ORIGINAL RESEARCH REPORT

Molecular Docking of *Crocus sativus* Phytochemicals against Inducible Nitric Oxide Synthase and Phosphodiesterase-9 in Heart Failure Preserved Ejection Fraction

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ABSTRACT

Background: Heart failure preserved ejection fraction (HFpEF) is a complex disease associated with metabolic disease as a risk factor. A previous study found an association between iNOS and PDE-9 with inflammation and myocardial fibrosis in HFpEF. **Objective**: This study aimed to identify the potential phytochemicals of *Crocus sativus* (Saffron) that can inhibit protein iNOS and PDE-9 based on a molecular docking study. **Material and Method**: A total of fifty phytochemicals were obtained from Dr. Duke's Phytochemical Database and IJAH IPB. Those phytochemicals were screened by using the PyRx application and followed with Lipinski's Rule of Five screening by using SWISS Adme. Ten phytochemicals with lowest binding energy for each protein were docked and visually analyzed using Autodock 4.2 and BIOVIA Discovery Studio Visualizer 2016. **Result**: The best binding energy between protein-phyto-chemicals were -9.17 kcal/mol and -8.55 kcal/mol for iNOS and -9.17 kcal,/mol and -9.08 kcal/mol for PDE-9. **Conclusion:** Delphinidine and malvidin are the recommended inhibitors against iNOS and PDE-9 and must be investigated in further research.

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Highlights

1. Heart failure preserved ejection fraction increases the mortality rate every year.
2. Definitive therapy for HFpEF is not well identified.
3. *Crocus sativus* phytochemical has anti-inflammatory and cardioprotective effects due to iNOS and PDE-9 inhibition.

BACKGROUND

Heart failure preserved ejection fraction (HFpEF) is a health problem with an increasing mortality rate yearly (Oktay, et al., 2013). More than 3 million Americans suffer from HFpEF, which is often misdiagnosed. HFpEF patients has a 5-year survival rate of 35% lower than cancer jor organs such as the heart, kidney, and lung, macro and microvascular inflammation, myocardial fibrosis, and disruption of the cardioprotective cGMP/PKG pathway. The whole process will have an effect on left ventricular stiffness and heart filling capacity, particularly under situations of high activity (Priksz et al., 2018; Shah, et al., 2020).

The heart and inflammatory cells will create inducible nitric oxide synthase when metabolic illness is established (Priksz, et al., 2018; Yu, et al., 2018). Consequently, XBP1s levels decrease and oxidative stress increases, particularly in the heart (Yu, et al., 2018). In addition, the presence of iNOS is considered to be able to strengthen the adhesion of plaque due to atherosclerosis in blood vessels, which has an impact on vascular dysfunction and ischemia of various organs (Daiber, et al., 2017). The systemic inflammatory process will have an impact on the activation of the cGMP/PGK pathway, which plays a cardioprotective role. In HFpEF, this pathway is overactivated, so that one of the products of the cGMP/PGK pathway, namely phosphodiesterase-9, will be excessively induced, and PDE- 9 will inactivate the cGMP/PGK pathway. Inactivation of the cGMP/PGK pathway results in increased levels of the protein titin resulting in myocardial stiffness (Borlaug & Paulus, 2011; Priksz, et al., 2018).

Crocus sativus is a part of the Iridaceae family and a traditional medicinal plant. *Crocus sativus* has reportedly been utilized as an anti-inflammatory, antioxidant, hypolipidemic, and cardioprotective agent (Gohari, et al., 2013). Previous study stated that the definitive therapy for HFpEF is not well identified and recent study investigated that iNOS and PDE-9 are potential to be targeted as anti inflammation and myocard fibrosis in HfpEF (Chowdhury, et al., 2018).

OBJECTIVE

We conducted this study to predict phytochemicals from *Crocus sativus* that potential to inhibit iNOS and PDE-9 proteins as targets for the HFpEF therapy by molecular docking. Hopefully, the result of this study can be the initiator for future in vitro and in vivo studies to investigate the inhibitory effects of iNOS and PDE-9 using *Crocus sativus.*

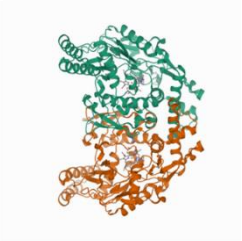
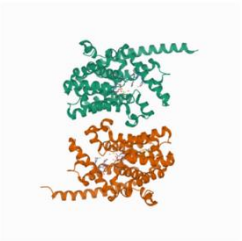
MATERIAL AND METHOD

This study was conducted using a laptop with Windows 10 software, Intel Core i3 and i5 and 4GB of RAM. The applications used are Autodock 4.2, Avogadro 2.0, Pyrx 0.9x, Biovia Discovery Visualizer 2016, and OpenBabel 2.4.1.

Protein selection

Target proteins were determined using the Protein Data Bank (https://www.rcsb.org/). The selected proteins are iNOS and PDE-9 with PDB ID 3e7G and 6a3N that shown in Table 1. The native ligands of 3E7G and 6A3N are AT2 and 9Q9, respectively as seen from the small molecule portion of both proteins via the PDB site.

Table 1. Target protein with active site (Protein Data Bank)

Protein target	PDB ID	3D structure	Native ligand	Active site
Human iNOS with inhibitor AR-C95791, Resolution 2.20 Å	3e7G		AT2 [**ethyl 4-[(4-methylpyridin-2-yl)amino]piperidine-1-carboxylate]**	ILE265, ARG266, TRP346, TYR347, MET374, TRP463, ILE462, MET120, SER118, VAL352, PHE369, MET120, TRP463, ILE462, SER118, VAL352, GLY371, GLU377, ARG382, ARG388
Crystal structure of the PDE9 catalytic domain in complex with inhibitor 2 Resolution 2.60 Å	6a3N		**9Q9 [1-cyclopentyl-6-({(2R)-1-[(3S)-3-fluoropyrrolidin-1-yl]-1-oxopropan-2-yl}amino)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one]**	PHE441, ALA452, GLN453, PHE456, GLU406, TYR424, LEU420, PHE251, HIS252, HIS256, ASN405, ILE403, ASP402, SER404

Phytochemicals selection

A total of fifty candidates for phytochemical compounds were selected through Dr. Duke's Phytochemical and Ethnobotanical Databases (https://phytochem.nal.usda.gov/phytochem/searchand) and Indonesia Jamu Herbs (IJAH IPB) (http://ijah.apps.cs.ipb.ac.id/#/home). Then, the SMILES of each compound from PubChem site (https://pubchem.ncbi.nlm.nih.gov/) was screened based on Lipinski's rule of five using the SWISS-ADME site (http://www.swissadme.ch/index.php). Further screening was carried out using the PyRx 0.9x application to determine the binding energy between the phytochemical compounds and the target protein.

The ten compounds with lowest binding energy are listed in Table 2. Compounds that met violation amount < 2 based on Lipinski's criteria and had the lowest binding energy were further analyzed using Autodock 4.2.

Validation, docking, and visualization

Protein and compound preparation were required in using Autodock 4.2. The protein preparation was needed to separate the native ligand from the target protein, then the H2O was removed from the protein. Ligand preparation was began with changing the file name from .sdf to .pdbqt using the OpenBabel 4.2.1 application, then the ligand was optimized using the Avogadro 2.0 application. Thereafter, protein validation was carried out by separating the protein only and native ligand (AT2 and QOP9) using Autodock 4.2 to determine the appropriate grid box coordinates. Protein only was added with polar-only hydrogens and Kollman Charges, while protein's native ligand and ligand must be added polar-only hydrogens, merge nonpolar, and computing gasteiger.

The grid box coordinate obtained for protein 3E7G and AT2 was X. 40 Y. 34 Z. 36, grid center X. 55.232 Y. 21.838 Z. 78.677. The grid box coordinate for protein 6A3N and native ligand 9Q9 was X.40 Y.34 Z.36, grid center X. -29.929 Y. 45.252 Z. -11.069. The docking process used the grid box and grid center as mentioned above, and fifty times genetic algorithm were run. Then, the results, such as binding

affinity and inhibition constant in .dlg files were compared between protein-native ligand and protein-10 ligands. The two-dimensional complexes visualization was provided by BIOVIA Discovery Studio Visualizer 2016 to identify the hydrogen and amino acid bond.

Table 2. Ligand screening result based lipinski rule of five and pyrx

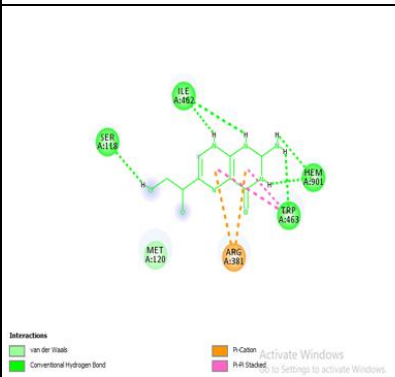
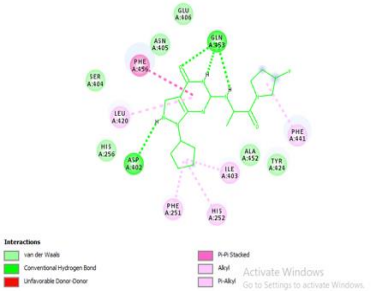
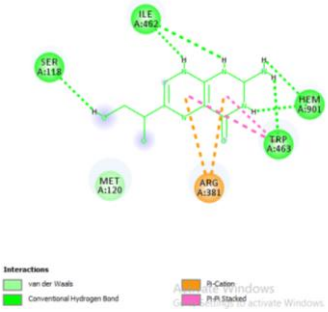
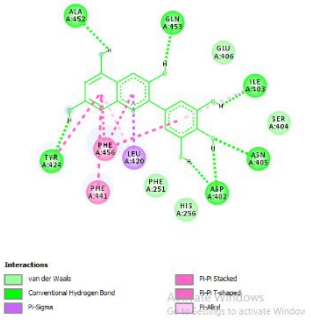
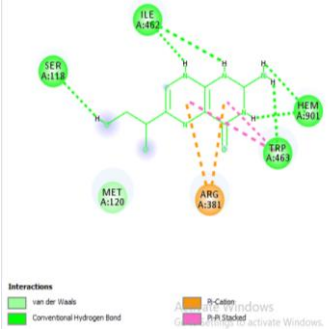
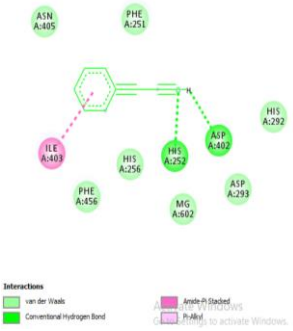
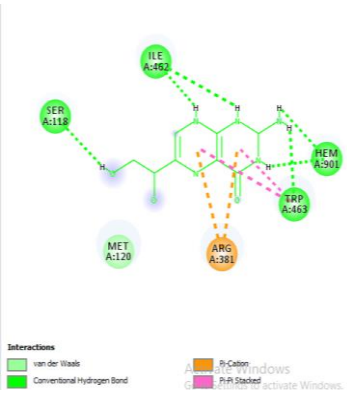
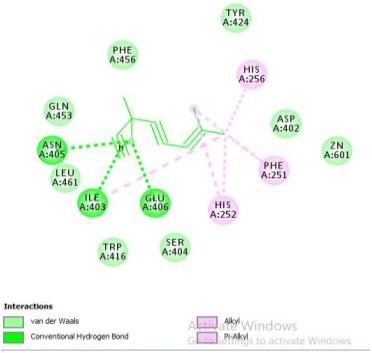
Ligand	PubChem ID	Formula molecule	Lipinski Rule of five		GIT Absorption	Pyrx iNOS binding energy	Pyrx PDE-9 binding energy	Ligand	PubChem ID	Formula molecule	Lipinski Rule of five
			Weight molecule	H-bond acceptor	H-bond donor	LogP ≤4.15		Violation			
Alpha-pinene	CID_6654	C10H16	136.23 g/mol	0	0	4.29	1	Low	-3.6	-4.9	
Alpha-terpinene	CID_7462	C10H16	136.26	0	0	3.27	0	Low	-4.1	-5.3	
Beta-phenylethanol	CID_6054	C8H10O	122.16	1	1	1.87	0	High	-5.1	-4.9	
Beta-pinene	CID_14896	C10H16	136.23	0	0	4.29	1	Low	-4.8	-5.3	
Delphinidine	CID_128853	C15H11ClO7	338.7	7	1	0.03	1	High	-5.9	-6.1	
Fenchone	CID_14525	C10H16O	153.23	1	0	2.3	0	High	-4.7	-4	
Gamma-terpinene	CID_7461	C10H16	136.23	0	0	3.27	0	Low	-4.6	-4	
Linalool	CID_6549	C10H18O	154.25	1	1	2.59	0	High	-4.9	-3.9	
Malvidin	CID_159287	C17H15O7	331.3	7	4	0.28	0	High	-3.2	-5.1	
Oleic Acid	CID_445639	C18H34O2	282.46	2	1	4.57	1	High	-4.9	-3.5	

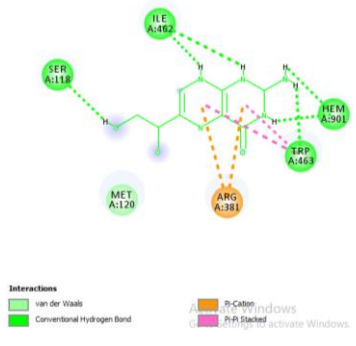
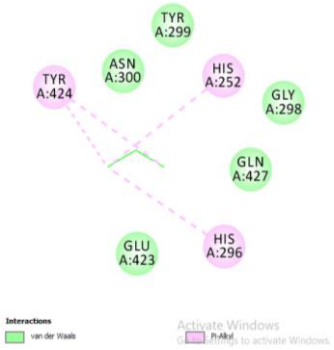
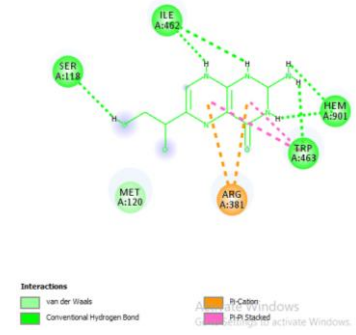
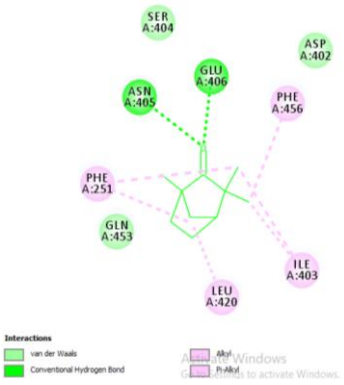
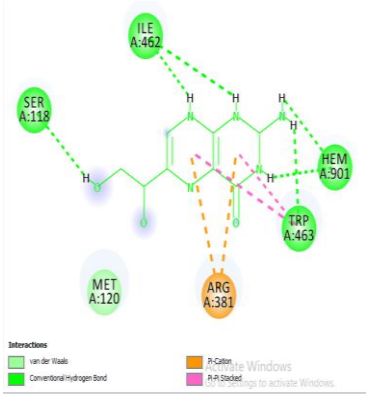
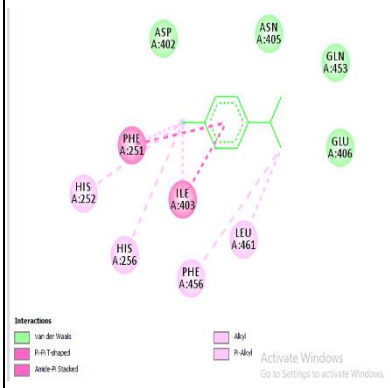
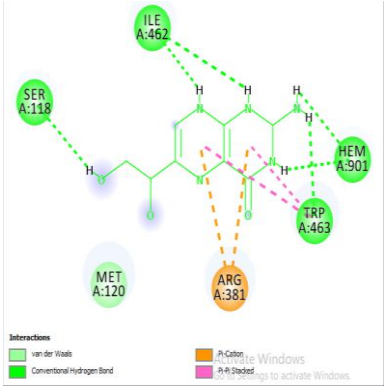
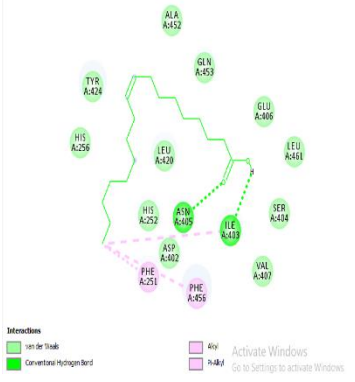
RESULT

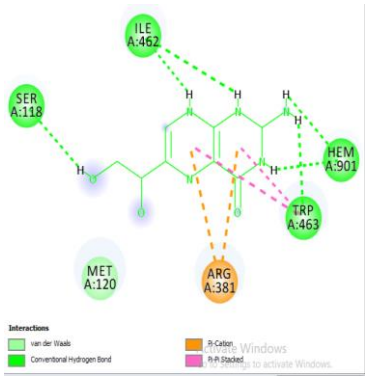
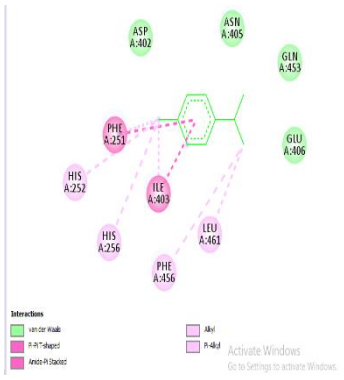
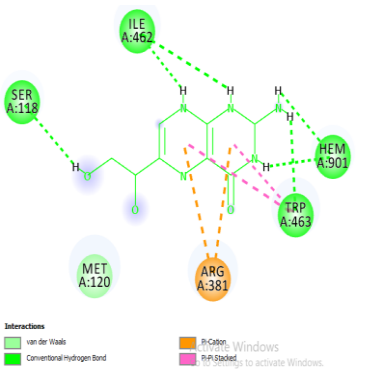
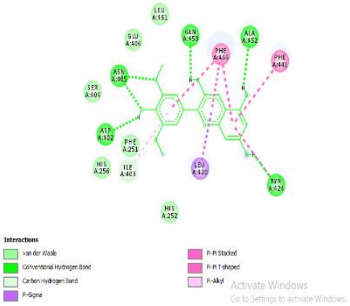
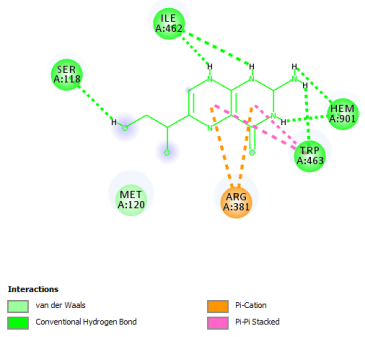
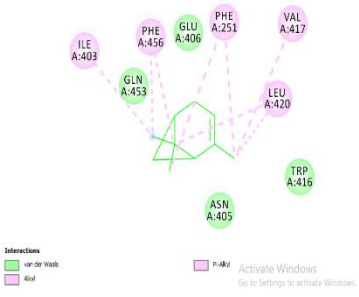
Ten from fifty phytochemicals with PyRx lowest binding energy and <2 Lipinski-rule of five's violation have been selected for docking using Autodock 4.2. Four ligands have one violation based on Lipinski rule of five, such as alpha pinene, beta pinene, delphinidine, and oleic acid. with Log P < 4.15. Beta phenylethanol, delphinidine, fenchone, linaloon, malvidine, and oleic acid have high GIT absorption, while other ligands have low absorption as shown in Table 2.

The molecular docking results between 3e7G and 6a3N proteins are shown in Table 3. The control variables in this study were the protein and native ligand binding energy and inhibitor constanta. There were no phytochemicals that had binding energy more negative than the native ligand (3e7G and AT2; -9.69 kcal/mol and 6a3N and QOP; -9.8 kcal/mol). CID_128853 phytochemical had the lowest binding energy from all ligands. CID_6054 phytochemicals had 2nd lowest binding energy (-8.55 kcal/mol) to 3a7G and (-9.08 kcal/mol) to 6a3N.

Table 3. 2D Molecular docking result

PubChem ID	3e7G (iNOS)		6a3N (PDE-9)	
Native Ligand (AT2 & QOP)		ΔG = -9.69 kcal/mol IC = 66.17 nM H-bond = 6		ΔG = -9.8 kcal/mol IC = 65.82 nM H-bond = 3
CID_128853		ΔG = -9.17 kcal/mol IC = 189.19 nM H-Bond = 6		ΔG = -9.17 kcal/mol IC = 188.90 nM H-Bond = 7
CID_6054		ΔG = -6.09 kcal/mol IC = 34.59 mM H-Bond = 6		ΔG = -4.65 kcal/mol IC = 388.32 mM H-Bond = 2
CID_6549		ΔG = -5.43 kcal/mol IC = 104.61 mM H-Bond = 6		ΔG = -5.23 kcal/mol IC = 144.70 mM H-Bond = 3

PubChem ID	3e7G (iNOS)		6a3N (PDE-9)	
CID_14896		ΔG = -3.05 kcal/mol IC = 5.81 mM H-Bond = 6		ΔG = -2.33 kcal/mol IC = 19.74 mM H-Bond = 0
CID_14525		ΔG = -8.16 kcal/mol IC = 1.04 mM H-Bond = 6		ΔG = -6.47 kcal/mol IC = 17.97 mM H-Bond = 2
CID_7461		ΔG = -6.46 kcal/mol IC = 18.33 mM H-bond = 6		ΔG = -5.39 kcal/mol IC = 111.08 nM H-bond = 0
CID_4456339		ΔG = -6.7 kcal/mol IC = 12.25 mM H-bond = 6		ΔG = -5.01 kcal/mol IC = 211.64 nM H-bond = 1

PubChem ID	3e7G (iNOS)		6a3N (PDE-9)	
CID_7462		ΔG = -6.23 kcal/mol IC = 27.06 mM H-bond = 6		ΔG = -5.38 kcal/mol IC = 113.41 nM H-bond = 0
CID_159287		ΔG = -8.55 kcal/mol IC = 54.211 ηM H-bond = 6		ΔG = -9.08 kcal/mol IC = 222.23 ηM H-bond = 4
CID_6654		ΔG = -7.9 kcal/mol IC = 1.63 µM H-bond = 6		ΔG =-6.14 kcal/mol IC = 31.64 ηM H-bond = 0

DISCUSSION

The native ligands obtained from 3e7G and 6a3N were respectively AT2 and 9Q9 with active sites as shown in Table 2. Ten phytochemicals had violation <2 based on Lipinski Rule-of-five screening and negative binding energy with both target proteins such as alpha pinene, alpha terpinene, beta-phenylethanol, beta pinene, delphinidine, fenchone, gamma terpinene, linalool, malvidin, and oleic acid. High levels of 3e7G are related to the severity of heart failure. Therefore, the treatment of heart failure with the target of inhibiting 3e7G is currently a research trend (Chowdhury, et al., 2018; Yu, et al., 2018). Phosphodiesterase is an enzyme that plays a role in hydrolysis of nucleotide adenosine 3',5'-cyclic monophosphate (cAMP) and/or cyclic guanosine 3',5'-cyclic monophosphate (cGMP) related to heart disease (Hashimoto, et al., 2018). Inhibitory action of 6a3N as target therapy can maximize the effectiveness of current heart failure treatments, especially those that have an antifibrotic or anti-apoptotic role (McMurray & Docherty, 2019).

CID_128853 and CID_159287 had the lowest binding energy to both protein (-9.17 kcal/mol-; 8.55 kcal/mol and -9.17 kcal/mol; -9.08 kcal/mol). The next sequences were CID_14525, CID_6654,

CID_7461, CID_445639, CID_7462, CID_6054, CID_6549, and CID_1489 with the higher binding strengths respectively listed in Table 3. CID_128853 and CID_159287 compounds's inhibition constants with iNOS were 188.9 nM and 54. nM, while with PDE-9 were 188.90 nM and 222.23 nM. Both compounds had an inhibition constant under 250 nM, which means that they had potential inhibition against 3e7G and 6a3N protein (Carlson, et al., 2008). Hydrogen and van der waals bond may affect the binding energy strength. Interaction between phytochemical with CID_128853 and protein 3e7G had equal hydrogen bond to native ligand that were shown in Table 3. It shows that there are 7 hydrogen bonds on CID_128853 and protein 6a3N. CID_128853 had the same amino acid bond compared to 3e7G's native ligand. They were Ser:118, Ile:462, Met:120, Arg:381, Trp:463, Hem: 901. CID_128853 had amino acid bond with Ala:452, Gln:453, Glu:406, Ile:403, Ser:404, Asn:405, Asp:402, HisL256, Phe:251, Leu:420, Phe:456, Phe: 441, Tyr:424 in 6a3N interaction. The amino acid bond was less than 6a3N and its native ligand, and it influenced the higher CID_128853 and 6a3N binding energy.

CID_128853 is a compound that is effective as anti-inflammatory by inhibiting oxidative stress, platelet aggregation and inducing prostaglandins and leukotrienes. The results of in vitro studies showed that the administration of CID_128853 in combination with other compounds resulted in endothelial cells with reduced 3e7G production. It is known to have an anti-apoptotic effect on the endothelium (Martin, et al., 2003).

CID_159287 is a plant pigment that plays a role in protecting plants from UV radiation and microbial infection. It was explained that malvidin had an inhibitory effect on lipopolysaccharide binding and nuclear factor kappa B (NFkB), the binding that has a pro-inflammatory effect. When inflammation is inhibited, minimal fibrosis occurs. CID_159287 has a positive effect on metabolic diseases such as obesity, diabetes, hypertension, and cardiovascular disease (Bognar, et al., 2013). From the docking result, CID_159287 had both protein binding energy closely to native ligand (3e7G: -8.55 kcal/mol and 6a3N: -9.08 kcal/mol). CID_159287 had <250 nM inhibitor constanta to both proteins. The amount of hydrogen and amino acid bond between 3e7G and CID_159287 was the same compared to a native ligand. CID_159287 and 6a3N had interaction with Tyr:424, Leu:420, His: 252, Ile: 403, His: 256, Phe: 251, Asp:402, Ser:404, Asn:405, Glu:406, Leu:461, Gln:453, Phe:456, Ala:452, Phe: 441, Leu:420 and 4 hydrogen bonds.

Another phytochemical with CID_14525 had binding energy with 3e7G protein (-8.16 kcal/mol) but (-6.47 kcal/mol) with 6a3N. It may be affected by the amount of the amino acid bond. Biovia Studio Visualizer showed that there were 6 amino acids bonds directly to ligand-protein. CID_14525 is an organic compound that belongs to monoterpenoids and ketones. An in vivo study stated that CID_14525 acts as an anti-inflammatory in microbial infection (Özbek, 2007). Therefore, fenchone can play a role in the wound healing process (Keskin, et al., 2017). Our result showed that the CID_14525's inhibitor constant to both proteins was more than 250 nM. CID_ 6654 and CID_14896 are monoterpene compounds that are insoluble in water and ethanol but soluble in fat (Salehi, et al., 2019).

CID_14896 has been shown to have anti-inflammatory effects and can fight H2O2, which functions as a modulator of oxidative stress. In clinical use, these two compounds work more as antimicrobials. On the heart itself, they have a positive effect as an inhibition of the inflammatory process due to endocarditis. However, CID_ 6654 is known to inhibit the binding of lipopolysaccharide and NKfB so that it has an impact on reducing 3e7G levels (Kim, et al., 2015). This is in line with the findings of this study, which showed that alpha pinene had good binding to the 3e7G protein. CID_7462 compound is known as one of the alternative treatments for hypertension from tea products (Santos, et al., 2011). An in vivo study describes CID_7462 given to mice induces potassium ions in blood vessels that act as vasodilators (Lee, et al., 2021).

CID_445639 is considered to work on endothelial protective effects due to systemic inflammatory processes in metabolic diseases with insulin resistance, reducing the process of apoptosis and instability of fatty plaques (Perdomo, et al., 2015). Other phytochemical with CID_6549 has potent anti-inflammatory through the decreasing of LPS-induced tumor necrosis and interleukin-6 production (Huo et al., 2013). In addition, an in vivo study suggested that it plays a role in lowering blood pressure by exerting a direct effect on vascular smooth muscle causing vasodilation (Anjos, et al., 2013). A study stated that CID_6054 can provide antioxidant, anti-inflammatory, anti-platelet, and anti-atherogenic activity in vitro and animal models (Tejada, et al., 2017).

Strength and limitations
This study's findings may serve as the basis for future in vitro and in vivo investigations of the inhibitory effects of iNOS and PDE-9 on Crocus sativus. This study is limited to identify one substance.

CONCLUSION

Delphinidine and maldividine are the two potential inhibitors for iNOS and PDE-9 from fifty *Crocus sativus'*s phytochemicals based on their closely binding energy compared to native ligand. The efficacy for HFpEF with iNOS and PDE-9 as definitive target therapy must be investigated by further researchers involving in vitro, in vivo, and advanced clinical trials.

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All authors have no conflict of interest.

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This research doesn't have ethical permission.

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Author Contribution
All authors have contributed to all processes in this research, including preparation, data gathering, analysis, drafting, and approval for publication of this manuscript.

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