraction was adjusted to 1.0 mg/mL using a culture medium for antioxidant and anti-diabetic analysis (Siahbalaei and Kavoosi, 2021).

2.5. Assessment of macrophage cell viability

Dulbecco modified eagle's medium (DMEM) enriched with streptomycin (100 μg/mL), penicillin (100 U/mL), glutamine (2.0 mM), and fetal bovine serum (FBS, 10%) was used to grow the hematopoietic mouse macrophage cell line (J774.A1). For 24 h at 37 °C, macrophage cells (1.4×10^4) were planted in the well of tissue culture plates in the presence of essential oil or oil emulsions (0.00–200 μg/mL). The supernatant was then substituted with MTT solution (0.5 mg/mL) then incubated at 37 °C in the dark for 4 h. For dissolve formazan crystals, the MTT solution was substituted with DMSO. Finally, using a microplate reader, the light absorbance of plates was measured at 492 nm (BioTek, USA). The concentrations that were cytotoxic on the cells were not used to evaluate the anti-oxidative and anti-hyperglycemic effects.

2.6. Macrophage cell line culture in the presence of glucose and LPS

As previously mentioned, the mouse macrophage cell line was cultivated in DMEM supplemented with penicillin, streptomycin, glutamine, and FBS. For one day, macrophage cells (2×10^6) were planted in tissue culture plate wells. The adherent cells are treated for 24 h at 37 °C with non-cytotoxic levels of glucose (20 mM) or LPS (1.0 μg/mL) and essential oil (20 μg/mL) or oil (20 μg/mL). Following that, treated cells were extracted for additional experiments such as measuring hydrogen peroxide and NOX, SOD, and CAT activity, as well as RNA extraction (Kavoosi and Teixeira da Silva, 2012).

2.7. Determination of hydrogen peroxide

The ferrous oxidation-xylenol orange (FOX) reagent is used to assess the amount of hydrogen peroxide in the macrophage cell line's culture medium using hydrogen peroxide as standard. The FOX solution contained 110 mM perchloric acid, 0.250 mM xylenol orange, and 0.250 mM ferrous ions. The culture medium (0.9 mL) was mixed with methanol (0.1 mL), and the mixture was incubated at room temperature for 30 min. After adding the FOX reagent (0.9 mL), vertexing was performed, and the mixture was incubated for additional 30 min. Afterwhich, the light absorbance of the mixtures was measured at 560 nm (Li et al., 2017).

2.8. Determination of NOX activity

Sodium dodecyl sulfate solution (1.0%) is used to lyse macrophage cells. NOX activity was measured using a sodium phosphate (100 mM, pH 7.5) reaction solution containing dithiothreitol (1.0 mM) and NADH (100 mM). After adding 500 μL of cell lysate to 500 μL of the reaction solution, the reaction began. Light absorbance at 345 nm was used to monitor the disappearance of NADH. The decomposition of 1.0 μM NADH per minute is known as one unit of NOX activity (Kim et al., 2017).

2.9. Determination of SOD activity

To evaluate SOD activity, the inhibition of nitroblue tetrazolium (NBT) reduction by SOD was determined. The reaction mixture contains sodium phosphate buffer (0.20 mL of 100 mM, pH 7.5), which contains NBT (50 M), EDTA (100 M), sodium carbonate (50 mM), methionine (12 mM), and riboflavin (10 M). Light absorbance was estimated at 560 nm after the cell lysate (0.20 ml) was added to the reaction mix and incubated for 15 min. A decrease in light absorbance corresponds to the percent of SOD activity. One unit of SOD equals the amount of enzyme used to avoid a 50% reduction in NBT (Aminizadeh et al., 2020).

2.10. Determination of CAT activity

The activity of a CAT was measured by measuring the decomposition of hydrogen peroxide in the presence of cell lysate at 240 nm. A sodium phosphate solution (0.50 mL of 100 mM) containing hydrogen peroxide (10 mM) was used in the reaction mixture. After adding 0.50 mL of cell lysate to the reaction mixture and incubating for 10 min, light absorbance at 240 nm was determined. The reduced light absorbance in the cell lysate indicates CAT activity. One unit of CAT is the amount of CAT that decomposes 1.0 μM hydrogen peroxide per minute (Aminizadeh et al., 2020).

2.11. Total RNA and real-time PCR analysis

Total RNA was obtained in RNase-free vials using Cinagen's RNX-plus solution (Tehran, Iran). From 1.0 μg of RNA, first-strand DNA was synthesized using Fermentas' first-strand DNA reagent (Hanover, MD). Real-time PCR analysis was performed using specific primers (Table S1 in the supplemental file). The amplification cycles were completed in the thermal cycler (Line-Gene, Bioer Technology Co., Hangzhou, China). To calculate the relative expression of target and control genes, the threshold cycle (CT) method is used. The Line-gene K program was used to measure the CT value for each sample. The 2-ΔΔCT formula stated in the literature was used to calculate RNA fold-expression (Rao et al., 2013).

2.12. Molecular modeling

The chemspider database was used to download the 3D structures of estragole, linoleic acid, and linolenic acid for molecular simulation. The Protein Data Bank was used to download the 3D structures of NADPH oxidase (1K4U), superoxide dismutase (1EM1), catalase (1F4J), NRF2 (3ZGC), and NF-kB (1A3Q). The Scripps Research Institute's Auto

Table 1
Chemical composition (percent area) of essential oil and oil fraction from *A. foeniculum*.

Compounds	Formula	EO	FA
Pentanoic acid	$C_5H_{10}O_2$	0.00 ± 0.00	0.48 ± 0.03
Benzoic acid	$C_7H_6O_2$	0.00 ± 0.00	0.48 ± 0.04
Salicylic acid	$C_7H_6O_3$	0.00 ± 0.00	0.35 ± 0.02
Estragole	$C_{10}H_{12}O$	97.0 ± 2.25	16.22 ± 1.25
Eugenol	$C_{10}H_{12}O_2$	0.35 ± 0.04	2.36 ± 0.06
Caryophyllene	$C_{15}H_{24}$	1.42 ± 0.05	0.00 ± 0.00
Cadinene	$C_{15}H_{24}$	0.36 ± 0.03	0.00 ± 0.00
spathulenol	$C_{15}H_{24}O$	0.33 ± 0.03	0.00 ± 0.00
Hexadecanoic acid	$C_{16}H_{32}O_2$	0.00 ± 0.00	12.62 ± 1.10
9,12,15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	0.00 ± 0.00	32.10 ± 1.65
9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	0.00 ± 0.00	12.04 ± 1.00
9-Octadecenoic acid	$C_{18}H_{34}O_2$	0.00 ± 0.00	8.73 ± 0.73
Octadecanoic acid	$C_{18}H_{36}O_2$	0.00 ± 0.00	3.71 ± 0.45
Eicosanoic acid	$C_{20}H_{40}O_2$	0.00 ± 0.00	4.94 ± 0.40
Docosanoic acid	$C_{22}H_{44}O_2$	0.00 ± 0.00	3.53 ± 0.37
Tetracosanoic acid	$C_{24}H_{48}O_2$	0.00 ± 0.00	1.80 ± 0.38
Total		99.5 ± 2.30	99.4 ± 2.20
Fatty acid		0.00 ± 0.00	79.95 ± 1.86
Terpenes		99.5 ± 2.40	19.41 ± 0.74
SFA		0.00 ± 0.00	26.60 ± 1.03
UFA		0.00 ± 0.00	52.87 ± 1.54
MUFA		0.00 ± 0.00	8.73 ± 0.70
PUFA		0.00 ± 0.00	44.13 ± 1.65
Omega-9		0.00 ± 0.00	8.73 ± 0.56
Omega-6		0.00 ± 0.00	12.04 ± 1.00
Omega-3		0.00 ± 0.00	32.10 ± 1.77
Omega-6/Omega-3		0.00 ± 0.00	0.38 ± 0.04

The main component of essential oil was estragole. The main chemical composition in the oil fraction were 9, 12, 15-octadecatrienoic acid (linolenic acid), estragole, hexadecanoic acid (palmitic acid), 9, 12-octadecadienoic acid (linoleic acid), and 9-octadecenoic acid (oleic acid).

DockTools-1.5.6/Vina was used to do the molecular docking. The ligand and receptor files were converted to PDBQT format, with polar hydrogen atoms inserted and water molecules removed. Finally, using PYMOL software, all figures were visualized, and the docking scores were recorded as binding free energy (ΔG) (Rizvi et al., 2013).

2.13. Statistical analysis

The information is shown in three different replicates. SPSS used the Tukey test to see whether there were any major variations between the data.

3. Results and discussion

3.1. Chemical composition of AFEO

The major chemical compounds of AFEO, according to GC-MS data, were methyl chavicol (97%) and caryophyllene (1.4%) (Table 1). AFEO is mostly made up of estragole (methyl chavicol), which is consistent with previous reports (Hashemi et al., 2017; Omidbaigi and Mahmoodi, 2010).

3.2. The chemical composition of the oil fraction

Linolenic acid (9,12,15-octadecatrienoic acid), estragole, palmitic acid (hexadecanoic acid), linoleic acid (9,12-octadecadienoic acid), and oleic acid (9-octadecenoic acid) were the main chemical components of AFoil (Table 1). Terpenoids (mostly estragole) and fatty acids make up the oil fraction. The main fatty acids are polyunsaturated omega-3 fatty acids, including 9,12,15-octadecatrienoic acid. Polyunsaturated fatty acids, especially omega-3, are frequently linked to polar lipids in various membranes, helping to maintain membrane fluidity under various conditions (Ferreri et al., 2010). The ratio of omega-6/omega-3 fatty acids in the AFoil is less than one. According to our findings, the AFoil is a rich source of essential fatty acids and the effective omega-6/omega-3 ratio for use as food supplements. From the standpoint of nutritional principles, a well-balanced omega-6/omega-3 fatty acid ratio is critical for lowering the prevalence of chronic diseases and preventing and treating obesity disorders (Simopoulos, 2008). Because of the high intake of omega-6 fatty acid-rich vegetable oils compared to omega-3 fatty acid-rich sources like fish and nuts, the ratio of omega-6 to omega-3 fatty acids in typical western diets is high (DiNicolantonio and O'Keefe, 2018). Polyunsaturated omega-3 fatty acids, which can suppress thrombosis and inflammation, are elevated in prostaglandin E3, thromboxane A3, prostacyclin I3, and leukotriene B5 (Shramko et al., 2020). Polyunsaturated omega-6 fatty acids, on the other hand, increase

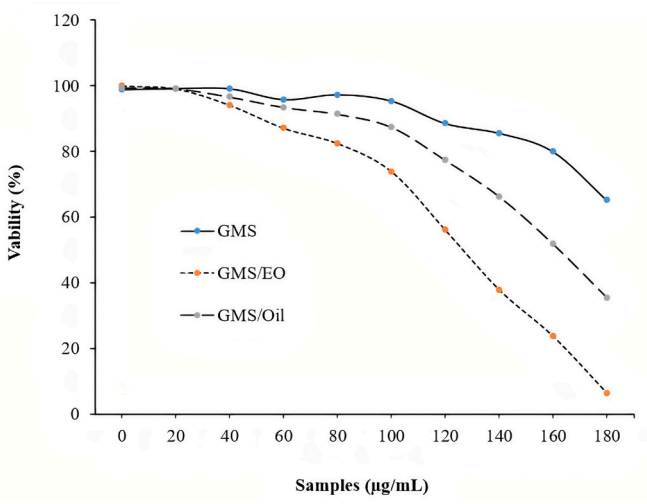

Fig. 1. Viability of macrophages cell line in the presence of glycerol monostearate (GMS), GMS- *A. foeniculum* essential oil (GMS/EO), and GMS-*A. foeniculum* oil (GMS/Oil).

Table 2
Modulatory effects of essential oil (20 µg/mL) and oil fraction (20 µg/mL) from *A. foeniculum* on ROS production and NADH oxidase (NOX), superoxide dismutase (SOD), and catalase (CAT) activity and related mRNA expression in LPS-stimulated macrophages.

Oxidant marker	Control	LPS	LPS/essential oil	LPS/oil
H_2O_2 (nM)	25.1 ± 2.2^c	47.5 ± 2.7^c	22.5 ± 1.7^b	20.5 ± 1.5^b
NOX activity (U/mL)	12.6 ± 1.5^a	31.1 ± 2.5^b	17.5 ± 2.2^a	13.7 ± 2.3^a
SOD activity (U/mL)	15.2 ± 2.0^b	22.4 ± 2.3^a	31.2 ± 2.2^c	28.6 ± 2.0^c
CAT activity (U/mL)	17.8 ± 1.4^b	23.8 ± 2.0^a	32.3 ± 2.0^c	29.8 ± 2.5^b
NOX22 mRNA (fold change)	1.0 ± 0.0^a	25.3 ± 2.2^d	18.5 ± 1.3^b	13.6 ± 1.0^b
NOX40 mRNA (fold change)	1.0 ± 0.0^a	17.4 ± 1.4^c	11.7 ± 0.8^b	10.5 ± 0.6^b
NOX47 mRNA (fold change)	1.0 ± 0.0^a	30.3 ± 2.0^c	18.4 ± 1.5^b	15.6 ± 1.2^b
NOX67 mRNA (fold change)	1.0 ± 0.0^a	23.4 ± 1.8^c	15.3 ± 1.2^b	14,6 ± 1.3^b
SOD mRNA (fold change)	1.0 ± 0.0^a	7.0 ± 0.5^b	12.4 ± 1.0^c	17.5 ± 1.5^d
CAT mRNA (fold change)	1.0 ± 0.0^a	8.0 ± 0.7^b	15.8 ± 1.6^c	16.5 ± 1.4^c
NF-kB mRNA (fold change)	1.0 ± 0.0^a	14.2 ± 1.3^c	9.6 ± 0.8^b	7.9 ± 0.7^b
NRF2 mRNA (fold change)	1.0 ± 0.0^a	9.4 ± 1.0^b	14.7 ± 1.2^c	16.3 ± 1.5^c

The values are expressed as means for three replicate experiments. Mean values with different letters within a column are significantly different by the Tukey test ($p < 0.05$).

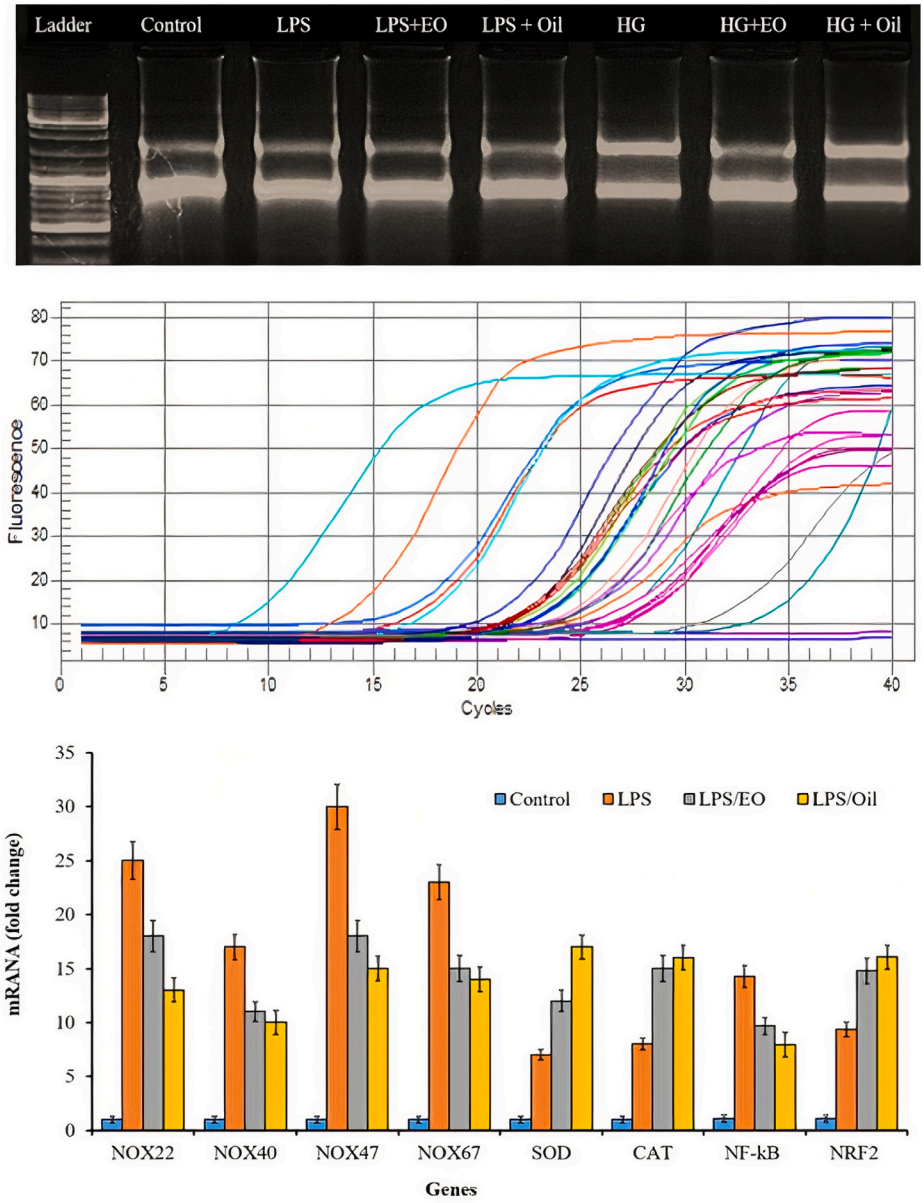

Fig. 2. Modulatory effects of essential oil (EO) and oil from *A. foeniculum* on NADH oxidase (NOX), superoxide dismutase (SOD), and catalase (CAT) expression in LPS-stimulated macrophages. LPS strongly induced NOX expression in the macrophage cells.

proinflammatory and prothrombotic mediators such as prostaglandin E2, prostacyclin I2, thromboxane A2, and leukotriene B4 (Innes and Calder, 2018). Because of its high omega-3 fatty acid content, AFoil can inhibit inflammatory reactions.

3.3. Cytotoxicity of AFEO or AFOil

The MTT assay revealed that GMS, AFEO, and AFoil inhibited J774A.1 cell viability in a concentration-dependent manner, with cytotoxicity significantly increasing at concentrations greater than 100 μg/mL (Fig. 1). AFEO had a greater cytotoxicity potential than the AFoil. Lipophilic components including GMS, AFEO, and AFoil penetrate the cytoplasmic and organelle membranes, disrupting and damaging the cytoplasmic and mitochondrial membranes, particularly causing cellular cytotoxicity (Jeena et al., 2020). This event can cause apoptosis or necrosis, resulting in protein and DNA damage as well as cytotoxic effects. GMS, AFEO, and AFoil have little impact on the cytoplasmic and mitochondrial membranes at low concentrations. On the other hand, these compounds may destroy and permeabilize mitochondria at high concentrations, oxidize to pro-oxidant, damage DNA and proteins, and eventually cause cell death (Jeena et al., 2020).

3.4. Expression and activity of oxidative markers in LPS-treated cells

Untreated cells had low levels of hydrogen peroxide, NOX mRNA, NOX activity, SOD mRNA, SOD activity, CAT mRNA, and CAT activity. Hydrogen peroxide, NOX mRNA, NOX activity, SOD mRNA, SOD activity, CAT mRNA, and CAT activity dramatically increased after incubation with LPS (Table 2 and Fig. 2). The hydrogen peroxide level, NOX mRNA, and NOX activity were substantially reduced when LPS-stimulated cells were treated with AFEO and AFoil fractions (Table 2 and Fig. 2). In LPS-stimulated cells, the AFEO and AFoil increased SOD activity, SOD mRNA, CAT activity, and CAT mRNA (Table 2 and Fig. 2). Our findings indicated a direct antagonistic relationship between LPS and AFEO and AFoil in the development of hydrogen peroxide and NOX activation. On the other hand, our findings indicate that LPS, AFEO, and

Table 3
Modulatory effects of essential oil (20 μg/mL) and oil fraction (20 μg/mL) from *A. foeniculum* on ROS production and NADH oxidase (NOX), superoxide dismutase (SOD), and catalase (CAT) activity and related mRNA expression in hyperglycemia (HG)-stimulated macrophages.

Diabetic marker	Control	HG	HG/essential oil	HG/oil
H_2O_2 (nM)	24.4 ± 2.0^{b}	43.3 ± 2.5^{c}	32.5 ± 2.2^{b}	25.2 ± 2.2^{b}
NOX activity (U/ml)	14.4 ± 1.6^{a}	35.3 ± 2.6^{b}	21.2 ± 1.6^{a}	14.6 ± 1.5^{a}
SOD activity (U/mL)	18.5 ± 2.0^{a}	23.4 ± 1.7^{a}	25.7 ± 2.0^{a}	27.2 ± 2.5^{bc}
CAT activity (U/mL)	16.5 ± 1.7^{a}	25.7 ± 2.0^{a}	30.3 ± 2.5^{b}	33.2 ± 2.9^{c}
NOX22 mRNA (fold change)	1.0 ± 0.0^{a}	24.4 ± 1.8^{d}	17.6 ± 1.2^{c}	13.5 ± 1.0^{b}
NOX40 mRNA (fold change)	1.0 ± 0.0^{a}	18.5 ± 1.3^{c}	12.7 ± 0.8^{b}	10.6 ± 0.7^{b}
NOX47 mRNA (fold change)	1.0 ± 0.0^{a}	33.4 ± 2.0^{c}	20.6 ± 1.8^{b}	18.8 ± 1.6^{b}
NOX67 mRNA (fold change)	1.0 ± 0.0^{a}	23.5 ± 1.7^{c}	14.6 ± 1.1^{b}	13.7 ± 1.4^{b}
SOD mRNA (fold change)	1.0 ± 0.0^{a}	7.0 ± 0.6^{b}	10.6 ± 0.8^{c}	13.5 ± 1.2^{d}
CAT mRNA (fold change)	1.0 ± 0.0^{a}	11.4 ± 0.9^{b}	19.5 ± 1.6^{c}	22.3 ± 2.0^{c}
NF-kB mRNA (fold change)	1.0 ± 0.0^{a}	16.5 ± 1.2^{c}	10.7 ± 0.8^{b}	8.3 ± 0.6^{b}
NRF2 mRNA (fold change)	1.0 ± 0.0^{a}	8.2 ± 0.7^{b}	14.4 ± 1.1^{c}	15.3 ± 1.3^{c}

The values are expressed as means for three replicate experiments. Mean values with different letters within a row are significantly different by the Tukey test ($p < 0.05$).

AFoil have a positive synergistic effect on SOD and CAT activation.

The molecular mechanisms by which LPS activates the NOX cascade are properly appreciated. The binding of LPS to TLR-4 causes NOX and superoxide synthesis to be activated (Sirokmány et al., 2016). NOX generates a superoxide anion from molecular oxygen, converted to hydrogen peroxide by SOD, which is then eliminated by CAT (Whaley-Connell et al., 2019). Inflammation and diabetes disorders require a balance of NOX, SOD, and CAT activities to determine the steady-state superoxide anion and hydrogen peroxide. Mitogen-activated protein kinases (MAPK) are stimulated by superoxide anion and hydrogen peroxide. MAPKs stimulate the synthesis of other proteins such as interleukins and cytokines, and enzymes like cyclooxygenase, superoxide dismutase, and catalase, by activating NF-kB (Kim et al., 2017). In current research on superoxide production in stimulated macrophages, actual inhibitory mechanisms of AFEO and AFoil can be attributed to the reduction of NOX function and the inhibition of TLR4 pathway in the LPS signaling cascade like NF-kB and NRF2 (Yousefian et al., 2019). According to our findings, AFEO and AFoil reduce NOX function while increasing SOD and CAT activities, implying that AFEO and AFoil have the potential to reduce superoxide generation and have a good anti-oxidative capability in cells. Nonetheless, more information about the molecular mechanisms of the anti-inflammatory capacity of fatty acid is required to be identified (Pegoraro et al., 2021).

3.5. Expression and activity of oxidative markers in HG-treated cells

Untreated cells had low levels of hydrogen peroxide, NOX mRNA, NOX activity, SOD mRNA, SOD activity, CAT mRNA, and CAT activity. Hydrogen peroxide, NOX mRNA, NOX activity, SOD mRNA, SOD activity, CAT mRNA, and CAT activity dramatically increased after incubation with HG (Table 3 and Fig. 3). The hydrogen peroxide amount, NOX mRNA, and NOX activity were substantially reduced when HG-stimulated cells were treated with AFEO and AFoil fraction (Table 3 and Fig. 3). In HG-stimulated cells, the AFEO and AFoil increased SOD activity, SOD mRNA, CAT activity, and CAT mRNA (Table 3 and Fig. 3). Our findings indicated a direct antagonistic relationship between HG and AFEO and AFoil in the formation of hydrogen peroxide and NOX activation. On the other hand, our findings indicate that HG, AFEO, and AFoil have a positive synergistic effect on SOD and CAT activation.

Hyperglycemia is the most common complication of diabetes mellitus, and it can stimulate superoxide and hydrogen peroxide synthesis by stimulating PKC, the polyol pathway, the hexosamine pathway, and protein glycation (Lahlou, 2013). By chemical and functional modification of nucleic acid, protein, carbohydrate, and lipid components of cells, superoxide contributes to inflammation, cancer, diabetes, neurodegenerative, and cardiovascular disease (Kurutas, 2015). Diabetes and other metabolic disorders are caused by oxidative stress and inflammation. Antioxidant and anti-inflammation studies, such as modulation of superoxide forming (NOX), superoxide removal (SOD), hydrogen peroxide removing (CAT), and another free radical detoxification enzyme, are critical in the quest for anti-diabetic substances (Shi et al., 2020). HG activates NOX expression, NOX function, and superoxide production in human umbilical artery endothelial cells (Taye et al., 2010), monocyte cells (Rajamani and Jialal, 2014), and human microvascular retinal endothelial cells (Cheng et al., 2019) through TLR2 and TLR4 activation and downstream signaling pathways, similar to the LPS signaling cascade.

TLRs and other downstream factors such as NK-kB are activated by HG, which provides the basis for NOX activation and superoxide formation (Urner et al., 2020). As a result, inhibiting NOX expression and activity may be a therapeutic approach to avoid oxidative stress in diabetic patients. AFEO and AFoil, a normal and plant-derived antioxidant that prevents NOX while inducing SOD and CAT, can be used to recover the antioxidant capacity of cells. More study is required to confirm whether these inhibitory effects reduce NOX expression or other downstream NOX signaling elements such as NF-kB or other transcription factors (Yousefian et al., 2019). The redox property of AFEO or AFoil can play a role in NOX inhibition. A reducing agent, such as NADH, is required for the NOX enzyme to function. The optimal activity of NOX is related to the redox potential of the cells and the ratio of NADPH to $NADP^{+}$. By disrupting the ratio of NADPH to NADP+ and redox potential, AFEO and AFoil, like antioxidants and electron donors, will affect NOX function (Zhang et al., 2018).

3.6. Expression of NF-kB and NFR2

In macrophages, HG and LPS increased the expression of the transcription factors NF-kB and NRF2. Treatment with AFEO and AFoil reduced NF-kB expression while increasing NRF2 expression in the treated cells, demonstrating that AFEO and AFoil have different modulatory effects on NF-kB and NFR2 (Tables 2 and 3). Our findings indicated that LPS and HG and AFEO, and AFoil have direct antagonistic effects on NF-kB expression. On the other hand, our findings show that LPS, HG, AFEO, and AFoil have a strong synergistic effect on NFR2 expression (Tables 2 and 3). According to our findings, AFEO and AFoil substantially reduced NF-kB expression while increasing NRF2 expression in the treated cells, demonstrating that AFEO and AFoil have different modulatory effects on NF-kB and NFR2.

The inhibition of NF-kB and upregulation of NRF2 was believed to be responsible for the antioxidant and anti-diabetic effects of AFEO and AFoil. Oxidative stimuli such as HG and LPS activate NRF2 and NF-kB. In unstimulated cells, the NF-kB-IKB complex is the inactive form of NF-kB. Phosphorylation of IKB with protein kinases leads to the release of IKB and activation of NF-kB, causing the inflammatory response and oxidative product to strengthen (Sivandzade et al., 2019). Under normal situations, the NRF2-Keap1 complex is the inactive form of NRF2. Oxidation of sulfhydryl groups of Keap1 in the oxidative tension leads to the release and activation of NRF2. The NRF2 activation enhancements antioxidant defenses, effectively neutralizing superoxide and hydroperoxide formed by the NF-kB pathway (Tu et al., 2019). In our

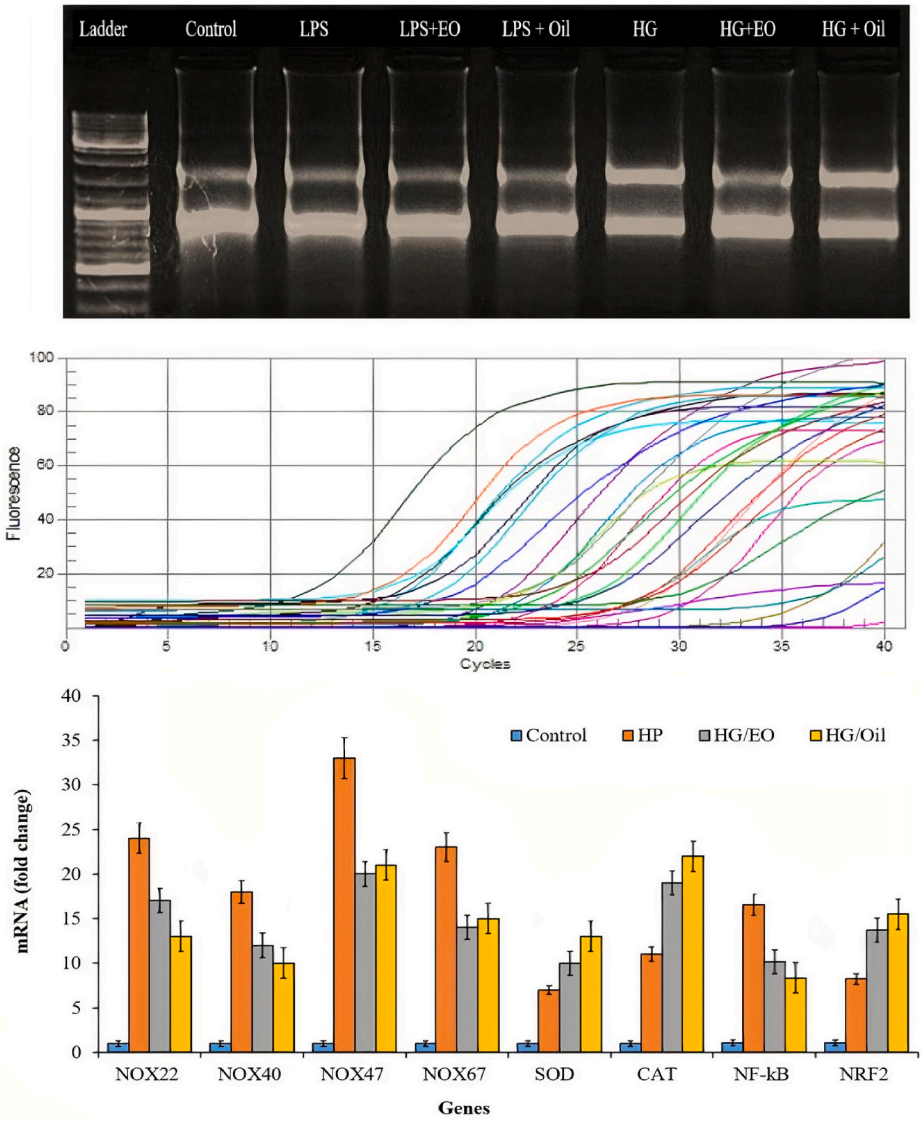

Fig. 3. Modulatory effects of essential oil (EO) and oil from *A. foeniculum* on NADH oxidase (NOX), superoxide dismutase (SOD), and catalase (CAT) expression in hyperglycemia (HG)-stimulated macrophages. Hyperglycemia induced NOX expression in the macrophage cells.

experiment, AFEO and AFoil inhibited NF-kB and activated the NRF2 pathway, suggesting that they may be useful as a preventative and therapeutic agent for diabetic and inflammation-related oxidative stress.

3.7. Molecular modeling of protein with estragole, linolenic acid, and linoleic acid

Estragole is bonded to a pocket binding site by hydrogen bonds, Van der Wall's bonds, and pi-Alkyl interactions, as shown by molecular docking of NOX. Linoleic and linolenic acids bind to the same hydrophobic pocket of NOX using van der Waals interactions, hydrogen bonds, and pi-alkyl interactions. Estragole, linolenic acid, and linoleic acid docked to 1K4U with binding energies of −4.3, −3.9, and −4.1, respectively (Fig. 4).

According to SOD molecular docking, linoleic acid attaches to a pocket binding site using hydrogen bonds, van der Waals bonds, and pi-Alkyl and pi-anion interactions. With hydrogen bonding, and van der Waals, and pi-alkyl interactions, estragole and linolenic acid bind to the same hydrophobic pocket of SOD. Estragole, linolenic acid, and linoleic acid dock to SOD with binding energies of −4.4, −2.4, and −3.1, respectively (Fig. 5).

According to CAT molecular docking, estragole binds to a pocket binding site using van der Waals bond, pi-Alkyl interactions, and hydrogen bonding. Hydrogen bonds, van der Waals, and pi-alkyl interaction are used by linoleic acid and linolenic acid to bind to the same hydrophobic pocket of CAT. Estragole, linolenic acid, and linoleic acid docked to CAT with binding energies of −4.1, −3.7, and −4.8, respectively (Fig. 6).

Estragole binds to a pocket binding site with hydrogen bonds, Van der Wall's bonds, and pi-alkyl interactions, according to NRF2 molecular docking. Linoleic and linolenic acids bind to the same hydrophobic pocket of NRF2 using van der Waals, pi-alkyl interactions, and hydrogen bonding. Estragole, linolenic acid, and linoleic acid have binding energies of −4.8, −4.7, and −4.4, respectively, for docking to NRF (Fig. 7).

According to NF-kB docking, linolenic acid binds to a pocket binding site with van der Waals, pi-Alkyl, pi-anion interactions, and hydrogen bonding. With van der Waals, and pi-alkyl interactions, and hydrogen bindings, estragole and linoleic acid bind to the same hydrophobic pocket of NF-kB. Estragole, linolenic acid, and linoleic acid docked to NF-kB with binding energies of −4.8, −4.7, and −4.6, respectively (Fig. 8).

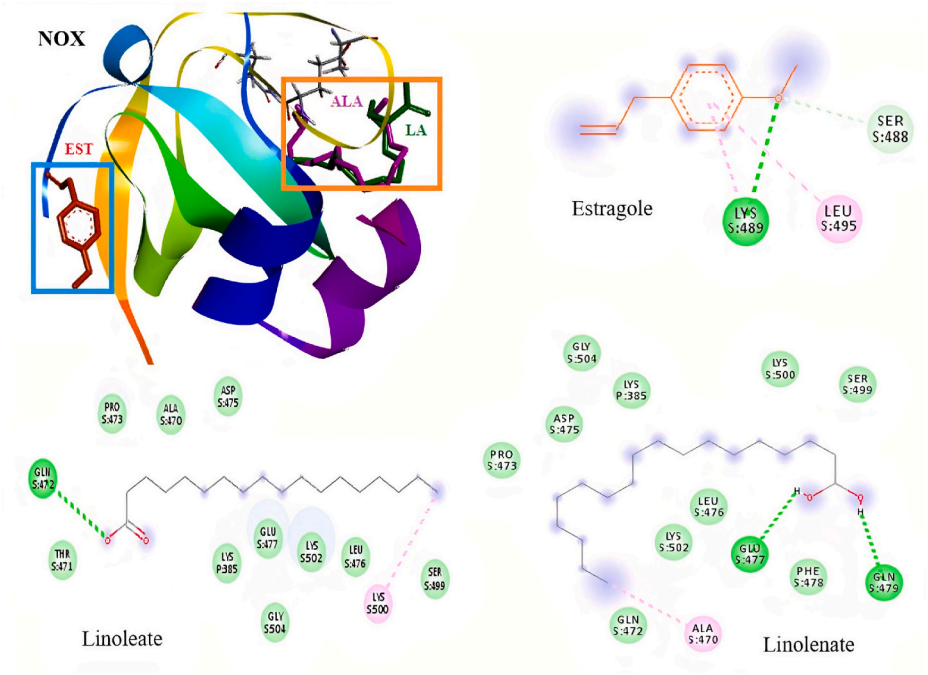

Fig. 4. The interaction types of NOX with estragole, linolenate, and linoleate and 2D images of the docked conformations of the selected ligands with the NOX residues at binding pocket. Hydrogen bond (dark green), carbon-hydrogen bond (light blue), Pi-Alkyl (light pink), and van der Waals (light green) interactions are by colored circles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

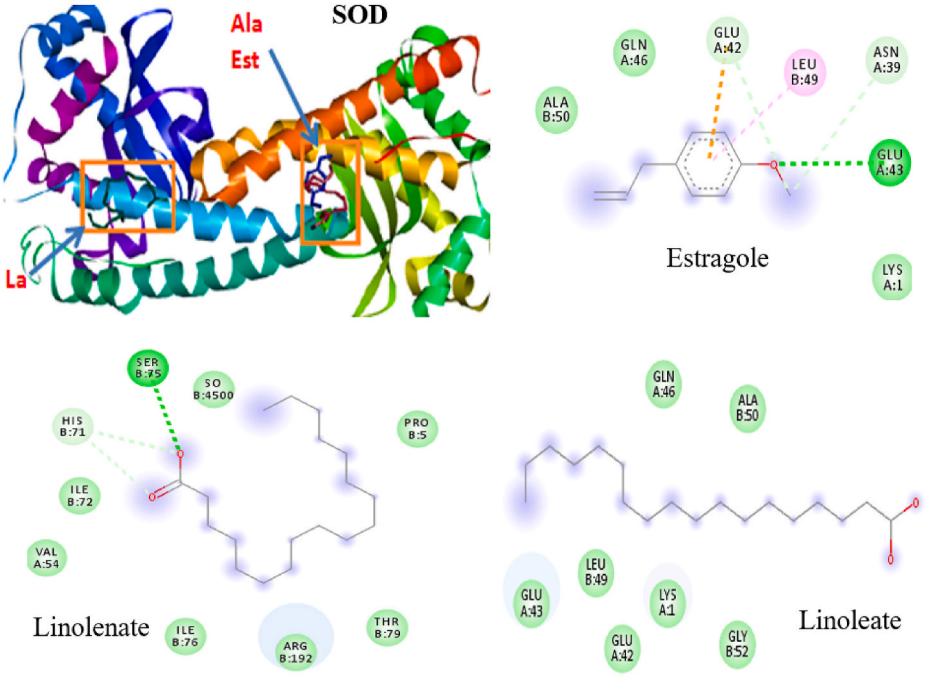

Fig. 5. The interaction types of SOD with estragole, linolenate, and linoleate and 2D images of the docked conformations of the selected ligands with the SOD residues at binding pocket. Hydrogen bond (dark green), carbon-hydrogen bond (light blue), Pi-Alkyl (light pink), Pi-Anion (orange), and van der Waals (light green) interactions are by colored circles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

According to these results, the key ingredient in AFEO was estragole, and the major chemical components of the AFoil are linolenic acid, estragole, palmitic acid, linoleic acid, and oleic acid. In HG and LPS-treated macrophages, AFEO and AFoil decreased NOX activity while increasing SOD and CAT activity. In terms of anti-hyperglycemic and anti-oxidative activity, AFoil with estragole and omega-3 fatty acids performed much better than AFEO with estragole. Estragole, linoleic acid, and linolenic acid bind to various hydrophobic pockets of NOX, SOD, CAT, NFR2, and NF-kB using hydrogen bonds, van der Waals bonds, pi-alkyl and pi-anion interactions, and with different binding energies, according to molecular docking studies. AFEO and AFoil can reduce oxidative stress markers by down-regulating superoxide-

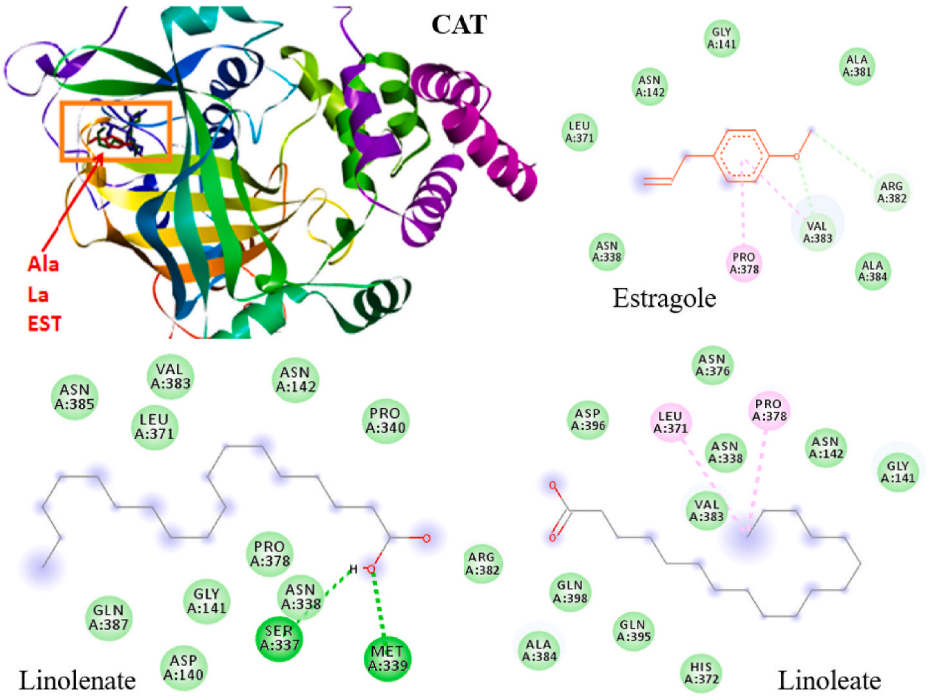

Fig. 6. The interaction types of CAT with estragole, linolenate, and linoleate and 2D images of the docked conformations of the selected ligands with the CAT residues at binding pocket. Hydrogen bond (dark green), carbon-hydrogen bond (light blue), Pi-Alkyl (light pink), and van der Waals (light green) interactions are by colored circles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

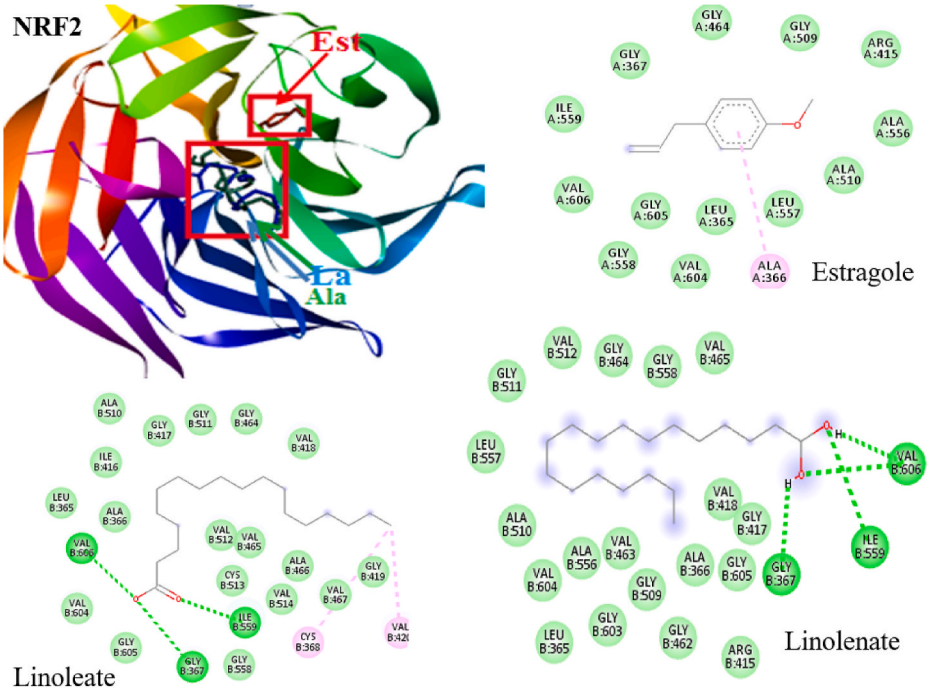

Fig. 7. The interaction types of NRF2 with estragole, linolenate, and linoleate and 2D images of the docked conformations of the selected ligands with the NRF2 residues at binding pocket. Hydrogen bond (dark green), carbon-hydrogen bond (light blue), pi-alkyl (light pink), and van der Waals (light green) interactions are by colored circles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

producing enzymes and up-regulating superoxide-removing enzymes at the gene and protein levels. These findings show that AFEO or AFoil can completely overpower oxidative responses, indicating potent antioxidant properties. The ability of AFEO and AFoil to modulate NOX, SOD, and CAT on stimulated macrophages and suppress superoxide development to some extent was similar.

To summarize, AFoil can be used to treat oxidative damage in diabetes and other inflammatory conditions by acting as an antioxidant. The AFoil can be used in healthcare applications and the treatment of hyperglycemia and oxidative stress. However, more practical research is required to clarify the molecular mechanism of estragole and omega-3 fatty acids on oxidative stress amelioration. Estragole's kinetic

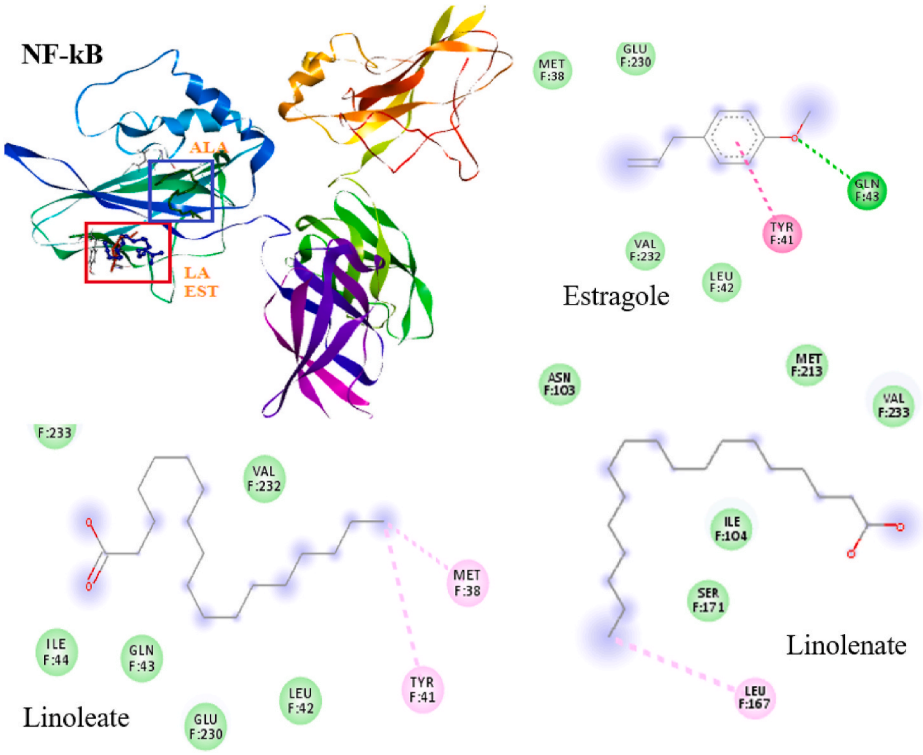

Fig. 8. The interaction types of NF-kB with estragole, linolenate, and linoleate and 2D images of the docked conformations of the selected ligands with the NF-kB residues at binding pocket. Hydrogen bond (dark green), carbon-hydrogen bond (light blue), pi-Alkyl (light pink), and van der Waals (light green) interactions are by colored circles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mechanism in the inhibition or activation of NOX, SOD, and CAT, namely competitive, non-competitive, and uncompetitive inhibition, or allosteric activation, must also be revealed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114814.

Author contribution

Fatemeh Najafi and Gholamreza Kavoosi were responsible for the study's concept and design, data analysis and interpretation, manuscript drafting, critical revision for important intellectual content, and statistical analysis.

Ethical approval

There were no human subjects or animal experiments in our study.

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