



## ISOLATION AND COMPUTATIONAL STUDIES OF $\gamma$ -SITOSTEROL FROM *ELAEOCARPUS GRANDIFLORUS* AND ITS ANTI-DIABETIC AND ANTI-HYPERLIPIDEMIC ACTIVITIES

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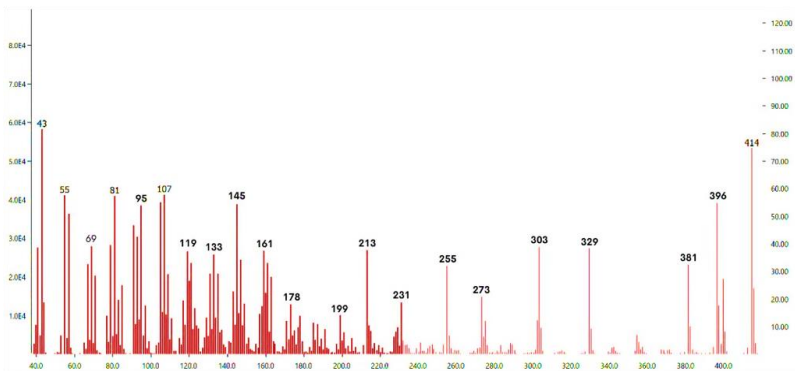
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Anyang-Anyang leaves (*Elaeocarpus grandiflorus*) have been used traditionally by the indigenous people of Indonesia to treat several ailments, including diabetes and hyperlipidemia. The current study aimed to isolate and identify the major constituent of less polar fraction of leaves of *E. grandiflorus* leaves by chromatographic, spectrometric, and spectroscopic protocols. An *in silico* approach was performed to evaluate the isolated compound's potency as an antidiabetic and antihyperlipidemic agent. The phytochemical study successfully



isolated a major compound and was univocally identified as  $\gamma$ -sitosterol based on EI-MS and 1D-NMR spectral data analysis. The computational study revealed that  $\gamma$ -sitosterol **1** possesses strong binding affinity against glucosidase (3A4A) and lipase (1LPA) protein with a binding affinity energy value of  $-9.8$ ,  $-10.4$  kcal/mol, respectively. These affinities were stronger than a standard drug, acarbose ( $-9.1$  kcal/mol against 3A4A protein) and orlistat ( $-7.4$  kcal/mol against 1LPA protein). Overall, these findings support the traditional claims of *E. grandiflorus* as an antidiabetic and antihyperlipidemic agent.

### INTRODUCTION

The Indonesian government has established a National Research Master Plan 2017–2045 with several focuses, including the development of

pharmaceutical industries based on Indonesian natural resources, medicinal plants<sup>1</sup>. The indigenous people of Indonesia inherited medicinal knowledge on the use of plants to treat several ailments through generations.<sup>2</sup>

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Nevertheless, the need to develop Indonesian traditional herbal medicine into scientifically proven dosage forms are necessary to ensure its safety and efficacy met the Government standard then available into formal health services<sup>1</sup>. Initial data curation regarding the uses of herbal medicine by ethics throughout the archipelagic country of Indonesia successfully curated, collected, and collated herbal medicine and their uses by means of ethnopharmacological data under the Ministry of Health project (RISTOJA).<sup>1</sup> One of important Indonesian medicinal plant species is *Elaeocarpus grandiflorus* in which the indigenous people of Indonesia have prepared the leaves to treat diabetes and hyperlipidaemia. *Elaeocarpus grandiflorus* (Anyang-Anyang) is an evergreen tree up to 25 meters tall, in which the tree is geographically distributed in Southeast Asian including Indonesia, Malaysia, Myanmar, Singapore, and the Philippines.<sup>3</sup> The indigenous people of Indonesia have been used decocted leaves and stem bark to cure arrays of infective ailments such as fever, boils and female related diseases. Leaves decoction was also traditionally prepared to lower sugar blood level.<sup>3</sup>

Previous study on the same genus, *Elaeocarpus mastersii* King obtained from Sumatra-Island Indonesia, crude extract of root, stem bark, and leaf constituted phenolic compound with significant antioxidant activities.<sup>4</sup> Furthermore, crude methanol extract of root, stem bark, leaf exhibited inhibitory activity against  $\alpha$ -glucosidase with IC<sub>50</sub> value of 60.57±2.84, 14.56±1.20, 96.36 ± 4.67 µg/mL, respectively (acarbose IC<sub>50</sub> value of 0.13 ± 0.00 µg/mL).<sup>4</sup> An *in vivo* study on Indian originated *Elaeocarpus variabilis* revealed aqueous extract treatment at 600 mg/kg dose to possess anti-hyperlipidaemia activity with total cholesterol (TC), triglyceride (TG), low density lipoprotein-cholesterol (LDL), high density lipoprotein-cholesterol (HDL) values of 155.3±0.94, 132.9 ± 6.5, 84.51±0.37, 41.3±0.56 mg/dl, respectively. These data were comparable to positive control, atorvastatin (10mg/kg dose), with TC, TG, LDL and HDL values of 146.6 ± 5.2, 129.0 ± 7.0, 74.59±0.56, 41.2±0.17 mg/dl, respectively.<sup>5</sup> It has been accepted that plants of the same genus are likely to possess similar biosynthesis pathways, leading to the production of similar secondary metabolite

molecular scaffolds with similar pharmacological activities.

Our previous research on leaves of *E. grandiflorus* revealed that the leaves contained alkaloid, flavonoid, polyphenol, and terpenoid class compounds, in which the methanol crude extract exhibited moderate antibacterial activity against *Escherichia coli* with IC<sub>50</sub> value of 360.97 µg/mL.<sup>6</sup> In the current study, this important anti-diabetic and antihyperlipidemic Indonesian medicinal plant was phytochemically studied using exhaustive chromatographic, spectrometric (GCMS) and spectroscopic (NMR) experiments, and its major constituent was screened for anti-diabetic and antihyperlipidemic activities through an *in silico* experiment involving a docking protocol against human glucosidase and lipase enzymes.

## RESULTS AND DISCUSSION

### Isolation and characterization of $\gamma$ -sitosterol

The current study focused on terpenoid components of leaves of *E. grandifloras*, where the crude terpenoid-containing fraction was generated by back extraction of the methanol crude extract with hexane. Isolation and purification of terpenoids was initiated by loading *n*-hexane extract into column chromatography with a gradient solvent mixture of hexane, dichloromethane, and ethyl acetate. Of the 240 total fractions collected, fractions 131–139 with crystalline indicated a moderately pure based on thin layer chromatography (TLC) monitor (Fig. 2). Preparative TLC successfully produced pure compounds as white needle crystals and was confirmed as a single spot at TLC analysis (Fig. 1).

Gas chromatography tandem with electron impact mass spectrometry (GCMS) was performed on the pure compound (**1**), which depicted a clean mass spectrum (Fig. 2) with a positive ion at 414 *m/z* representing the mother ion of molecular formula C<sub>29</sub>H<sub>50</sub>O. Database comparison against the National Institute of Standards and Technology (NIST) mass spectral library identified the isolate as  $\gamma$ -sitosterol based on similarities in the peaks pattern at 414, 396, 381, 329, 303, 273, 255, 231, 173, 161, 145, 133, 119, 107, 95, 81, 69, 55, and 43 *m/z*.

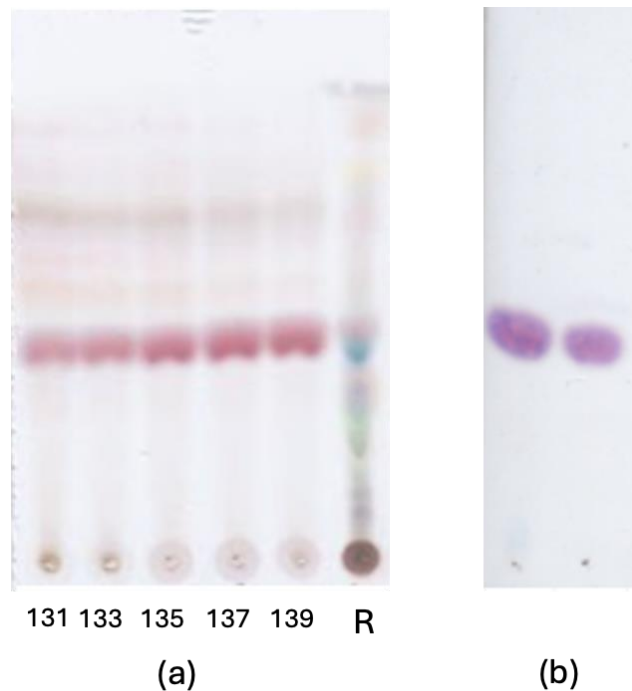


Fig. 1 – Chromatogram of: a) fraction 131–139; b) re-purified fraction in which both TLC were developed using n-hexane: ethyl acetate (3.4:0.6) and stained using vanillin reagent with purple colour indicating terpenoid present. Reference (R) was used as a chemical constituent profile monitor.

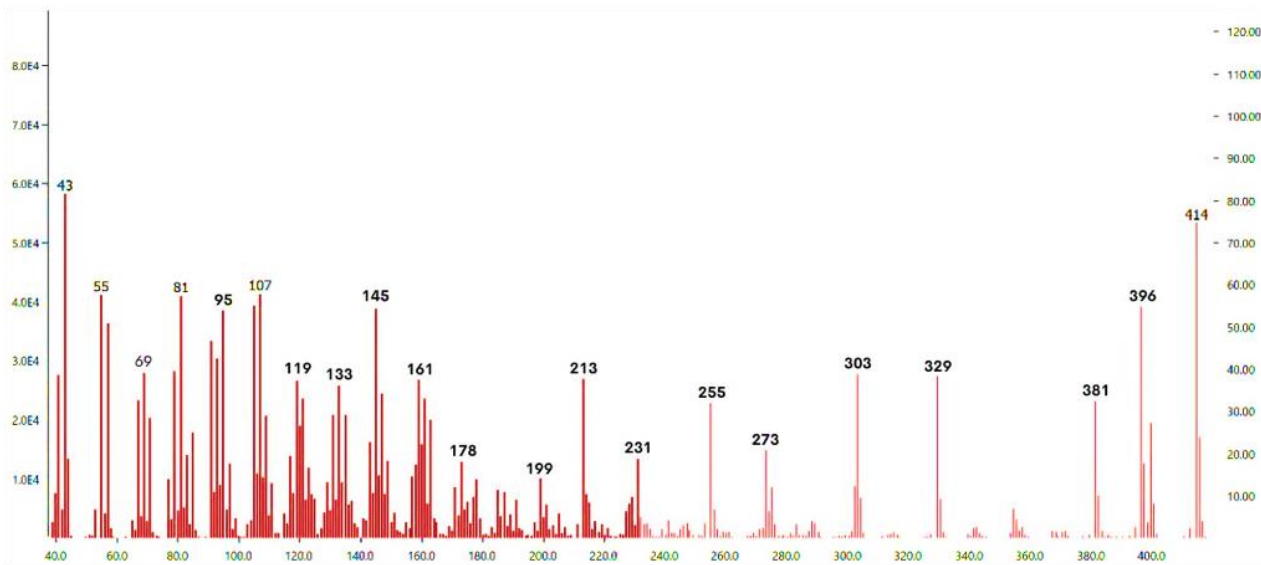


Fig. 2 – Mass spectrum obtained from isolated terpenoid of n-hexane fraction of leaves of *Elaeocarpus grandiliflorus*.

Further spectroscopic analysis using  $^1\text{H-NMR}$  spectral data analysis confirmed the key features of  $\gamma$ -sitosterol, including an alkene signal at  $\delta_{\text{H}}$  5.35 (m) corresponding to H-6 and oxymethine proton signal at  $\delta_{\text{H}}$  3.54 (d) for H-3. In addition, the spectrum showed proton signals at  $\delta_{\text{H}}$  1.01 (s, H-19),  $\delta_{\text{H}}$  0.92 (d, H-21),  $\delta_{\text{H}}$  0.84 (d, H-26),  $\delta_{\text{H}}$  0.83 (s, H-27),  $\delta_{\text{H}}$  0.86 (s, H-29), dan  $\delta_{\text{H}}$  0.68 (s, H-18) for six methyl groups (Fig. 3). In addition,  $^{13}\text{C-NMR}$  spectral analysis showed signals for

29 carbon atoms including for six methyl carbons ( $\delta_{\text{C}}$  19.83,  $\delta_{\text{C}}$  19.41,  $\delta_{\text{C}}$  19.03,  $\delta_{\text{C}}$  18.78,  $\delta_{\text{C}}$  11.99, dan  $\delta_{\text{C}}$  11.86), eleven methylene ( $\delta_{\text{C}}$  42.30, 39.77, 37.25, 33.94, 31.9, 31.67, 28.26, 26.05, 24.31, 23.06 and 21.08), nine methine ( $\delta_{\text{C}}$  121.74, 71.82, 56.76, 56.04, 50.12, 45.83, 36.15, 31.9 and 29.13), and three quaternary carbon atoms ( $\delta_{\text{C}}$  140.76, 42.3 and 36.51) (Fig. 3). Overall, the NMR spectral data were in agreement with the existing literature spectra of typical  $\gamma$ -sitosterol -sitosterol.<sup>7</sup>

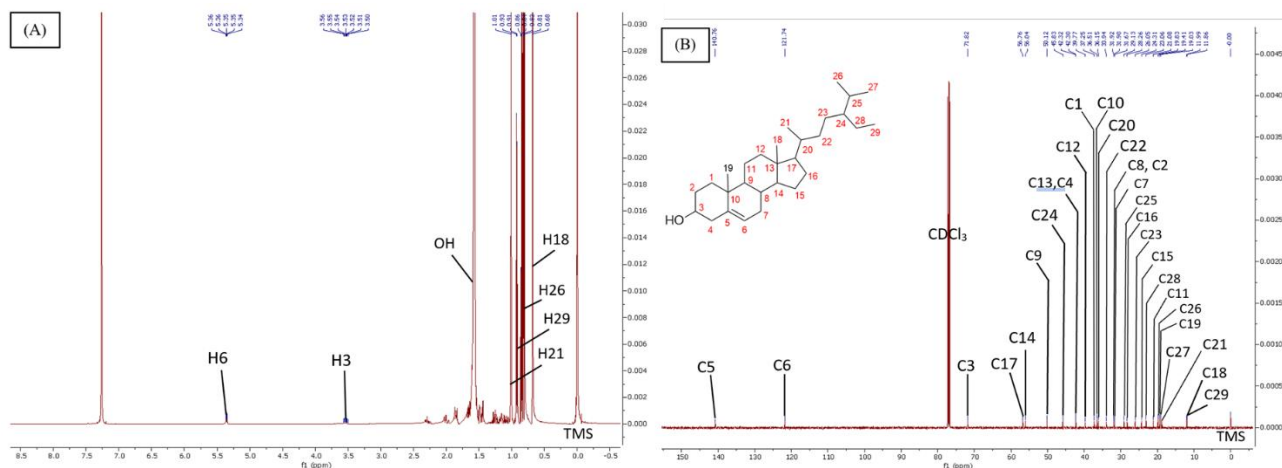


Fig. 3 – a)  $^1\text{H}$ -NMR spectrum of isolate **1** ( $\gamma$ -sitosterol) recorded in  $\text{CDCl}_3$  at 500 MHz; b)  $^{13}\text{C}$ -NMR spectrum of isolate **1** ( $\gamma$ -sitosterol) recorded in  $\text{CDCl}_3$  at 125 MHz.

### Computational Studies

The molecular docking experiment was initiated with method validation by performing re-docking of the natural ligand present in the protein-ligand complex structure. The results are shown as RMSD

values of 2,082 Å (RCSB PDB ID: 1LPA) and 1,911 Å (RCSB PDB ID: 3A4A), indicating that the molecular docking protocol used is valid (Fig. 4). These RMSD values represented no significant discrepancies in the geometrical conformation in the active pocket of the proteins.

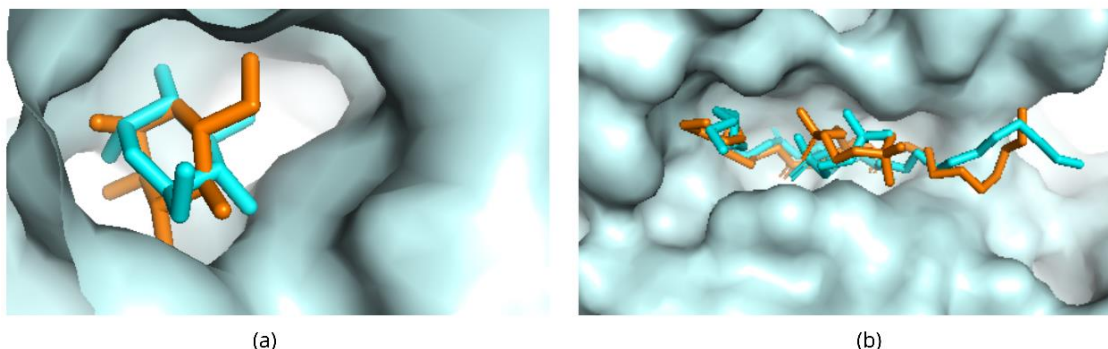


Fig. 4 – 3D visualization of docking validation a) crystallized GLC (alpha-D-glucopyranose) showed with blue color and re-docked GLC (alpha-D-glucopyranose) showed with orange color in the active site of  $\alpha$ -glucosidase protein (RCSB PDB ID: 3A4A) b) crystallized PLC (Diundecyl phosphatidyl choline) showed with blue color and re-docked PLC (Diundecyl phosphatidyl choline) showed with orange color in the active site of human lipase protein (RCSB PDB ID: 1LPA).

Molecular docking predicts the optimal orientation of one ligand in relation to another when bound together to form a complex of which, the orientation provides information about the binding affinity or strength of the association between two molecules (ligand-receptor), typically measured using binding affinity scores expressed in kcal/mol. In this study,  $\gamma$ -sitosterol demonstrated maximum binding energy  $-10.4$  kcal/mol against human lipase protein (RCSB PDB ID: 1LPA) and maximum binding energy  $-9.8$  kcal/mol against  $\alpha$ -glucosidase protein (RCSB PDB ID: 3A4A). In comparison, positive control drug molecules, orlistat and acarbose, exhibited

binding affinities  $-7.4$  and  $-9.1$  kcal/mol, respectively. These results suggest that  $\gamma$ -sitosterol possesses significant binding affinities, which could contribute to their possible efficacy as a potential inhibitory agent against selected receptors and could be further considered as a promising drug candidate.

According to the molecular docking analysis,  $\gamma$ -sitosterol exhibited hydrogen bonding interactions with both  $\alpha$ -glucosidase (RCSB PDB ID: 3A4A) and human lipase (RCSB PDB ID: 1LPA) target proteins. Interestingly, both sterol and positive control were properly allocated into the active site as shown in the Fig. 5.



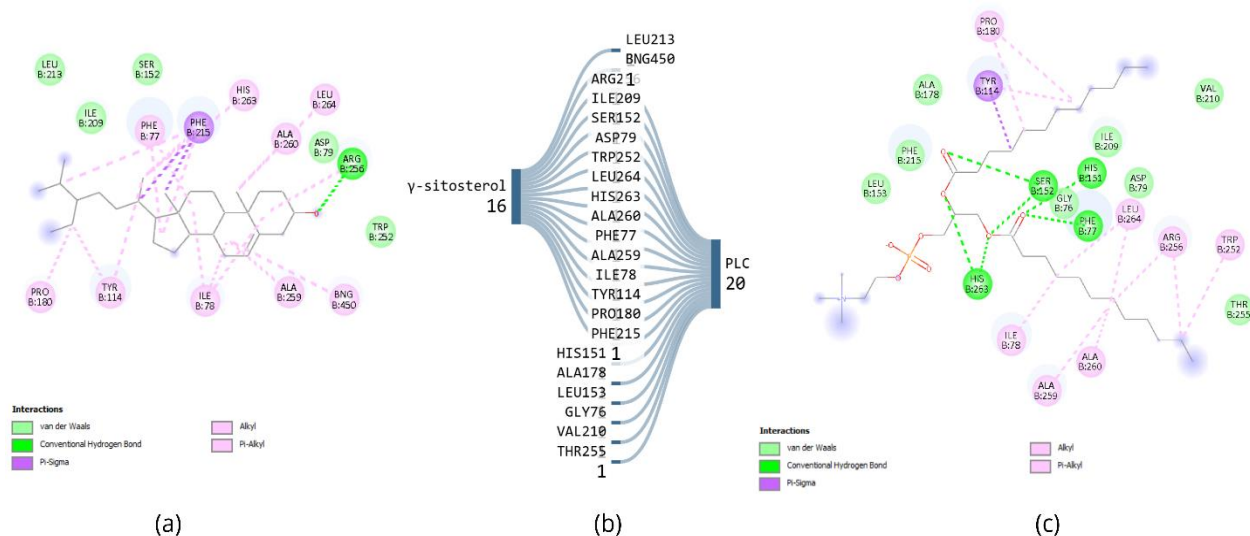


Fig. 7 – 2D visualization of docking results of compounds: (a)  $\gamma$ -sitosterol; b) Sankey diagram of amino acid residues  $\gamma$ -sitosterol and PLC co-crystal ligands against 1LPA; c) PLC co-crystal ligands

Moreover, docking results for the  $\alpha$ -glucosidase protein (RCSB PDB ID: 3A4A) revealed that both  $\gamma$ -sitosterol and a standard drug, acarbose interacted with common amino acid residues, including GLU277, ASP352, GLU411, PHE159, ARG442, PHE178, ARG213, VAL216, GLN279, ASP242, SER157, SER240, LEU313, PHE314, PRO312, ARG315, HIS280, LYS156, PHE303, TYR158 (Figure 8). Likewise, docking analysis on the human lipase protein (RCSB PDB ID: 1LPA) depicted that both  $\gamma$ -sitosterol and a standard drug, orlistat interacted with shared residues such as ARG256, LEU213, ILE209, SER152, ASP79, TRP252, LEU264, HIS263, ALA260, PHE77, BNG450,

ALA259, ILE78, TYR114, PRO180, PHE215 (Fig. 9). These similar interaction patterns indicate that  $\gamma$ -sitosterol may possess a binding profile analogous to positive controls, potentially leading to similar pharmacological activities. These observed molecular interactions covering hydrogen bonds, pi-alkyl, pi-cation, and hydrophobic interactions underscored the specificity and strength of ligand binding to the target enzyme. These interactions contributed significantly to the stability and binding affinity of the complexes, highlighting the potential of  $\gamma$ -sitosterol as a promising  $\alpha$ -glucosidase protein (RCSB PDB ID: 3A4A) and human lipase protein (RCSB PDB ID: 1LPA) inhibitors.

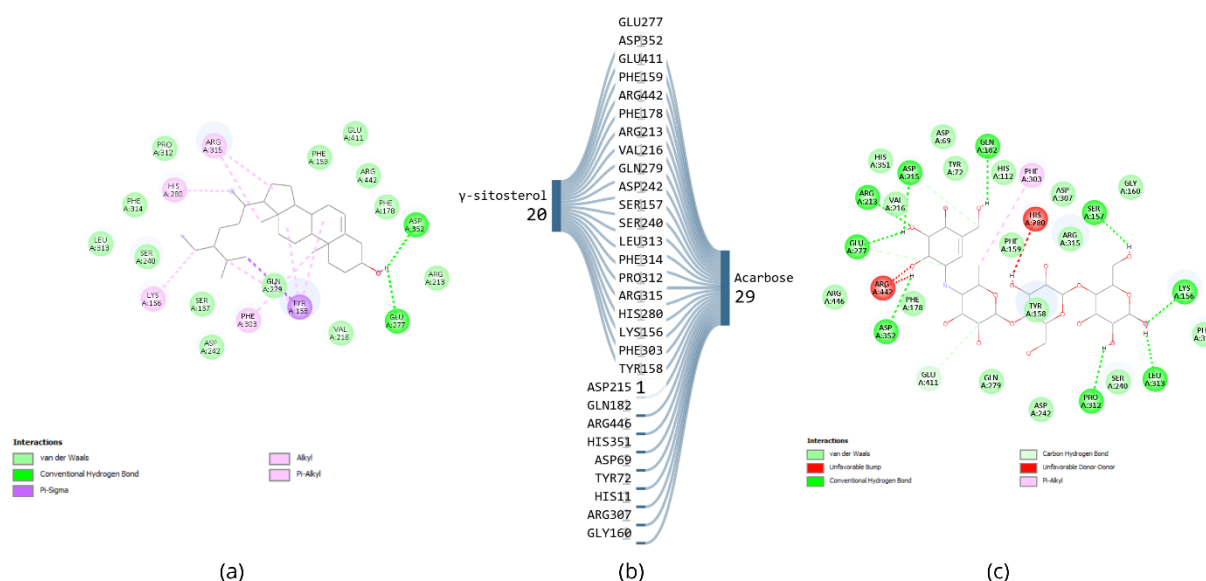


Figure 8. 2D visualization of docking results of compounds: a)  $\gamma$ -sitosterol; b) Sankey diagram of amino acid residues  $\gamma$ -sitosterol and acarbose against 3A4A; c) acarbose.

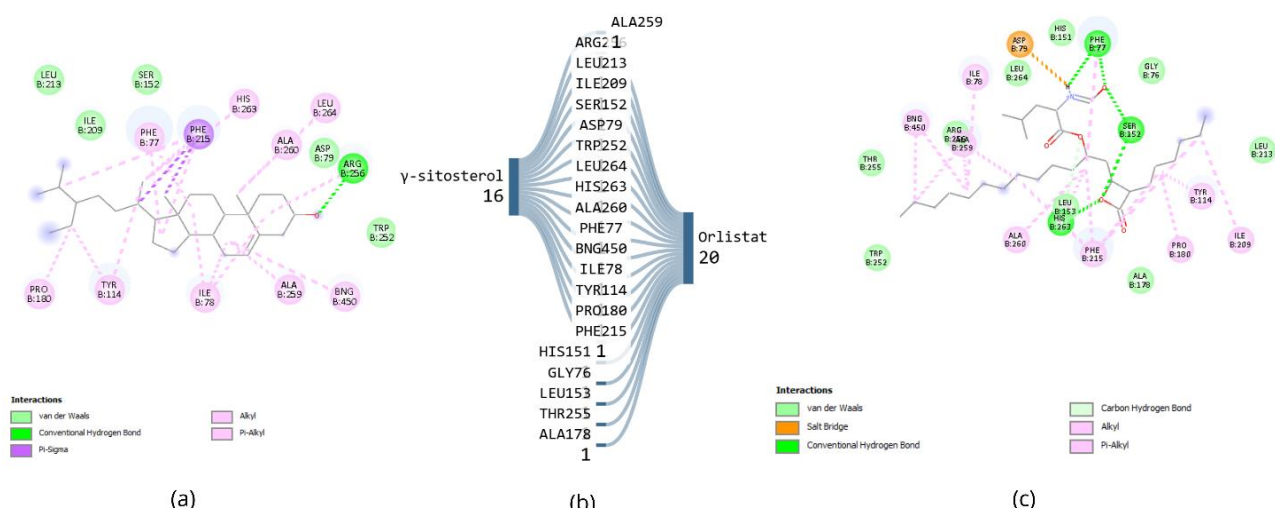


Fig. 9 – 2D visualization of docking results of compounds: a)  $\gamma$ -sitosterol; b) Sankey diagram of amino acid residues  $\gamma$ -sitosterol and orlistat against 1LPA; c) orlistat.

## EXPERIMENTAL

The isolation was performed on column chromatography using silica gel 0.063–0.200 mm (Sigma-Aldrich). The purification of compounds was performed by TLC analysis and preparative TLC using aluminum-precoated TLC silica gel 60 F254 plates (20 × 20 cm, 0.22 mm) purchased from Merck. The molecular elucidation of the pure isolate was conducted based on spectroscopic data analysis ( $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$ ) and spectrometric data analysis (EIMS). NMR data were recorded on JEOL-ECZ 500 R with 500 MHz for  $^1\text{H-NMR}$  and 125 MHz for  $^{13}\text{C-NMR}$  analysis in deuterated chloroform ( $\text{CDCl}_3$ ) with tetramethylsilane (TMS) as reference. 1D-NMR was recorded on a JEOL-ECZ 500 R with a frequency of 500 MHz for  $^1\text{H-NMR}$  and 125 MHz for C-NMR analysis. NMR spectra were processed and interpreted using MestReNova 6.0.2-5475 software. Mass Spectrometry (MS) was measured on a Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent 19091S-433: 93.92873 DB-5MS UI 5% Phenyl Methyl Silox column (30 m x 250  $\mu\text{m}$  x 0.25  $\mu\text{m}$ )) tandem with Electron Impact (EI) (5977A MSD). MS spectral data were analyzed using Openchrom 1.5.0 "McLafferty" software. General solvents, *n*-hexane, dichloromethane, ethyl acetate, and methanol were purchased from Merck.

### Plants Sample

*E. grandiflorus* leaves samples were collected from the Technical Implementation Unit Laboratory

of Herbal Materia Medica Batu, East Java, Indonesia, and were transported to the Drug Utilization and Discovery Research Group (DUDRG), Faculty of Pharmacy, University of Jember-Indonesia in July 2022 under accession code ELG.

### Extraction and Constituent Isolation

The leaf powder (200 g) was soaked in methanol (7x 700 mL) for six hours at a magnetic stirrer. Filtrate was obtained using Buchner funnel in which the solution was then vacuum dried to produce crude leaf extract (73.48 g). To the crude extract, a liquid-liquid fractionation performed using *n*-hexane in order to produce *n*-hexane fractions (2.099 g). A portion of *n*-hexane fraction (1.5 g) was subjected to silica gel column chromatography (4-cm diameter, 30-cm length) and was eluted with *n*-hexane (0.5 L), *n*-hexane:DCM (75:25 0.5 L), *n*-hexane:DCM (50:50 0.5 L), *n*-hexane:DCM (25:75 0.5 L), DCM (0.5 L), DCM:EtOAc (75:25 0.5 L), DCM:EtOAc (50:50 0.5 L), DCM:EtOAc (25:75 0.5 L), and EtOAc (0.5 L) to produce 240 fractions. Fractions with crystalline present (F131–141, 20.321 mg) were pooled and re-purified using preparative thin layer chromatography (Prep-TLC, *n*-hexane: EtOAc (3.4:0.6)) to produce white needle crystal analysis ELG-M2 (4.928 mg), which was identified as  $\gamma$ -sitosterol.

*Stigmast-5-en-3 $\beta$ -ol (24S)- dan 24 $\beta$ -etilkoolest-5-en-3 $\beta$ -ol or  $\gamma$ -sitosterol 1*, was obtained as needle white crystal; 4.928 mg (0.03 mg/g dried sample);

<sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>,  $\delta$ , ppm): 5.35 (m, H6), 3.54 (d, H3), 1.01 (s, H19), 0.92 (d, H21), 0.86 (s, H29), 0.84 (d, H26), 0.83 (s, H27), 0.68 (s, H18). <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>,  $\delta$ , ppm): 140.76 (C5), 121.74 (C6), 71.82 (C3), 56.76 (C17), 56.04 (C14), 50.12 (C9), 45.83 (C24), 42.32 (C13), 42.30 (C4), 39.77 (C12), 37.25 (C1), 36.51 (C10), 36.15 (C20), 33.94 (C22), 31.9 (C2), 31.67 (C7), 29.13 (C25), 28.26 (C16), 26.05 (C23), 24.31 (C15), 23.06 (C28), 21.08 (C11), 19.83 (C26), 19.41 (C19), 19.03 (C27), 18.78 (C21), 11.99 (C18), 11.86 (C29); EIMS: 414 *m/z* (M)<sup>+</sup>, 399 *m/z* (M-15)<sup>+</sup>, 396 *m/z* (M-18)<sup>+</sup>, 381 *m/z* (m-33)<sup>+</sup>, 329 *m/z* (M-85)<sup>+</sup>, 273 *m/z* (M-141)<sup>+</sup>, 255 *m/z* (M-159)<sup>+</sup>, 231 *m/z* (M-183)<sup>+</sup>, 213 *m/z* (M-201)<sup>+</sup>, 163 *m/z* (M-251)<sup>+</sup>, 145 *m/z* (M-269)<sup>+</sup>, 85 *m/z* (M-329)<sup>+</sup>.

### Computational Studies

The investigation was conducted using AutoDock Vina v1.2.3 on  $\alpha$ -glucosidase protein (RCSB PDB ID: 3A4A) with acarbose as positive control and on human lipase protein (RCSB PDB ID: 1LPA) with orlistat as positive control. All crystallographic water and small molecules were removed, and hydrogen atoms and charges were added with MGLTools software 1.5.7. Prior to docking, molecular energy minimization and format conversion to pdbqt were performed using MM2 Chem3DBio and MGLTools software 1.5.7. Docking validation was performed at active site of 3A4A (xyz coordinate 21.5188, -7.7018, 23.5542 with overall gride size 40 Å) and 1LPA (xyz coordinate 19.4449, 21.3561, 39.5246 with overall gride size 40 Å). The 2D and 3D interactions of the best docking conformation were imported into the Discovery studio visualizer in order to identify interactions between the ligands and the receptor protein. The resulting ligand-protein interactions and binding affinities were then observed and compared against the standard ligand.

### CONCLUSION

Major terpenoid compound from *n*-hexane fraction of Anyang-Anyang leaves (*E. grandiflorus*) was successfully isolated and identified as  $\gamma$ -sitosterol 1 based EIMS, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectral data analysis. Computational work suggested that  $\gamma$ -sitosterol possesses significant binding affinity against human glucosidase and lipase proteins, whose activities were comparable to a standard drug, acarbose and orlistat. These findings provided scientific background for the use of the leaves of *E. grandiflorus* as antidiabetic and antihyperlipidemic agents. Nevertheless, further bioassays, such as *in vitro* and *in vivo* model, are required to confirm the claims.

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