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Original Article

Phytocompounds of *Nigella sativa* seeds extract and their neuroprotective potential via EGR1 receptor inhibition: A molecular docking study

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Abstract

Bioactivity of *Nigella sativa* seed extract has the potential as a neuro-protector, offering its promising utility in the clinical setting for brain injury management. This study aimed to identify the phytocompounds contained in the extract of *N. sativa* seeds and further screen their respective neuronal anti-inflammatory activities in silico. The extract of *N. sativa* seeds was prepared through successive maceration using non-polar to polar solvents (n-hexane and ethanol, respectively). The phytocompounds in the ethanolic extract were initially identified through qualitative analysis and further analyzed with gas chromatography-mass spectrometry (GC-MS). The spectral data were compared with the compound library for identification. The identified phytocompounds were then simulated computationally for their binding affinities toward the active pocket of early growth response-1 (EGR1) receptor (PDB: 14r2a). We found that the ethanolic extract of *N. sativa* seeds were predominantly constituted of hexadecanoic acid, ethyl ester (17.15%); linoleic acid ethyl ester (15.0%); octadecanoic acid (13.26%); and ethyl oleate (10.38%). The binding affinity of the phytocompounds ranged from -7.49 kcal/mol (methyl palmitoleate) to -14.31 kcal/mol (9-hexadecanoic acid, methyl ester), with 12 compounds having binding affinity < -10 kcal/mol. In conclusion, ethanolic extract of *N. sativa* seeds are rich with fatty acids that have active as anti-inflammatory and may exert neuronal protection by inhibiting EGR1 receptor. Studies using animal models to confirm the activity are warranted.

Keywords: Anti-inflammation, black cumin, neuroprotection, GC-MS, phytochemical screening

Introduction

Nigella sativa or black cumin, also known as *jintan hitam* in Indonesian, has been used as a home remedy to maintain health and treat various diseases worldwide [1, 2]. The seeds contain rich bioactive compounds such as camphene, t-anethole, -pinene, -terpinene, limonene, sabinene, -cymene, -terpinolene, -cymene-8-ol, carvacrol, longipinene, camphor, linaloolcis, sabinenehydrate, longifolene, dihydro carveol, thymol, 4-terpineol, carvone, thymoquinone, -

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thujone, and thujone [2, 3]. Some of well-studied antioxidants derived from the *N. sativa* include thymoquinone, dithymoquinone, thymohydroquinone, and thymol [2, 3]. Potent xenobiotics are also witnessed to be contained in this plant, namely nigellon and glutathione [4, 5]. The foregoing phytoconstituents have been associated with antioxidant, antihistamine, anti-inflammatory, immunomodulatory, antitussive, antidiabetic, antihypertension, antipyretic, analgesic, anticancer, and antimicrobial effects [4-7].

As a neuroprotective agent, the seed oil of *N. sativa* has been reported effective in protecting nerve cells from the damage induced by free radicals deriving from non-enzymatic lipid peroxidation and deoxyribose degradation [8]. In a traumatic brain injury rat model, extract of *N. sativa* was suggested to act as anti-inflammation and neuroprotective agent [9]. Medicinal benefits of this plant for nerve cells have been explored for traumatic brain injury, Alzheimer's and Parkinson's in vivo or in vitro [10]. In another study, *N. sativa* has been proposed as a protector of lipopolysaccharide-induced synaptic plasticity [6]. Additionally, its effect on improving learning and memory, especially for those with Alzheimer's disease has been reported [2].

Based on the foregoing explanations, herein, we intend to examine the efficacy of *N. sativa* seeds extract as an adjuvant to propofol in attenuating pathologic brain inflammation following traumatic injury. However, a preliminary study is required to confirm the phytoconstituents of the *N. sativa* seeds extract used in the present study. Further, no studies have explored the potential of *N. sativa* seeds extract in targeting transcription factors involved in the neuronal inflammation. The aim of this study was to investigate the potential of the *N. sativa* seeds extract in inhibiting the receptor of early growth response 1 (EGR1) by molecular docking. EGR1 is a transcription factor with a role in expressing genes of pro-inflammatory cytokines and chemokines, that further recruits inflammatory cells to the injured site [11, 12]. Molecular docking itself has been considered as an efficient computational approach to predict the drug's targets [13, 14].

Methods

Materials

N. sativa seeds were obtained from the herbal shops in East Java, Indonesia. Other materials included n-hexane, ethanol, Dragendorf reagent, Mayer reagent, and Wagner reagent, Liebermann-Burchard reagent, HCl, Mg, $FeCl_3$, gelatin, H_2SO_4, potassium hydroxide 5%, and chloroform. Otherwise stated, all materials were procured as analytical grade from Merck (Selangor, Malaysia).

Extraction and phytocompounds identification

N. sativa seeds was extracted with successive maceration method, started from using non-polar solvents to polar ones (n-hexane and ethanol, respectively). Twenty-five grams sample of *N. sativa* seed powder was macerated in Erlenmeyer flask containing n-hexane (200 mL) at 200 rpm and room temperature. Extraction was carried out for 24 hours and repeated three times. Thereafter, the residues were re-dried before re-macerated with ethanol, with the same maceration procedure as used in n-hexane. Upon completion, the organic solvent was evaporated under vacuum at a rotary evaporator to produce a crude extract paste. The ethanolic extract was used in the following investigations.

Phytocompound identification

Qualitative analysis of the phytochemical contents was carried out for identifications of flavonoids, saponins, alkaloids, tannins and total polyphenol levels using the methods reported previously [15, 16]. The presence of alkaloids was considered positive if the extract was reactive to one of the reagents, namely Dragendorf, Mayer, and Wagner. Steroids and terpenoids (or triterpenoids) were tested using Liebermann-Burchard reagent. Saponins were observed by the presence of stable foam after the extract solution was shaken rigorously. The extract was positive containing polyphenols if the color change observed after the addition of HCl and Mg.

Flavonoids were determined using $FeCl_3$. The presence of quinones was investigated by Borntrager's test using potassium hydroxide 5% on the extract that had been priorly separated with chloroform. As for Tannins, their presence was investigated by a combination of gelatin and H_2SO_4.

Semi-quantitative analysis was further carried out using gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies 7890GC/5975MS, Santa Clara, CA, USA). The ethanol-dissolved extract was injected through sample injector, run through the GC—MS system with oven temperature of 45°C, injection temperature of 280°C, and pressure of 51.5 kPA. In the column, the flow rate was set at 1 mL/min with a total flow of 14 mL/min in the whole system. Linear velocity of the system was 36.2 cm/s. Each separated compound was analyzed in the MS, and the spectral data were compared with that from the library of National Institute of Standards and Technology.

Retrieval and preparation of the protein and ligan structures

Phytocompounds identified in the extract of *N. sativa* seeds acted as the ligand in the molecular docking simulation. The 3D structure of each compound was retrieved as PDB (.pdb) files from OpenBabel chemical format converter (http://www.cheminfo.org/), after its 'simplified molecular input line entry system' (SMILES) was inserted. The SMILES used in this analysis was obtained from the PubChem (https://pubchem.ncbi.nlm.nih.gov/), and are presented in details in **Table 1**. As for the protein, the EGR1 receptor (PDB: 14r2a), downloaded from protein data bank (PDB, https://www.rscb.org/), acted as the target. Optimization of the ligand and protein was carried out by hydrogen atoms insertion, partial charge addition, and energy minimization, carried out on PyRx program. For the protein, missing atoms were repaired on the same computing program.

Table 1. PubChem ID and SMILES of the phytocompounds acting as ligands in the molecular docking

Compounds	PubChem ID	SMILES' structure
Methyl laurate, dodecanoic acid, methyl ester	8139	CCCCCCCCCCCC(=O)OC
4-(3-Methoxycarbonylpropyl)-4-butanolide	559216	COC(=O)CCCC1CCC(=O)O1
t-Butylhydroquinone	16043	CC©©C1=C(C=CC(=C1)O)O
Ethyl laurate, dodecanoic acid, ethyl ester	7800	CCCCCCCCCCCC(=O)OCC
2(3H)-Furanone, 5-heptyldihydro-(CAS)	7714	CCCCCCCC1CCC(=O)O1
Methyl tetradecanoate	31284	CCCCCCCCCCCCCC(=O)OC
Ethyl myristate, tetradecanoic acid, ethyl ester	31283	CCCCCCCCCCCCCC(=O)OCC
Methyl palmitoleate	643801	CCCCCCC=CCCCCCCCC(=O)OC
9-Hexadecenoic acid	5282745	CCCCCCC=CCCCCCCCC(=O)O
Methyl palmitate, hexadecanoic acid, methyl ester	8181	CCCCCCCCCCCCCCCC(=O)OC
Ethyl 9-hexadecenoate	5364759	CCCCCCC=CCCCCCCCC(=O)OCC
Hexadecanoic acid, ethyl ester	12366	CCCCCCCCCCCCCCCC(=O)OCC
9,12-Octadecadienoic acid, methyl ester	8203	CCCCCC=CCC=CCCCCCCCC(=O)OC
Elaidic acid, 9-octadecenoic acid, methyl ester	5280590	CCCCCCCCC=CCCCCCCCC(=O)OC
Methyl stearate	8201	CCCCCCCCCCCCCCCCCC(=O)OC
Linoleic acid ethyl ester	5282184	CCCCCC=CCC=CCCCCCCCC(=O)OCC
Ethyl oleate	5363269	CCCCCCCCC=CCCCCCCCC(=O)OCC
Octadecanoic acid, ethyl ester	8122	CCCCCCCCCCCCCCCCCC(=O)OCC
Docosahexaenoic acid (control)	445580	CCC=CCC=CCC=CCC=CCC=CCC=CCC C(=O)O

Molecular docking simulation

The docking sites were determined by following molecular operating environment (MOE) protocol [17]. The determined docking grid dimensions were X: 40, Y: 40, and Z: 40 located in the center of the protein molecule, where the spacing of 0.375 Å employed. The docking protocol was run on Autodock Vina 1.1.2 [18]. The probable interactions between the ligands (phytocompounds from *N. sativa* seeds) and the protein (EGR1 receptor) with the best docking score were visualized on Discovery Studio 2021 and PyMol V.2.5.1 software. Due to the absence

of native ligand, the validation was carried out using docosahexaenoic acid as suggested by the findings from a previous study [19].

Results

Results from qualitative screening of phytocompounds

Results from the qualitative screening of phytocompounds contained in the ethanolic extract of *N. sativa* seed are been presented in **Table 1**. In alkaloids tests, the extract was reactive to Dragendorf and Mayer reagents, but not to Wagner reagent. The presence of saponins, tannins, flavonoids, steroids, and polyphenols were confirmed positive by these tests. As for quinones and triterpenoids, they were not observable in the qualitative screening.

Table 1. Qualitative phytochemical screening test results of *N. sativa* seed extract

Phytocompound group	Observation
Alkaloids	
Dragendorf	Reactive
Mayer	Reactive
Wagner	Non-reactive
Saponins	Reactive
Tannins	Reactive
Flavonoids	Reactive
Steroids	Reactive
Quinones	Non-reactive
Polyphenols	Reactive
Triterpenoids	Reactive

Phytocompounds identified by GC-MS

Identification of phytocompounds was also performed using semi-quantitative method, GC-MS, where the chromatogram has been presented in **Figure 1**. The ethanolic seed extract was found to predominantly contain hexadecanoic acid, ethyl ester based on the spectral area with relative abundance of 17.15% (**Table 2**). Linoleic acid ethyl ester (15.0%); 9,12-octadecadienoic acid (13.26%); ethyl oleate (10.38%); methyl palmitate, hexadecanoic acid methyl ester (8.27%); and ethyl laurate, dodecanoic acid ethyl ester (8.20%) were compounds with high relative abundance in the *N. sativa* seeds extract. Hexadecanoic acid, ethyl ester or also known as ethyl palmitate has its derivatives detected in GC-MS analysis, including methyl palmitoleate, 9-hexadecenoic acid (0.61%), methyl palmitate, hexadecanoic acid, methyl ester (8.27%) and ethyl 9-hexadecenoate (0.7%) (**Table 2**).

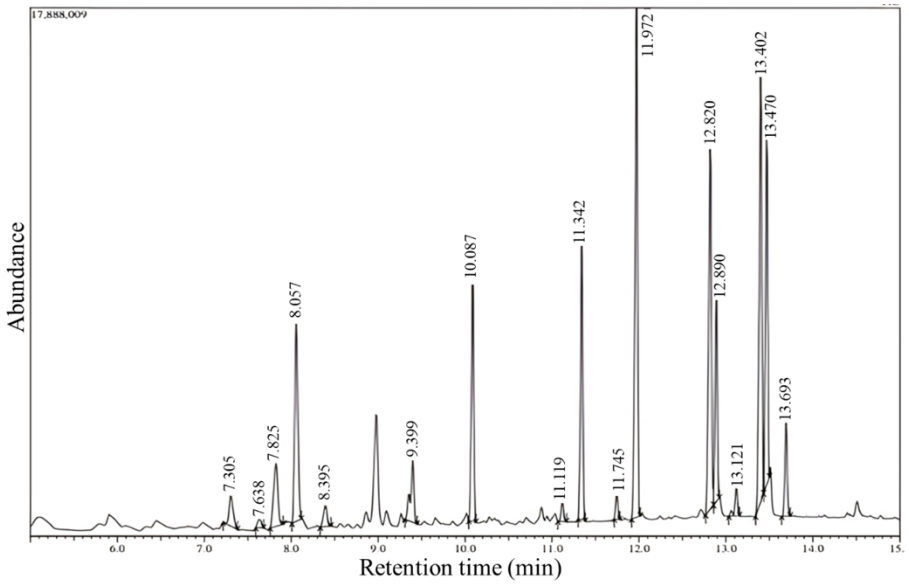

Figure 1. Chromatogram of gas chromatography-mass spectrometry (GC-MS) from the phytocompounds analysis on *Nigella sativa* seed extract.

Table 2. The result from gas chromatography-mass spectrometry (GC-MS) of ethanolic extract from *Nigella sativa* seeds

Peak #	Retention time (min)	Area (%)	Height (%)	Area/ height	Name
1	7.305	1.58	1.04	3.21	Methyl laurate, dodecanoic acid, methyl est
2	7.638	0.43	0.28	3.25	4-(3-Methoxycarbonylpropyl)-4-butanolide
3	7.825	3.38	2.17	3.31	t-Butylhydroquinone
4	8.057	8.20	6.80	2.55	Ethyl laurate, dodecanoic acid, ethyl ester
5	8.395	0.94	0.69	2.88	2(3H)-Furanone, 5-heptyldihydro- (CAS) .g
6	9.399	2.84	2.13	2.82	Methyl tetradecanoate
7	10.087	7.64	8.19	1.98	Ethyl myristate, tetradecanoic acid, ethyl ester
8	11.119	0.61	0.67	1.91	Methyl palmitoleate, 9-Hexadecenoic acid
9	11.342	8.27	9.50	1.84	Methyl palmitate, hexadecanoic acid, methyl ester
10	11.745	0.70	0.81	1.83	Ethyl 9-hexadecenoate
11	11.972	17.15	17.61	2.06	Hexadecanoic acid, ethyl ester
12	12.820	13.26	12.43	2.26	9,12-Octadecadienoic acid, methyl ester
13	12.890	5.79	6.98	1.76	Elaidic acid, 9-octadecenoic acid, methyl ester
14	13.121	1.02	0.93	2.32	Methyl stearate
15	13.402	15.00	14.65	2.17	Linoleic acid ethyl ester
16	13.470	10.38	11.90	1.85	Ethyl oleate
17	13.693	2.82	3.22	1.86	Octadecanoic acid, ethyl ester

Ligand-protein interactions

The docking results of the respective interactions between phytocompounds in the *N. sativa* seeds extract and EGR1 receptor are been presented in **Table 3**. All of the phytocompounds, mostly fatty acids and their respective derivatives, were found to exceed a minimum 5 kcal/mol threshold for possible interactions. When compared to control (docosahexaenoic acid; binding affinity = -10.32 kcal/mol), there were 12 compounds having close or even higher scores. The highest docking score was obtained by the interaction involving 9-hexadecanoic acid, methyl ester (-14.31 kcal/mol). The compound binds to EGR1 receptor in the active pocket with two hydrogen bonds formed with DG 6 and Arg 357. Interestingly, Arg 357 was also the amino acid involved in the interaction of EGR1 receptor with control. The 3D and 2D presentations of the simulated interactions between EGR1 receptor and the phytocompounds of interest (are presented in **Figure 2** and **Figure 3**.

Table 3. Predicted binding affinity of *Nigella sativa* seeds extract phytocompounds with early growth response 1 (EGR1) receptor

Name	Binding affinity (kcal/mol)	Amino acids involved in H-bond
Methyl laurate, dodecanoic acid, methyl ester	-10.58	Ser 380
4-(3-Methoxycarbonylpropyl)-4-butanolide	-9.31	Ser 380, Asp 381
t-Butylhydroquinone	-9.92	-
Ethyl laurate, dodecanoic acid, ethyl ester	-10.36	-
2(3H)-Furanone, 5-heptyldihydro- (CAS) .g	-9.59	-
Methyl tetradecanoate	-7.49	-
Ethyl myristate, Tetradecanoic acid, ethyl ester	-11.65	Ser 380
Methyl palmitoleate	-12.35	
9-Hexadecanoic acid, methyl ester	-14.31	DG 6, Arg 357
Methyl palmitate, hexadecanoic acid, methyl ester	-12.96	-
Ethyl 9-hexadecanoiate	-11.49	-
Hexadecanoic acid, ethyl ester	-10.15	Arg 357
9,12-Octadecadienoic acid, methyl ester	-8.17	-
Elaidic acid, 9-octadecenoic acid, methyl ester	-11.94	-
Methyl stearate	-13.67	Arg 357 (n=2)
Linoleic acid ethyl ester	-13.19	-
Ethyl oleate	-13.11	-
Octadecanoic acid, ethyl ester	-9.59	-
Docosahexaenoic acid (control)	-10.32	DG 8, Arg 357

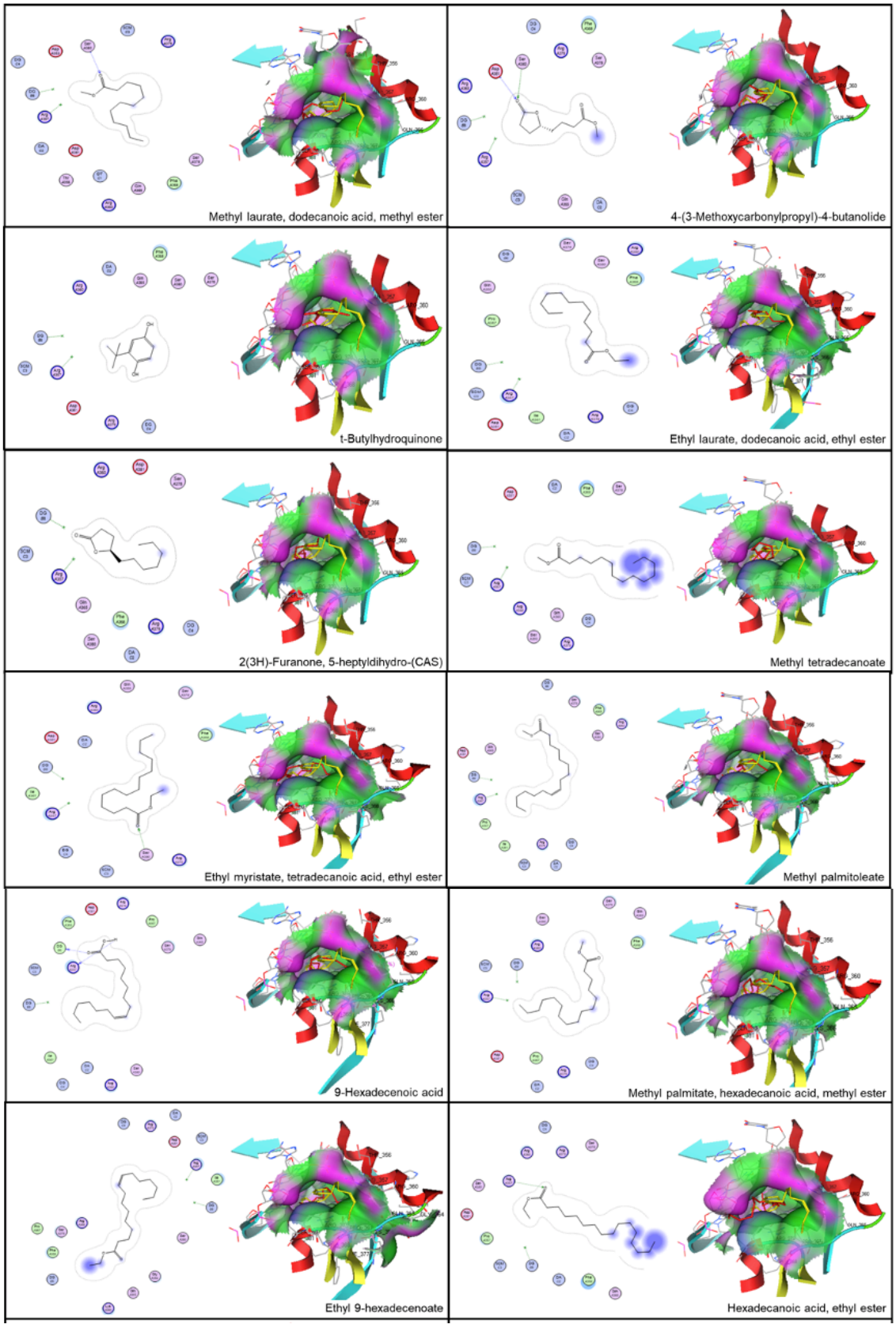

Figure 2. Simulated interactions of early growth response 1 (EGR1) receptor with phytocompounds of *Nigella sativa* seeds extract presented in 3D and 2D.

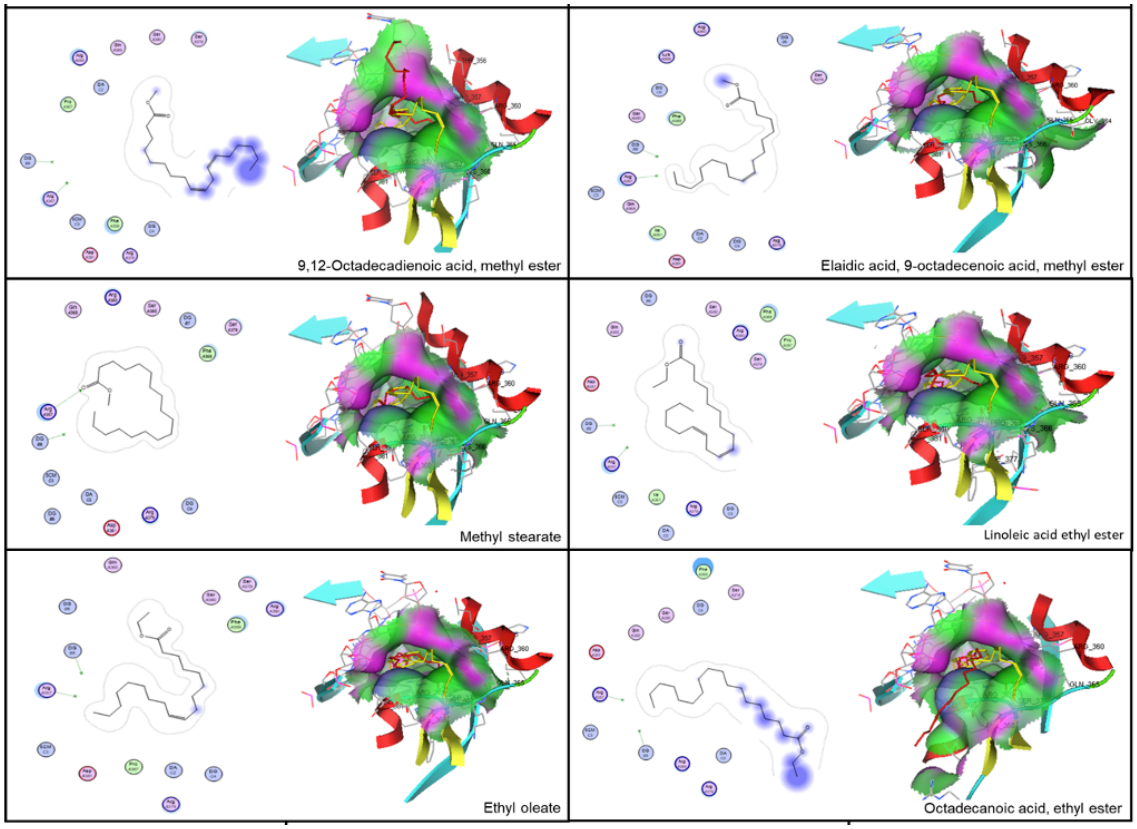

Figure 3. Simulated interactions of early growth response 1 (EGR1) receptor with phytocompounds of *Nigella sativa* seeds extract presented in 3D and 2D.

Discussion

Our findings suggest the extract from *N. sativa* seeds contained alkaloids, saponins, tannins, flavonoids, steroids, and polyphenolic compounds. Compared to a previous study, the seeds contained 8 saponins, 10 alkaloids, 10 flavonoids, 6 phenolics, and 18 fatty acids, but no steroids were found [20]. Different extraction methods used between the present study and the previous study might be the reason for this difference [20]. Flavonoids and polyphenols contained in *N. sativa* extract function as antioxidants that neutralize free radicals contributing to protective effects on traumatic cells or tissue damage [21, 22]. Anti-inflammatory activities have also been associated with alkaloids, terpenoids, and saponins, and contained in the seed extract [23, 24]. In addition, saponins have been reported to exert neuroprotective, anti-inflammatory, and antioxidant activities [25]. Together with flavonoids and other phenolic compounds, the extract is potentially capable of reducing necrosis and regenerating β cells [26, 27].

In the present study, the GC-MS results suggest that the extract is rich with fatty acids and their respective derivatives. A common saturated fatty acid, hexadecanoic acid, ethyl ester or ethyl palmitate, has been shown in limited evidence to exert anti-inflammatory activities. Ethyl palmitate has also been reported to be associated with anti-inflammatory of *Aspergillus arcoverdensis* extract in vitro [28]. Another compound found in the extract with high abundance in the present study was hexadecanoic acid, methyl ester or methyl palmitate. In rats, ethyl palmitate and methyl palmitate were capable of reducing inflammatory cytokines (tumor necrosis factor-α and interleukin-6) and inhibiting the infiltration of neutrophils [8]. Moreover, hexadecanoic acid, methyl ester has been suggested as neuroprotective agent with efficacy in cerebral blood flow modulation [29].

Herein, the molecular docking simulation revealed that the 9-hexadecanoic acid, methyl ester is found to be a potent inhibitor for EGR1 receptor. The binding between the protein and this compound is higher as compared to that of docosahexaenoic acid (control). In a previous study, docosahexaenoic acid, one of omega-3 fatty acids, has been reported to inhibit

inflammation via a downregulation of EGR1 expression through extracellular signal-regulated kinase signaling pathway [19]. Inhibition of EGR1 receptor may block the activity of EGR1 which involves activation and promotion of inflammatory gene expressions. EGR1 plays a role in inflammatory pain, reported by a study using a mice model with peripheral injury [30]. In mice with depression-like behaviors, miR-26a-dependent expression of EGR1 is responsible for promoting the release of inflammatory factors and the apoptosis of hippocampal neurons [11]. In stroke conditions, the overexpression of EGR1 is responsible for focal ischemic brain damage, attributed to inflammation and neuronal damage [12]. Taken altogether, the phytocompounds contained in the *N. sativa* seeds extract could act as EGR1 receptor inhibitors providing neuroprotection upon injuries.

Limitations of this study include the extract may contain impurities affecting the phytochemical identification when analyzed qualitatively with reagents or semi-quantitatively with GC-MS. Secondly, the comparison of the docking scores is not based on the native ligand since studies on EGR1 receptor inhibitors are still limited. Thirdly, the validation using molecular dynamics was not carried out. Last but not least, as a classical limitation in computational study that neglects physiological influences and other inhibitory mechanisms, the findings herein should be further confirmed in vitro or in vivo.

Conclusions

Extract from *N. sativa* seeds contained alkaloids, saponins, tannins, flavonoids, steroids and polyphenolic compounds which could potentially act as antioxidant, anti-inflammatory, and neuroprotective agents. Further analysis using GC-MS, the extract predominantly possessed anti-inflammatory fatty acids such as methyl palmitate, ethyl palmitate, and linoleic acid. Analysis using molecular docking simulation revealed that 9-hexadecanoic acid along with other fatty acids contained in the extract could inhibit EGR1 receptor, which is indicative of their neuroprotective effects.

Ethics approval

Not required.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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Underlying data

All data underlying the results can be requested from the corresponding author.

How to cite

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