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Inhibition of IgE and IgG1 expression in BALB/c mice and clionasterol potential bioactivity evaluation in *Dioscorea alata* extract, in vivo and in silico studies

Sri Nabawiyati Nurul Makiyah[1,*], Sri Tasminatun[2], Muhammad Sasmito Djati[3], Muhaimin Rifa'i[3], Widodo[3]

[1]*Histology and Molecular Biology Department, School of Medicine, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, Indonesia;* [2]*School of Pharmacy, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, Jl. Brawijaya, Tamantirto, Kasihan, Bantul, Daerah Istimewa Yogyakarta 55183;* [3]*Biology Department, Faculty of Mathematics and Natural Science, Brawijaya University, Malang, East Java, Indonesia*

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Abstract

The prevalence of allergic diseases worldwide is rising dramatically in both developed and developing countries. Dioscorea alata (DA) contains flavonoids and saponin steroid which have potential as anti-allergic agents. This research aims to examine the anti-allergy potential of the ethanol extract of *Dioscorea alata* (EEDA) through IgE and IgG1 levels and to investigate the bioactivity of the phytocompound of hexane extract of *D. alata* (HEDA) in silico. Allergy-induced BALB/c mice used Ovalbumin and were treated with EEDA for 30 days. The levels of B220IgE and B220IgG1 were evaluated. In silico analysis was conducted with HEDA, phytocompound from previous research, 3D structure from PubChem (https://pubchem.ncbi.nlm.nih.gov/), Chemspider (http://www.chemspi-der.com/), Bioactivity analysis on Molinspiration (https://www.molinspiration.com/), PassOnline (http://www. way2drug.com/passonline/predict.php) webservers, target proteins from SwissTarget (http:// www.swisstargetprediction.ch/), STRING (https://string-db.org/), and analyzed with Cytoscape ver 3.9.1. The docking analysis utilizes Vina, a built-in tool in PyRx 0.9, and the results are visualized using Biovia Discovery Studio. B220IgE and B220IgG1 levels decreased in the EEDA treatment significantly ($p<0,05$). The bioactivity predictions showed the activity of enzyme inhibitors, anti-inflammation agents, and cholesterol antagonists. Clionasterol, 9,12-octadecadienoic, and triosanol-1 are the phytocompounds with the highest average bioactivity scores. PSEN1, NR1C1, MAPK14, PTGS2, NR1C2, F2, FABP1, KDR, CNR1, and UGT2B7 are target proteins. Molecular docking results showed that clionasterol had the lowest average binding energy, followed by caryophyllene oxide and 11-pentan-3-ylhenicosane. Clionasterol had a high potential to interact with PPAR-α, PPAR-γ, and MAPK14 as an inhibitor. There was a decrease in B220IgE and B220IgG1 levels in mice given EEDA. Furthermore, Clionasterol could potentially inhibit PPAR and MAPK14, potentially leading to anti-inflammatory activity.

Keywords: *Dioscorea alata*, clionasterol, immunoglobulin E, anti-allergic, in silico

1. Introduction

Dioscorea alata L. (DA) is a plant used to investigate due to its high phytocompound content and potential as a primary food ingredient. Research on the tubers of DA has been conducted to identify the activity of capturing reactive oxygen species, antioxidants, antidiabetic, anticlastogenic, and antiosteoporotic (Amarasekara & Wickramarachchi 2021; Makiyah et al. 2022; Peng et al. 2011; Wang et al. 2011). Meanwhile, early research studying DA as an antiallergic agent has so far been conducted by Makiyah et al. (2014). Therefore, a study related to the expression of immunoglobulin (Ig) E and G1 was carried out to investigate more deeply the potential of the flavonoid and saponin steroid (diosgenin) of DA (Jesus et al. 2016; Wang et al. 2023) on IgE. Flavonoid has potential as an antiallergic agent (Rakha et al. 2022). The antiallergic activity of saponin steroid (diosgenin) in vivo is associated with the suppression of IgE production (Huang et al. 2009). IgE is a crucial molecule as a mediator of allergic responses (asthma, rhinitis, food allergy, atopic dermatitis, etc.) (Gould & Sutton 2008). Moreover, the understanding of the IgE concept is that IgE will act as an initial response to antigen or allergen exposure and will change the immune response to IgG (whose dominant isotypes are IgG1 and IgG4) and IgA after repeated exposure to the same antigen or allergen. (Brazdova et al. 2015). Numerous studies have shown that the majority of food allergies are immunoglobulin E (IgE)-mediated type 1 hypersensitivity reactions, which depend on the antigen-specific differentiation of T helper (Th) 2 cells in the sensitization and degranulation phases and the cytokine production of mast cells and basophil cells in the phase effect (Kumar et al. 2012; Sampson 1999; Yu et al. 2016). However, because the allergy signaling pathway is

* Corresponding author. e-mail: nurul.makiyah@umy.ac.id.

complex, in silico analysis is required to identify target proteins that can be used to decrease allergies.

This research is essential because allergy is one of the diseases with quite a lot of prevalence. Globally, 240 – 550 million people may suffer from food allergy (Pawankar et al. 2013). Research models for inducing allergies in experimental animals have been carried out using Ovalbumin (Barlianto et al. 2013). Ovalbumin sensitization as an allergen in mice can increase IgE and IgG1 levels in blood serum (Saldanha et al. 2004). In this study, DA tuber simplicia was dissolved in 70% ethanol solvent and we also analyzed the data from hexane extraction in previous studies (Makiyah & Djati 2018) to further explore the compound's wider potential in silico. Extraction with a solvent is based on the polarity of the substance in the solvent (Leksono et al. 2018). In this research, ethanol is a polar solvent used to extract flavonoid compounds from *D. alata* tubers. Ethanol is a polar to semi-polar solvent and is universal so it can extract all classes of secondary metabolite compounds; it can also extract many polar, semi-polar compounds and some non-polar compounds (Sasidharan et al. 2011) including flavonoids, which are polar, and diosgenin, which is non-polar. Ethanol can dissolve flavonoid compounds (Hakim & Saputri 2020) in *D. alata* tubers. Diosgenin was obtained from GC-MS analysis of N-hexane extract (Arya et al. 2023). N-hexane is a solvent with a low polarity level, so it is able to extract more non-polar compounds in Dioscorea alata tubers, especially Diosgenin (Hotmian et al. 2021).

The mechanism of action of Dioscorea alata as an antiallergic agent is difficult to understand, because it contains many complex compounds and metabolites. By combining the discovery of potential actionable compound targets and the isolation of active compounds, systems pharmacology approaches together with high-throughput computational analyzes provide a powerful tool to study the underlying mechanisms of DA as an antiallergic agent.

This study aims to examine the effect of the ethanol extract of DA tuber (EEDA) on B220IgE and B220IgG1 levels in the spleen of the mice BALB/c model of gastrointestinal allergy. Also, it further explores the potential of the phytocompound in the hexane extract of DA tuber using in silico to provide a high-throughput computational explanation of phytocompound bioactivity at the molecular level.

2. Materials and Method

2.1. Dioscorea alata L. sample collection.

DA tubers were obtained from the Sumberrahayu area, Moyudan, Sleman, Yogyakarta Special Region. Taxonomic identification of *Dioscorea alata* L. has been carried out in the Plant Taxonomy Laboratory, Faculty of Biology, Gadjah Mada University, with certificate number 0368/S.Tb.

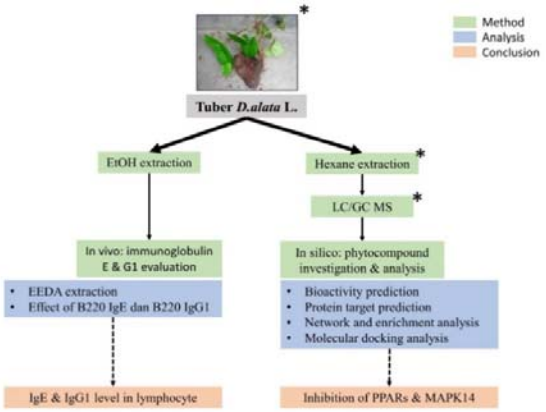


Figure 1. Graphical method for this study. ***** labeled from previous research (Makiyah & Djati 2018)

2.2. Dioscorea alata L. tuber ethanol extraction method

The preparation of ethanol extract of *DA* tubers (EEDA) refers to Badaring et al. (2020). The tubers were sliced and dried under exposure to sunlight covered with black cloth at a temperature of ± 30^0C and then mashed into simplicia. The simplicia was macerated with 70% ethanol with a simplicia: EtOH ratio of 1:70, room temperature, 5 x 24 hours. Remaceration was done using the same steps with a simplicia: EtOH ratio of 1:30 for 2 x 24 hours. Evaporation was carried out at 50ºC in a water bath using a vacuum pump evaporator.

2.3. Dosage formulation and mice model allergy with Ovalbumin

A dose conversion formula based on body weight determined the dose of the EEDA tubers (Ngatidjan 2007). The dose of EEDA was 0.17 g/kg body weight, 2.01 g/kg body weight, and 10.04 g/kg body weight orally in mice with a volume of 0.5 ml. The antihistamine drug used was Fexofenadine (Telfast®) at a dose of 0.4 mg/mice/day (Diding et al. 2008) in 0.5 ml of distilled water = 0.8 mg/ml. The dose of diosgenin is 200 mg/kg of body weight (Huang et al. 2010). Sixty-three male mice (*Mus musculus*) BALB/c strain, body weight ± 20 grams, age ± 6 weeks were divided into 7 groups (control; EEDA 0,00; EEDA 0,17; EEDA 2,01; EEDA 10,04; Drug (OAH), and diosgenin) with 9 mice in each group. Mice were kept in plastic cages measuring 30x40x20 cm. They were acclimated for 10 days in the Animal Physiology cage in the Biomol Building, floor I UB. Furthermore, for 30 consecutive days, groups I – VI were given treatment according to the group. (Table 1).

EEDA, OAH and diosgenin was induced for 30 days on day 1 until day 30. The mouse model of allergy to Ovalbumin, mice were injected intraperitoneally on day 15 with 0.15 cc of Ovalbumin in Al(OH)3/mouse from 2.5 mg of Ovalbumin dissolved in 7.75 ml of aluminum hydroxide and on day 22 with a dose of 0.15 cc of Ovalbumin in distilled water/mice from 2.5 mg of Ovalbumin dissolved in 10 ml of distilled water. On days 23 to 30, mice were exposed orally to 0.15 cc of Ovalbumin in distilled water from 2.5 mg of Ovalbumin in 2.5 ml of distilled water (Fischer et al. 2005). Mice are declared allergic if there is an increase in specific antibody levels, especially those related to Th2 immune responses

such as B220IgE and B220IgG2 after being induced by Ovalbumin. (Abril-Gil et al. 2015; Sun et al. 2013).

Table 1. Mice model and treatment

Group	Ovalbumin induction	EEDA dosage (g/kg bw/day)	Antihistamine drug dosage (mg)	Diosgenin (mg/kg bw/day)
Control	No	-	-	-
I	Yes	0	-	-
II	Yes	0.17	-	-
III	Yes	2.01	-	-
IV	Yes	10.04	-	-
V	Yes	-	0.4	-
VI	Yes	-	-	200

2.4. Flow cytometry analysis

Lymphocyte cells were isolated from the spleen of mice. The spleen was homogenized with PBS and filtered until the lymphocytes were collected in the PBS solution. Lymphocyte cell suspension was centrifuged at 2500 rpm at 4°C for 5 minutes. The supernatant obtained was discarded, and the cell suspension was resuspended with PBS and then homogenized to obtain a homogenate. The homogenate was separated based on the number of groups, and dye antibodies were added according to the protein used and standard procedures in the laboratory. The homogenate and antibody mixtures were incubated in the dark at 4°C for 20 minutes. Next, the mixture was centrifuged at 2500 rpm, temperature 4^0C, for 5 minutes. The resulting lymphocyte pellet was resuspended with 50 µL antibody (1:50) in sterile PBS, then transferred to a cuvette and mounted on a flow cytometry nozzle (BD FACS CaliburTM). The setting was done on the computer with the BD Cell Quest ProTM software and connected to the flow cytometer (acquiring mode). Flow cytometer results stored in the Cell Quest ProTM software were analyzed for the measured cytokine profile in the form of dot plots with percentage figures for the relative number of lymphocytes expressing the estimated cytokine profile and then recapitulated and analyzed (Rifa'i et al. 2023).

2.5. Hexane extract DA sample collection and GCMS method

The HEDA compound was collected based on the results of previous studies (Makiyah & Djati 2018). N-hexane extract of *DA* tuber for GC-MS test was prepared from DAE, which was dissolved in n-hexane (Merck) with the maceration method. GC-MS analysis of bioactive compounds from an n-hexane extract of *DA* L. tuber was carried out using a GC (Shimadzu Europa Gmbh) equipped with Column DB 1 (30 m long x 250µm diameter x 0.25 mm film thickness) and injection temperature 3100C as an injection split method. The mobile phase utilized Helium gas, and the stationary phase was a non-polar Agilent J&W DB-1 column with a flow rate of 39.3 mL/min. The column temperature was programmed with the initial temperature at 800C and held for 5 minutes, then increased to 3000C and held for 15 minutes. The Mass Spectrophotometer (MS) was operated on the electron impact model (EI, 70 Ev). The mass range was from 28-600 Atomic Mass Units (AMU). The results of the spectra of each peak were interpreted by the suitability method of the compound library in the database National Institute of Standards and Technology (NIST) and Wiley (Iheagwam et al. 2019).

2.6. Compound and protein 3D structure mining, bioactivity prediction, and ADME analysis

The 3D structure and canonical SMILES of HEDA were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and Chemspider (http://www.chemspider.com/). Furthermore, the physicochemical structure of the compound was analyzed through SwissADME (http://www.swissadme.ch/index.php) to determine the ADME score and drug-likeness based on Ro5. Bioactivity prediction of 33 compounds was carried out using the Molinspiration webserver (https://www.molinspiration.com/), and 6 compounds with the highest nuclear receptor ligand and bioactivity prediction scores of enzyme inhibitor were selected for further bioactivity analysis on the PassOnline webserver (http://www.way2drug.com/passonline/predict.php)(Daina et al. 2017; Filimonov et al. 2014).

2.7. Allergic Mice induced by Ovalbumin

The P I - P VI group mice were induced with an allergy model of Ovalbumin. Mice were sensitized and challenged intraperitoneally with Ovalbumin. They were injected intraperitoneally on day 15 with 0.15 cc Ovalbumin in Al(OH)3/mice from 2.5 mg Ovalbumin dissolved in 7.75 ml aluminum hydroxide and on day 22 with a dose of 0.15 cc Ovalbumin in distilled water/mice from 2.5 mg Ovalbumin which was dissolved in 10 ml of distilled water. On the 23rd to the 30th day, the mice were exposed orally to 0.15 cc of Ovalbumin in distilled water from 2.5 mg of Ovalbumin in 2.5 ml of distilled water (Fischer et al. 2005) with modification by Diding et al., 2008. On the 18th day, 25th day, and 31st day, three mice per group were sacrificed by neck dislocation. The mice were dissected, and their spleens were taken to isolate the lymphocytes.

2.8. Protein target prediction and network analysis

Eleven compounds (10-nonadecanone, 9-octadecenamide, caryophyllene oxide, clionasterol, ethyl oleate, 11-pentan-3-ylhenicosane, methyl linolelaidate, methyl octadec-10-enoate, 9,12-octadecadienol, palmitic acid, and tricosanol-1) were subjected to network analysis for further investigation of protein targets through the Swiss Target Prediction webserver (http://www.swisstarget prediction.ch/). The proteins with probability scores > 0 were taken to analyze further interactions, and the network formed through the STRING webserver (https://string-db.org/) (Szklarczyk et al. 2019). A total of 265 proteins were analyzed using STRING, with the parameter used being a high confidence score (0.700) on each edge connecting nodes. Proteins that had no interaction with the main network were selected so that there were 118 proteins for molecular docking analysis. Next, the STRING network data was analyzed in Cytoscape ver. 3.9.1. using the NetworkAnalyzer plugin to determine the score betweenness and closeness centrality and the degree of each node. Ten proteins with the highest betweenness centrality score and two additional proteins were selected for further analysis and became protein candidates for molecular docking analysis targets.

Functional annotation analysis in the form of Gene Ontology (GO) and Pathway (KEGG) was performed on the 118 proteins using the DAVID web server (https://david.ncifcrf.gov/tools.jsp) for their bioactivity in cells. Furthermore, the enrichment related to the bioactivity of 12 proteins was carried out using ClueGO with the following settings: Load marker, Homo sapiens [9606], GOterm and KEGG, Network specificity, medium, pV < 0.05 (Bindea et al. 2009; Shannon et al. 2003; Xia et al. 2014)

2.9. Molecular docking analysis

Six proteins were selected as docking target proteins and downloaded from the RCSB PDB (https://rcsb.org/) with 11 selected compounds, namely PPARA (PDB ID: 6LX4), PPAR-Γ (PDB ID: 4XLD), MAPK14 (PDB ID: 1DI9), PTGS2 (PDB ID: 5IKR), CYP19A1 (PDB ID: 5JKW), and HRH1 (PDB ID: 3RZE). The control compounds were standard drugs registered in the drug bank (https://go.drugbank.com/drugs). Sequentially, the control compounds used were Fenofibric Acid (CID: 64929), Rosiglitazone (CID: 445655), 4-[3-methylsulfanylanilino]-6,7-dimethoxyquinazoline (MSQ) (CID: 1714), Rofecoxib (CID: 5090), testosterone (CID: 6013), and Doxepin (CID: 667477) (Table 2). Protein was prepared using Biovia Discovery Studio ver 19.1.0.18287 to remove ligands, water, and Chimera ver. Alpha to construct the missing residue using a modeler (Webb and Sali, 2016). Molecular docking analysis was triplicated using AutodockVina on PyRx 0.9.7 with standard procedures. The binding site was determined based on the experimental results of interacted residue on PDB as seen through PDBsum (https://www.ebi.ac.uk/). Next, the binding energy score was recorded, and 3D visualization was performed using Biovia Discovery Studio ver 19.1.0.18287.

Table 2. The list of proteins and drugs used as a control group

No	Protein	PDB ID	Drug Name	Drug CID	Drug Pharmacology	Reference
1	PPARA	6LX4	Fenofibric acid	64929	Agonist	(Kamata et al. 2020)
2	PPAR-Γ	4XLD	Rosiglitazone	445655	Agonist	(Mirza et al. 2019) (Gelin et al. 2015)
3	MAPK14	1DI9	4-[3-methylsulfanylanilino]-6,7-dimethoxyquinazoline	1714	Inhibitor	(Shewchuk et al. 2000)
4	PTGS2	5IKR	Rofecoxib	5090	Inhibitor	(Nuvoli et al. 2021)
5	CYP19A1	5JKW	Testosterone	6013	Substrate	(Ghosh et al. 2018)
6	HRH2	3RZE	Doxepin	667477	Antagonist	(Shimamura et al. 2011)

2.10. Statistical analysis

Data in the form of B220IgE and B220IgG1 profiles in B cells were analyzed using the One-Way ANOVA test followed by the Tukey test (Pratiknya 2000). Statistical analysis of One-Way Anova and Two-Way Anova with Dunnett's multiple comparisons were utilized to provide statistical evidence based on the significance of the difference in scores obtained from the docking results.

2.11. Ethical Clearance

This research has received approval for ethical clearance by the Research Ethics Committee of the Universitas Muhammadiyah Yogyakarta with a certificate number 002/EP-FKIK-UMY.

3. Results

3.1. Effect of EEDA on body weight and B220IgE and B220IgG1 cytokine profiles

There was a change in body weight and cytokine profiles of B220IgE and B220IgG1 in the gastrointestinal tract's sensitivity, challenge, and allergic phases after treatment with *D. alata* tuber ethanol extract (EEDA) in Balb/C mice model of digestive tract allergy induced by Ovalbumin (Figure 2 and 3).

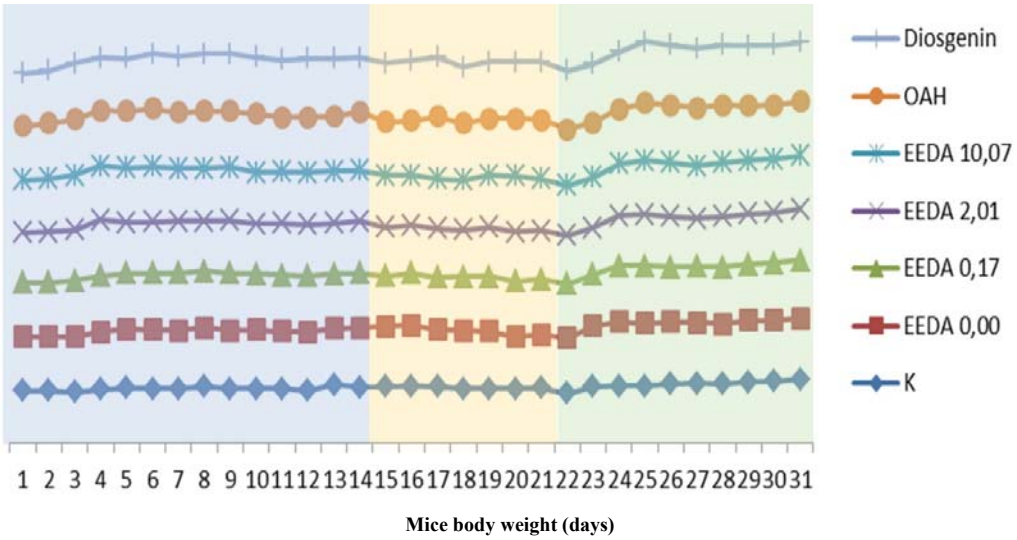


Figure 2. Comparison of representative mice body weight (g) in the sensitivity phase (blue area), challenge phase (yellow area) and digestive tract allergy phase (green area) after administration of *Dioscorea alata* L. tuber ethanol extract (EEDA) in the control group (K), negative control (KN), EEDA 0.17 g/kg; EEDA 2.01 g/kg; EEDA 10.04 g/kg, Antihistamines (OAH) and Diosgenin.

The body weight of the mice in this study increased every day for 30 consecutive days. The body weight of mice can be seen in the sensitivity phase, challenge phase, and allergic phase of the digestive tract after intraperitoneal induced Ovalbumin on day 15 and 22 and Ovalbumin induced orally on day 23 to day 30 (Figure 2). It indicated a slight decrease in body weight from day 16 to day 17, on day 23 to day 24, and on day 24 to day 26.

According to (Dourado et al. 2010), Ovalbumin sensitization in Balb/C mice increased IgE and IgG1 levels. During the sensitization phase, there was an increase in the cytokine profiles B220IgE and B220IgG1 in all groups induced by Ovalbumin. The highest profile of cytokine B220IgE and B220IgG1 was in the EEDA treatment group of 10.04 g/kg. Intraperitoneal injection of low doses of Ovalbumin as an antigen and aluminum hydroxide as an adjuvant resulted in increased formation of high levels of IgE antibodies (Aguilar-Pimentel et al. 2010; Barwig et al. 2010; He et al. 2015; Lee et al. 2013). In the challenge phase, there was an increase in the profile of cytokine B220IgE and IgG1 in all Ovalbumin-induced mice. Controls had the lowest profiles of B220IgE and B220IgG1 cytokines. The highest profile of B220IgE and B220IgG1 cytokines at 0.17 g/kg EEDA was higher than 0.00 g/kg EEDA which was only induced by Ovalbumin. In the allergic phase of the gastrointestinal tract, there was a significant decrease in the profile of cytokines B220IgE and B220IgG1 in EEDA and anti-histamine drugs ($p < 0.05$) (Figure 3). The profile of cytokine B220IgE and B220IgG1 was highest at 0.00 g/kg EEDA and Diosgenin. It showed that Ovalbumin as an allergen had made mice model allergies, proving that administering Ovalbumin can increase IgE and IgG1 levels of Balb/c mice in the sensitivity, challenge, and allergic phases of the digestive tract. One of the clinical symptoms of food allergy is increased levels of IgE and IgG1 (Gocki & Bartuzi 2016).

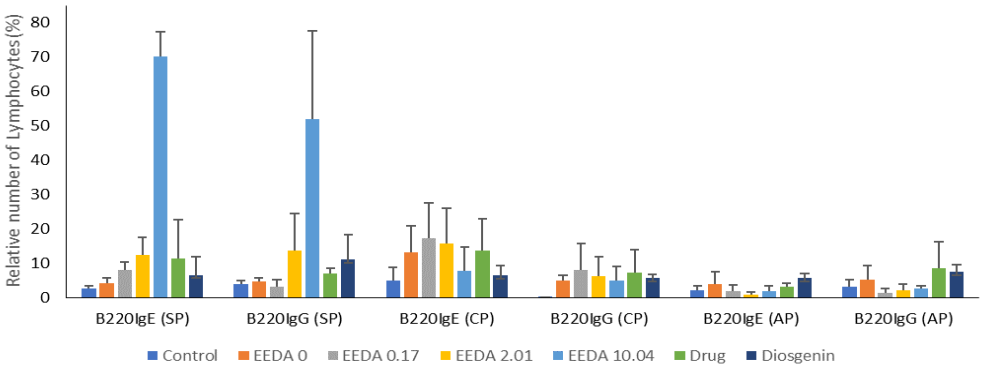


Figure 3. Comparison of the relative number of mice spleen lymphocytes expressing B220IgE and B220IgG1 in the sensitivity phase (A), challenge phase (B) and digestive tract allergic phase (C) after administration of EEDA in the control group, EEDA 0 g/kg as negative control, EEDA 0 .17g/kg; EEDA 2.01 g/kg; EEDA 10.04 g/kg, antihistamines drug and Diosgenin

3.2. Phytocompound based on GCMS and drug-likeness analysis

Research related to the potential of *D.alata* phytocompound is still interesting due to the active compound content and other macromolecules that make it suitable as a staple food. (Makiyah & Djati 2018; Makiyah et al. 2022; Salehi et al. 2019). Based on the GCMS results, 40 peaks were obtained with a total of 33 secondary metabolites from the hexane extract of *DA*

(HEDA) (Table 3). Based on the peaks, the 33 phytocompounds ranged from Tetradecane (13,324) to clionasterol (37,544). Hexadecanoic acid, ethyl ester, and 9-Octadecenamide (Z) were the constituents with the lowest abundances of 0.34% and 0.47%, while methyl palmitate and methyl stearate were the phytocompounds with the highest abundances of 11.59% and 11.75% as revealed from our previous studies (Makiyah & Djati 2018). Phytocompounds with the lowest molecular weight (168 Da) are 1-undecane, 7-methyl- and 1-nonene, 4,6,8-trimethyl. Clionasterol is a phytocompound with a molecular weight of 414 Da. All phytocompounds have a Rule of five (Ro5) violation of less than two, which means they have good drug-likeness. The Ro5 parameter is formulated to assess the absorbance potential of drugs orally based on their physicochemical structure (Leeson et al. 2021). However, it is less relevant to see the potential for drugs that are applied by injection or in other ways other than orally. Still, at least 90% of the compounds from the Available Chemical Directory follow the Ro5 parameter (Benet et al. 2016).

3.3. Bioactivity prediction result

An orally active drug clionasterol, 9,12-octadecadienoic, and tricosanol-1 are the compounds with the highest average probability scores (Tables 4 and 5). Specifically, clionasterol had the highest bioactivity score as a nuclear receptor ligand (0.734), 9,12-octadecadiol as an ion channel modulator (0.152), and tricosanol-1 with scores above the average on several bioactivities. Based on the high bioactivity scores of the nuclear receptor of ligands and enzyme inhibitors compared to other bioactivities, it can be concluded that the compounds contained in HEDA had high potential as nuclear receptor ligands and enzyme inhibitors so that from this bioactivity, six phytocompounds were investigated further in the next analysis.

Furthermore, we also predicted the bioactivity of the six phytocompounds using PassOnline as an anti-inflammatory, non-steroidal inflammatory agent, immunosuppressant, anti-hypercholesterolemic, and cholesterol antagonist. In anti-inflammatory bioactivity, 9,12-octadecadienoic, methyl linolelaidate, and caryophyllene oxide had scores that stood out from the other three phytocompounds. Furthermore, non-steroidal inflammatory agents were the bioactivities with the lowest average predictive scores of the other four bioactivities, followed by anti-hypercholesterolemic and immunosuppressant. Meanwhile, for the two bioactivities related to anti-cholesterol, clionasterol seemed to have the highest score of the other phytocompounds. The predictive score for the bioactivity of clionasterol was increased, indicating that this phytocompound was interesting to identify its anti-cholesterol role in further studies. (Table 4).

Table 3. Prediction of the active compounds of D.alata hexane extract based on GCMS analysis in previous studies (Makiyah & Djati 2018)

Line	Compound	Retention time (min)	Area (%)	MW	Formula	CAS	Lipinski #violations
1	Clionasterol	37.544	6.99	414	C29H50O	83-47-6	1
2	9,12-octadecadienol	23.598	2.67	266	C18H34O	1577-52-2	1
3	Tricosanol-1	24.053	5.4	340	C23H48O	3133-01-5	1
4	Methyl linolelaidate	22.936	4.03	294	C19H34O2	2566-97-4	1
5	Caryophyllene oxide	18.487	1.81	220	C15H24O	1139-30-6	0
6	11-Pentan-3-ylhenicosane	19.617	0.87	366	C26H54	55282-11-6	1

Table 4. Phytocompound bioactivity prediction by MolInspiration

No	Compound Name	GPCR Ligand	Ion Channel Modulator	Kinase Inhibitor	Nuclear receptor ligand	Protease Inhibitor	Enzyme Inhibitor	Mean
1	Clionasterol	0.136	0.045	-0.51	0.734	0.071	0.509	0.164
2	9,12-octadecadienol	0.152	0.152	-0.086	0.142	-0.03	0.306	0.106
3	tricosanol-1	0.08	0.021	0.006	0.126	0.088	0.102	0.071
4	methyl linolelaidate	0.147	0.069	-0.195	0.135	0.027	0.225	0.068
5	caryophyllene oxide	-0.081	0.144	-0.861	0.621	0.003	0.567	0.066
6	11-pentan-3-ylhenicosane	0.089	0.049	-0.107	0.113	0.138	0.061	0.057

Table 5. Phytocompound bioactivity prediction by PassOnline

No	Compound Name	Anti-inflammation	Non-steroidal anti-inflammatory agent	Immunosuppressant	Antihyper-cholesterolemic	Cholesterol antagonist
1	Clionasterol	0.467	0.000	0.762	0.960	0.957
2	9,12-octadecadienol	0.745	0.355	0.484	0.644	0.751
3	tricosanol-1	0.498	0.275	0.430	0.452	0.654
4	methyl linolelaidate	0.728	0.402	0.538	0.691	0.792
5	caryophyllene oxide	0.759	0.414	0.758	0.000	0.281
6	11-pentan-3-ylhenicosane	0.402	0.174	0.336	0.522	0.599
	Mean	0.600	0.270	0.551	0.545	0.672

3.4. Protein-protein network and enrichment analysis

Based on the results of network enrichment of 118 proteins, it is revealed that 10 proteins encoded by the genes Presenilin1 (PSEN1), nuclear receptor subfamily 1, group C, member 1 (NR1C1) or Peroxisome Proliferator Activated Receptor Alpha (PPARA), mitogen-activated protein kinase 14 (MAPK14), prostaglandin-endoperoxide synthase 2 (PTGS2) or Cyclooxygenase 2 (COX-2), nuclear receptor subfamily 1, group C, member 2 (NR1C2) or Peroxisome Proliferator Activated Receptor Gamma (PPAR-Γ), Coagulation Factor II (F2), Fatty Acid-Binding Protein-1 (FABP1), Kinase Insert Domain Receptor (KDR), Cannabinoid Receptor 1 (CNR1), and UDP-Glucuronosyltransferase-2B7 (UGT2B7) had a role as a hub on networks. The majority of proteins in the network were known to have a role in metabolic pathways, various signaling in cells, and several disease pathways, such as cancer and Alzheimers. The results of a functional analysis of the interactions between GOTerm and KEGG on 12 selected proteins (10 hub proteins, H1R (HRH1), and Aromatase (CYP19A1)) showed parental bioactivity related to the production of inflammatory-related molecules, regulation of fatty acid and small molecule metabolism, and oxidation of fatty acids (Figure 4). Six (6) proteins Peroxisome Proliferator Activated Receptor Alpha (PPARA), Peroxisome Proliferator Activated Receptor Alpha (PPAR-Γ), MAPK14, COX2, CYP19A1, H1R) were selected for molecular docking analysis as they had a dominant role in the inflammatory process and were proteins targeted by more than 3 phytocompounds (Nuvoli et al. 2021; Pawlak et al. 2015).

3.5. Molecular docking analysis result and visualization

The molecular docking analysis results demonstrate that the binding energy (kcal/mol) required for interactions between the six compounds and the six protein is higher on average than the binding energy necessary for interactions between the control compounds and the proteins (Table 5). However, clionasterol and caryophyllene oxide were the active compounds with the lowest average binding energy than control. Clionasterol has significantly lower binding energy than MSQ (CID: 1714) (P<.0001) and requires binding energy that is not significantly different from that of fenofibric acid (CID: 64929) and rosiglitazone (CID: 445655) to interact with PPARA and PPAR-Γ. Specifically, clionasterol has a high potential to interact with PPAR, CYP19A1, and MAPK14.

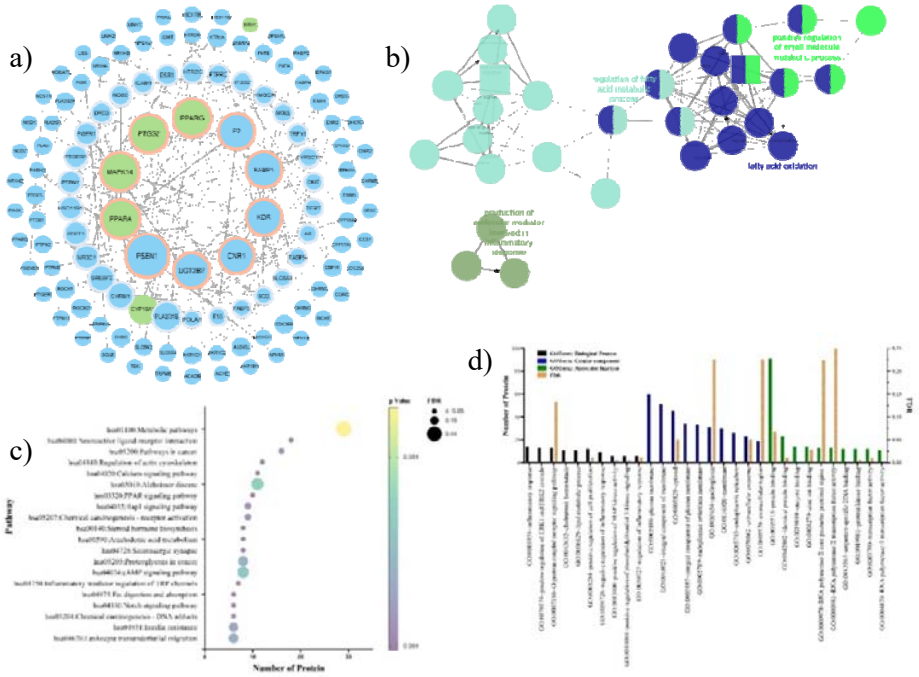


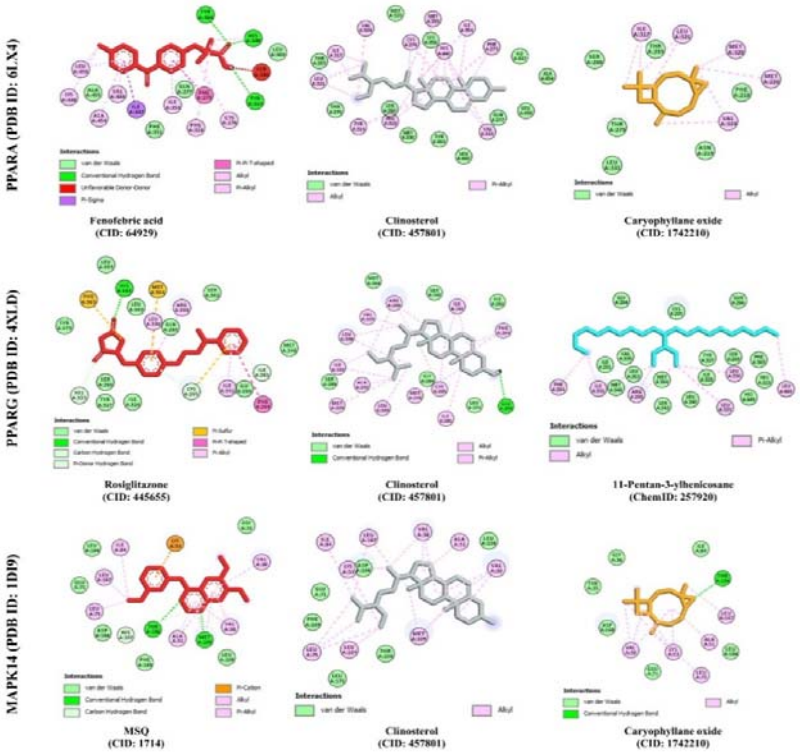
Figure 4. Target protein interaction network of 11 HEDA compounds and their biological activities. a) Network of 118 target proteins. The green proteins were the target proteins in molecular docking analysis. They were selected based on the literature regarding their bioactivity in inflammation and their suitability as proteins for molecular docking analysis. The size of the protein nodes scaled with the betweenness centrality (BC) score. 10 proteins with the highest BC scores had red border nodes. b) Bioactivity of 12 related protein genes (PSEN1, PPARA, MAPK14, PTGS2, PPAR-Γ, F2, FABP1, KDR, CNR1, UGT2B7, HRH1, and CYP19A1) based on GOTerm (round nodes) and KEGG pathway (square nodes). c) GOTerm enrichment analysis of 118 proteins. d) Pathway formed from 118 proteins based on KEGG.

Table 6. Protein and ligand binding energy (kcal/mol)

No	Ligand	PPARA	PPAR-Γ	MAPK14	PTGS2	CYP19A1	HRH1
1	Control	-9.67 ± 0.23	-8.70 ± 0.00	-6.73 ± 0.06	-8.40 ± 0.00	-10.10 ± 0.00	-10.83 ± 1.33
2	Clionasterol	-9.60 ± 0.00	-8.57 ± 0.06	-8.13 ± 0.12	-4.67 ± 0.06	-9.17 ± 0.23	-4.47 ± 0.90
3	Caryophyllene oxide	-7.23 ± 0.40	-6.30 ± 0.00	-6.97 ± 0.06	-6.00 ± 0.00	-7.10 ± 0.00	-7.77 ± 0.32
4	11-Pentan-3-ylhenicosane	-6.70 ± 0.17	-6.37 ± 0.15	-5.40 ± 0.17	-7.27 ± 0.06	-5.90 ± 0.10	-7.40 ± 0.10
5	Methyl linolelaidate	-6.67 ± 0.15	-6.37 ± 0.21	-5.07 ± 0.06	-6.97 ± 0.06	-6.03 ± 0.06	-7.40 ± 0.10
6	9,12-octadecadienol	-6.40 ± 0.10	-6.13 ± 0.31	-5.07 ± 0.12	-6.70 ± 0.10	-6.20 ± 0.17	-7.20 ± 0.00
7	Tricosanol-1	-6.43 ± 0.21	-5.90 ± 0.20	-4.87 ± 0.15	-6.60 ± 0.17	-5.90 ± 0.10	-7.33 ± 0.06

The visualization results on three proteins (PPARA, PPAR-Γ, and MAPK14) with low binding energy when interacting with HEDA compounds showed some interesting data. Clionasterol had a binding energy score that was not significantly different from binding energy control in PPARA and PPAR-Γ. Second, the number of hydrogen bonds formed during interaction did not always contribute to lowering the binding energy. (Figure 5; Supplementary table 1).

A



B

Figure 5. Ligand binding position and 2D interaction with the receptor. A) The location of the binding site of each receptor and the comparison of the interaction positions of each compound with the control B) Ligand interaction with receptor amino acid residues in 2D

4. Discussion

The body weight variable is a marker of the success of the gastrointestinal allergy model mice, indicated by diarrhea in mice characterized by watery feces and weight loss. This study successfully made mice models of canal allergy through a slight decrease in body weight after administration of the Ovalbumin allergen. It aligns with the results of research by Mollica et al. (2013). This study showed no difference in feed consumption between groups

of mice, but the mice treated with Ovalbumin lost a little weight due to hypercatabolism caused by the production of inflammatory cytokines (Cara et al. 1997; Dourado et al. 2010). This study did not evaluate whether the weight loss in the Ovalbumin treatment group was due to a decrease in feed consumption or water retention, as found in a (Moreira 2006) study on Ova-sensitized test animals.

The research results of Jan et al. (2007) also showed that the body weight of mice treated with Diosgenin doses of 200 mg/kg and 400 mg/kg did not differ significantly from the body weight of the control group mice. After the sensitization phase with Ovalbumin, re-exposure to the antigen causes local or systemic manifestations of food allergy. Systemic antigen sensitization using intraperitoneal adjuvants is conducted to induce antigen sensitization and food hypersensitivity responses to antigen challenges. This process provides essential insights into the mechanisms of the effector phase of food allergies. (Link et al. 2020; Yoo et al. 2014). Furthermore, the results of this study indicated that EEDA had a variety of active compounds that had the potential as anti-allergenic agents. It is proven that EEDA could reduce IgE and IgG1 levels of Balb/c mice in the allergic phase of the digestive tract. Ovalbumin stimulated an allergic reaction and repeated exposure to Ovalbumin activates CD4+ lymphocytes, stimulating B cells to increase IgE production. IgE is essential in developing allergic reactions (Akdis et al. 2020; Majewska-Szczepanik et al. 2016). Research by Mine et al., (2019) and Wu et al., (2020) showed that giving Ovalbumin orally to mice induced the production of Th2 and IgE cytokines. It will result in an immune response to allergic inflammation. One of the parameters to analyze the development of the allergic process is IgE levels. IgE binds to the FcεRI receptor on the surface of mast cells. In line with allergen exposure, inflammatory mediators are released to trigger allergic symptoms. Administration of EEDA doses of 0.17, 2.01, and 10.04 g/kg in allergic model BALB/c mice in the allergic phase of the gastrointestinal tract in this study reduced the profile of cytokines B220IgE and B220IgG1.

In addition, regarding hub proteins that are not the target of molecular docking analysis, the role of UGT2B7, PSEN1, F2, FABP1, and CNR1 in the inflammatory process is still under further research. UGT2B7 is an enzyme commonly found in the liver that plays an important role in phase II metabolic pathway of chemical compounds, including drugs and endogenous compounds. (Abdullah & Ismail 2018; Yang et al. 2017) Presenilin-1 (PSEN1) is a protein part of the γ-secretase complex which plays a role in the production of amyloid peptide and amyloid precursor protein (APP) which is widely recognized for its effect on Alzheimer's disease (AD) where more than 300 mutations have been known to be involved in the neurodegenerative pathway (Arber et al. 2021; Bagaria et al. 2022; Kelleher & Shen 2017). Besides, prothrombin (F2) is an unstable coagulation protein. It will be cleaved into a small functional protein called thrombin. Thrombin has pro-inflamm atory activity by activating protease-activating receptors on immune cells such as monocytes, dendritic cells, endothelium, and lymphocytes (Palta et al. 2014). Kinase Insert Domain Receptor (KDR), also known as vascular endothelial growth factor receptor-2 (VEGFR-2), is a receptor protein in the endothelium. KDR plays a major role in signaling processes through angiogenesis, cell proliferation, and neovascularization growth factors. (Modi & Kulkarni 2019; Yeo et al. 2019). Next, fatty acid binding protein-1 (FABP1) and Cannabinoid receptor-1 (CNR1) play a role in the endocannabinoid system (ECS). FABP1 plays an important role in lipid metabolism. It is a cytoprotectant and minimizes oxidative damage in hepatocytes (Schroeder et al. 2016; Wang et al. 2015). Meanwhile, CNR1 is abundant in cells in the cerebral cortex, basal ganglia, hippocampus, and cerebellum, which cause the brain to experience psychotropic effects due to the presence of cannabis. (Im 2013; Ye et al. 2019)

COX-2 (*PTGS2*) is an inducible enzyme whose excessive activity will exacerbate inflammation as it is the main source of prostaglandins (PGs) and is also associated with CYP19A1 related to inflammatory processes in malignant pleural mesothelioma (MPM) cells. CYP19A1 (aromatase) is a cytochrome P450 enzyme complex co-translated with COX-2 by expressing PKA-CREB-dependent genes (Ju et al. 2022; Nuvoli et al. 2021). Human histamine receptor H1 (HRH1) plays a role in signaling, resulting in allergies, anaphylaxis, asthma, and autoimmune disease. HRH1 inhibition can inhibit the TH2 response, form IL-4 and IL-13, and prevent airway inflammation and hyperactivity reactions by allergens (Wang et al. 2014). Peroxisome proliferator-activated receptors of alpha and gamma (PPAR-A and PPAR-Γ) are inducible nuclear transcription factors that can induce adipocytokine signaling pathways related to metabolisms, such as keto-lipogenesis, lipid, fatty acid, and cholesterol metabolism, adipocyte differentiation, and cell survival (Michalik & Wahli 2008; Pawlak et al. 2015; Tan et al. 2021). MAPK14 (Mitogen activation protein kinase-14), also known as p38 / p38a, regulates the biosynthesis of inflammatory cytokines such as TNF-a, IL-1, IL-6, and IL-1b. Furthermore, MAPK14 is an important protein in initiating inflammatory disorders, cardiovascular cases, neurodegenerative disease, and cancer, where inhibition of MAPK14 can be an alternative to overcome these disorders (Ali et al. 2021; Ariey-Bonnet et al. 2020; Madkour et al. 2021).

Clionasterol, also known as γ-sitosterol, is a phytosterol compound that is the epimer of β-sitosterol. Clonasterol has high potential as a hypolipidemic agent as it can have low binding energy when interacting with acetoacetyl thiolase, 3-(HMG-CoA) reductase, HMG-CoA synthase, squalene synthase, and oxide-squalene cyclase. In addition, the activity of clionasterol as an anti-hyperlipidemic was also shown by lowering blood cholesterol and triglyceride levels in streptozotocin-induced rats. (Balamurugan et al. 2015). Reducing cholesterol levels in the blood can reduce inflammatory activity, thereby reducing the possibility of diseases such as chronic inflammation, atherosclerosis, and obesity. (Tall & Yvan-Charvet 2015) Elective cytotoxicity activity by clionasterol from *Strobilanthes crispus* on colon cancer cell lines (Caco-2), liver cancer cell lines (HepG2), and hormone-dependent breast cancer cell lines (MCF-7) has low and high IC50 in normal cells (Chang liver cell line). Cytotoxicity occurs since clionasterol can induce apoptosis in Caco-2 and HepG2 and suppress the c-myc gene expression in all cell lines (Endrini et al. 2015). Another study evaluated the clionasterol-rich hexane fraction (CRHF2) of Caulerpa racemosa (CR) against particulate

matter-induced skin damage using a zebrafish model. CRHF2 of CR demonstrated superior protective activity by downregulating ROS and mitochondrial levels, inhibiting oxidative stress, and inhibiting mitochondrial-mediated apoptosis. This result supported the idea that clionasterol has the potential for pharmaceutical development to attenuate inflammation-related diseases. (Liyanage et al. 2022)

MAPK signaling plays an important role in cell differentiation, cell activation, cell proliferation, degranulation and migration of various immune cells (Duan & Wong 2006). MAPK signaling is involved in mast cells that regulate cytokine production in response to specific extracellular stimuli and then initiate biological reactions (Li et al. 2016). Apart from MAPK, Peroxisome proliferator-activated receptor-γ (PPAR-γ) also plays an important role in the response to allergies. PPAR-γ is a nuclear receptor that has emerged as an important regulator of multiple cell types involved in the inflammatory response to allergens; from airway epithelial cells to T Helper (TH) cells. Over the past 10 years, it has become clear that PPAR-γ is a critical component of the type 2 immune response to allergens. In particular, PPAR-γ is important in driving allergic responses in tissue, for instance by promoting IL-33R expression on TH2 cells and ILC2. PPAR-γ may be a critical environmental sensor that modulates allergic immune responses (Stark et al. 2021).

The molecular docking results were interesting as the binding sites were located in different locations even though they were close together. Clionasterol could interact with Phe273 residue as an active site with insignificant differences in binding energy with fenofebric acid, indicating it could interfere with the activation of PPAR-A. (Kamata et al. 2020). Rosiglitazone could form bonds with Cys285 and His323 residues, which were the active sites of PPAR-Γ but not with clionasterol or pentane (Gelin et al. 2015). Based on these results, clionasterol could potentially reduce drug use due to its efficacy as a PPARA and PPAR-Γ agonist. PPAR-γ agonists can mitigate allergic inflammation by suppressing pro-inflammatory gene expression in epithelial cells. PPAR-γ promotes type 2 immune responses by regulating lipid metabolism and inducing effector gene expression. Preclinical models implicate PPAR-γ in driving allergic inflammation (Stark et al. 2021). It was known from the interaction of MSQ with MAPK14, which formed three hydrogen bonds on His107, Thr106, and Met109 residues. They had higher binding energy than clionasterol, which did not form hydrogen bonds in its interactions, but hydrophobic (Met109) and van der Waals bonds (Thr106).

MAPK signaling plays an important role in cell differentiation, cell activation, cell proliferation, degranulation and migration of various immune cells (Duan & Wong 2006). MAPK signaling is involved in mast cells that regulate cytokine production in response to specific extracellular stimuli and then initiate biological reactions (Li et al. 2016). Apart from MAPK, Peroxisome proliferator-activated receptor-γ (PPAR-γ) also plays an important role in the response to allergies. PPAR-γ is a nuclear receptor that has emerged as an important regulator of multiple cell types involved in the inflammatory response to allergens; from airway epithelial cells to T Helper (TH) cells. Over the past 10 years, it has become clear that PPAR-γ is a critical component of the type 2 immune response to allergens. In particular, PPAR-γ is important in driving allergic responses in tissue, for instance by promoting IL-33R expression on TH2 cells. ILC2. PPAR-γ may be a critical environmental sensor that modulates allergic immune responses (Stark et al. 2021).

5. Conclusion

There was a decrease in IgE and IgG1 levels in mice given EEDA. Clionasterol in HEDA had the potential to become PPAR agonist and MAPK14 inhibitors due to its bioactivity as an anti-inflammatory. This research has provided information on the possible potential efficacy of the active and secondary compound content of DA. Further research for its development as a standard herbal medicine or health supplement requires more research and evaluation of the adverse effects that may result from long-term consumption.

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Attachment

Supplementary table 1. Binding energy which needed (kcal/mol)

Protein	Ligand	Binding Energy (kcal/mol)	Interacted residue					
			Hydrogen bond	Hydro-phobic bond	Electro-static bond	van der Waals bond	Unfavor-able bond	Others bond
PPARA	Control (Fenofebric acd)	-9.7 ± 0.23	TYR314 HIS440 TYR464	PHE273 CYC276 PHE318 ILE354 VAL444 ILE447 LYS448 ALA454 LEU456		GLN277 PHE351 ALA455 LEU460	SER280	
	Clionasterol	-9.6 ± 0.00		PHE273 CYS276 TYR314 ILE317 PHE318 LEU321 VAL324 ILE354 MET355 HIS440 VAL444		GLN277 THR279 SER280 THR283 MET320 MET330 LYS358 ILE447 ALA454 LEU456 LEU460 TYR464		
	Caryophyllene oxide	-7.2 ± 0.40		MET220 ILE317 MET320 LEU321 VAL324		PHE218 ASN219 THR273 SER280 THR283 LEU331		
PPARG	Control (Rosiglitazone)	-8.7 ± 0.00	ILE281 CYS285 HIS323 HIS449	PHE264 ARG288 LEU330 ILE341		GLY284 GLN286 SER289 ILE326 TYR327 SER342 MET348 LEU453 LEU469 TYR473		CYS285 (Pi-sulfur) PHE363 (Pi-sulfur) MET364 (Pi-sulfur)
	Clionasterol	-8.6 ± 0.00	GLU259	PHE264 ILE281 CYS285 ARG288 ALA292 ILE326 MET329 LEU330 LEU333 VAL339 ILE341 MET348		LEU255 ILE262 GLY284 SER289 SER342 MET364		

	11-Pentan-3-ylhenicosane	-6.4 ± 0.15		PHE264 ARG288 LEU330 LEU333 ILE341 LEU469		ILE281 GLY284 CYS285 GLN286 SER289 HIS323 ILE326 TYR327 VAL339 LEU340 SER342 MET348 LEU353 PHE363 MET364 HIS449
MAPK14	Control (MSQ)	-6.7 ± 0.06	THR106 HIS107 MET109	VAL30 VAL38 ALA51 LYS53 LEU75 ILE84 MET109	LYS53	GLY31 GLU71 LEU104 LEU108 ASP168 PHE169
	Clionasterol	-8.1 ± 0.12		VAL30 VAL38 ALA51 LYS53 LEU75 ILE84 LEU104 MET109 LEU167		GLU71 THR106 LEU108 ASP168 PHE169 LEU171
	Caryophyllene oxide	-7.0 ± 0.06	THR106	VAL38 ALA51 LYS53 LEU75 LEU167		THR35 GLY36 GLU71 ILE84 LEU104 ASP168

* The bold residue shows the same residue as the control.