Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Plectranthus amboinicus (Spreng.) Semi-purified Fractions with Selective β-Glucuronidase Inhibition Elucidated with gas chromatography-mass spectrometry and *in silico* docking

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Submitted: 05-Apr-2021 Revised: 26-May-2021 Accepted: 26-Jul-2021 Published: 15-Sep-2021

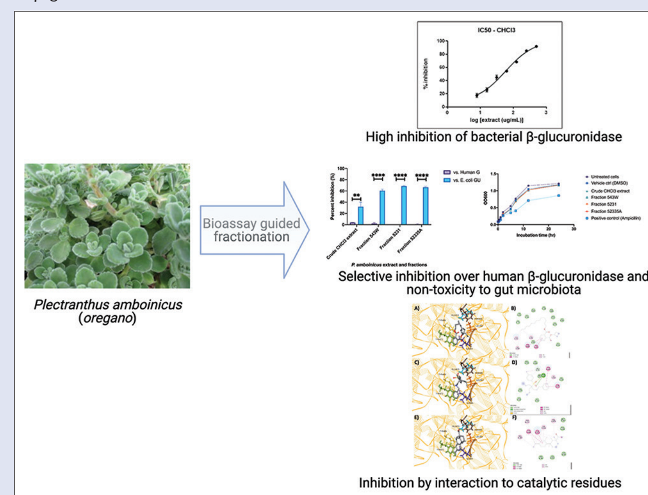
ABSTRACT

Background: *Plectranthus amboinicus* (Spreng.) is an herb commonly used in folk medicine and food by several Asian countries. The bioactivities of this medicinal plant have so far not been linked to a specific enzyme target. Bacterial beta-glucuronidases (β-GUS) expressed by human gut microbiota affect xenobiotic processing by reactivating toxic substances (e.g., anticancer drugs, nonsteroidal anti-inflammatory drugs, and food carcinogens) in the gut lumen. **Objectives:** An approach to alleviating the toxic effects of these compounds is by inhibiting bacterial β-GUS. **Materials and Methods:** We determined the *Escherichia coli* β-GUS inhibitory activity of *P. amboinicus* leaves using a bioassay-guided purification approach. The *P. amboinicus* chloroform extract was purified using normal-phase column chromatography to produce several fractions. The fractions were screened for *E. coli* β-GUS inhibitory activity using the 4-Methylumbelliferyl glucuronide (4-MUG) assay. Fractions with high activity were assayed further to determine toxicity against *E. coli* and selectivity compared to human β-GUS. Highly-active and highly-selective fractions were further characterized using gas chromatography-mass spectrometry (GC-MS) and *in silico* docking to identify specific compounds. **Results:** Assay-guided purification of the crude chloroform extract with β-GUS inhibitory activity (IC_{50} = 57.8 μg/mL) yielded four fractions with high activity: Fraction-543W (IC_{50} = 16.24 μg/mL), Fraction-5231 (IC_{50} = 3.087 μg/mL), and Fraction-52335A (IC_{50} = 12.93 μg/mL). The crude extract and fractions exhibited high selectivity for *E. coli* β-GUS against human β-GUS (P < 0.0001, α = 0.05). The antimicrobial assay of fractions showed no toxic effects on *E. coli*. GC-MS profiling of the active fractions identified the compounds present to be similar to essential oil extracts of *P. amboinicus* reported previously. Ranking of these compounds by *in silico* identified the compounds with high binding affinity: Phthalic acid, cyclobutyl tridecyl ester (-7.5 kcal/mol) from Fraction-543W, N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine (-8.0 kcal/mol) from Fraction-5231, and Dehydroabietic Acid (-7.9 kcal/mol) from Fraction-52335A. By comparing to binding modes of reported inhibitors, we show that these compounds also interact with active site residues Tyr469 and Tyr472, and with several residues in the β-GUS bacterial loop. **Conclusion:** Herein, we identified highly-active and highly-selective *E. coli* β-GUS inhibitors from *P. amboinicus* leaf chloroform extracts, utilizing a bioassay-guided purification coupled by metabolomics and *in silico* docking approach. This is the first report on the potential of *P. amboinicus* as selective inhibitor of *E. coli* β-GUSs.

Key words: *Escherichia coli*, gas chromatography-mass spectrometry, *in silico* docking, *Plectranthus amboinicus*, β-glucuronidase

SUMMARY

- *Plectranthus amboinicus*, locally known in the Philippines as oregano, is commonly used in folk medicine and food ingredient by several Asian countries. Although used in several ethnopharmacological applications as treatment to burns, bruises, headaches, and stomachaches, there has so far been no identified functional protein target of the phytochemicals contained in its extract. In this study, we aim to link its ethnopharmacological properties to its inhibition of bacterial beta-glucuronidases (β-GUS), specifically that of *Escherichia coli*. β-GUS are expressed by gut microbiota that affect xenobiotic processing of certain toxic substances. By the action of these enzymes, glucuronide-conjugated toxic substances that leave the gut lumen are reactivated by release of the glucuronide moiety. As such, through bioassay-guided purification coupled by gas chromatography-mass spectrometry characterization and *in silico* docking, we identified several compounds that could potentially inhibit bacterial β-GUS. Specifically, these are the compounds Phthalic acid, cyclobutyl tridecyl, N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine, and Dehydroabietic Acid. This is the first report on the potential of *P. amboinicus* as selective inhibitor of *E. coli* β-glucuronidase.



Abbreviations used: 4-MUG assay: 4-methylumbelliferyl assay; ANOVA: Analysis of variance; β-GUS: Beta-glucuronidase; $CHCl_3$ extract: Chloroform extract; CPT-11: Camptothecin-11; DMSO: Dimethyl sulfoxide;

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Cite this article as: Alvarez MR, Billones J, Lin CH, Heralde FIII. *Plectranthus amboinicus* (Spreng.) semi-purified fractions with selective β-glucuronidase inhibition elucidated with gas chromatography-mass spectrometry and *in silico* docking. Phcog Mag 2021;17:S268-77.

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E. coli: Escherichia coli; GC-MS: Gas chromatography-mass spectrometry; GPS: Global positioning system; IC_{50}: 50% Inhibitory concentration; LB Broth: Luria-Bertani broth; MPLC: Medium performance liquid chromatography; NaOH: Sodium hydroxide; NSAIDs: Nonsteroidal anti-inflammatory drugs; OD600: Optical density at 600 nm; *P. amboinicus: Plectranthus amboinicus*; PDB: Protein Data Bank; RMSD: Root mean square deviation; SN-38G: 7-Ethyl-10-hydroxycamptothecin glucuronide; UDP-glucuronyltransferase: Uridine diphosphate-glucuronyltransferase.

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DOI: 10.4103/pm.pm_149_21

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INTRODUCTION

Plectranthus amboinicus (Spreng), also called *Coleus aromaticus* (Benth.), *Coleus suganda* (Blanco), or *Plectranthus aromaticus* (Roxb.), is an aromatic succulent herb commonly used in folk medicine and food by several countries, including the Philippines (*Oregano*, *latai*, *suganda*), China (*da shou xiang*), India (*Pashan bhedi, Karpooravalli, Patharchur*), and Malaysia (*Daun bangun-bangun, Pokkok bangun-bangun*), among others.[1] In the Philippines, macerated fresh leaves are applied to burns, bruises, and insect bites. The leaves are also applied to the temples and forehead for headaches. An infusion or syrup from the leaves are used for dyspepsia or asthma. The Chinese also use the juice of the leaves with sugar for coughs. In Indo-China, it is employed as an infusion for asthma, chronic coughs, and epilepsy. Malays use the juice of the plant for stomach aches[2] Previously reported bioactivities of this plant species include antibacterial,[3-8] antifungal,[9] antiviral,[10,11] antitumorigenic,[12] anti-inflammatory,[12-14] antioxidant[7,15] activities and activity against digestive diseases such as diarrhea.[16-18]

Escherichia coli beta-glucuronidases (*E. coli* β-GUS) is a member of the Family 2 glycosyl hydrolases, which also includes human (human β-GUS), mice and rat β-GUSs, as well as *Clostridium acetobutylicum*, *Klebsiella pneumoniae* and *Lactobacillus bulgaricus* β-GUS.[19] These enzymes catalyze the hydrolysis of β-D-glucuronic acid residues from the non-reducing end of carbohydrates.[20] The *E. coli* β-GUS occurs as a tetramer composed of two asymmetric units, which in turn is composed of two monomers of 597 ordered residues. It contains several domains: The N-terminal domain (residues 1–180) is the sugar binding while the C-terminal domain (residues 274–603) forms an αβ barrel containing the active-site residues Glu413 and Glu504, and the region between the 2 domains show an immunoglobulin-like β-sandwich domain found in other Family 2 glycosyl hydrolases. In addition, it contains a 17-residue "bacterial loop" that is not found in the human form of the enzyme.[21] Bacterial β-GUSs expressed by gut microbiota affect xenobiotic processing in humans, by removing the glucuronic acid that are conjugated to xenobiotics (in a process called glucuronidation) in the liver by UDP-glucuronyltransferases.[22] This process essentially releases the original xenobiotic into the gut lumen, thus affecting the toxic properties of anticancer compounds such as Camptothecin-11 (CPT-11),[23-25] nonsteroidal anti-inflammatory drugs,[26,27] and food carcinogens.[28,29] Specifically, the release of these xenobiotics into the gut results in their toxic properties (*e.g., diarrhea, epithelial injury*). Thus, targeted inhibition of this bacterial β-GUS could ameliorate these toxic effects. Indeed, several studies have shown that inhibition of bacterial β-GUS alleviated nonsteroidal anti-inflammatory drugs-induced enteropathy in rats[27] and mice,[26] enhanced CPT-11 anticancer activity[30] and modulated CPT-11 toxic side effects.[21,23,31-33]

Herein, we present for the first time, data on the potential of *P. amboinicus* (Spreng.) crude extract and fractions as selective inhibitor of *E. coli* β-GUS. We also show that the chloroform extract and fractions obtained do not affect *E. coli* viability, which is important in preserving the integrity of gut microbiota. Finally, characterization of the extract was undertaken using gas chromatography-mass spectrometry (GC-MS) and the binding modes of the detected putative compounds were determined through *in silico* modeling.

MATERIALS AND METHODS

Materials and reagents

All reagents used were analytical grade. The solvents used for the extraction, gravity column, and medium-pressure liquid chromatography (MPLC) were from Duksan Pure Chemicals (Taipei). Dimethyl sulfoxide used for sample preparation in the assay was analytical grade and obtained from Fisher Chemical (Taipei). 4-Methylumbelliferyl glucuronide (4-MUG) substrate used in the *E. coli* β-GUS inhibition assay was from Sigma-Aldrich. *E. coli* cells for the antibacterial assay, purified *E. coli* and human β-GUS were kindly provided by Dr. Chun-Hung Lin (Academia Sinica, Taiwan).

Sample collection and extraction

P. amboinicus (Spreng.) leaves were obtained from Echague, Isabela, Philippines (GPS: 16.706292, 121.676219). *P. amboinicus* was authenticated by Michelle Alejado-San Pascual from Botany Division, Museum of Natural History, Laguna, Philippines. Voucher specimens (Accession number: #073652) were kept at the Herbarium of the University of the Philippines Los Baños. Gathered leaves of *P. amboinicus* were air-dried in the Biochemistry Laboratory, College of Medicine, and University of the Philippines Manila, Philippines, at room temperature. The dried leaves were ground using a mechanical blender to a coarse powder. For preliminary screening, 10 g of the powdered leaves were extracted in each of the following solvents at a ratio of 1 g sample: 10 mL solvent: Hexane, ethyl acetate, dichloromethane, chloroform, ethanol, methanol, and distilled water. After 12 h of soaking with occasional shaking, the resulting mixture was filtered. The extracts were concentrated using a rotary evaporator at 40°C and stored in aluminum foil covered preweighed glass vials and the solvents removed completely using a high-vacuum pump. Dried extracts were stored at 4°C. Upon determining the most active extract was the chloroform extract using the *E. coli* β-GUS inhibition assay screening, all 200 g of the plant material was extracted using the same procedure.

Escherichia coli beta-glucuronidases inhibition assay

E. coli β-GUS inhibition assay was performed after each round of purification of the extract, to constitute bioassay-guided fractionation. The assay was modified from the 4-MUG assay.[34] For the enzyme reaction, 5 μL of the extract (prepared in DMSO) or DMSO (negative

control) was added to 75 μL HEPES buffer and 10 μL enzyme (50 ng/mL). These were incubated at 37°C for 20 min. Then, 10 μL of 4-MUG substrate (1.5 mM) were added and incubated for another 15 min in 37°C. Afterward, 100 μL stop buffer (glycerin, 1.0 M NaOH) was added to terminate the reaction. The percent inhibition was estimated using fluorescence measurements (Ex: 365 nm, Em: 455 nm) and calculated using the following equation:

$$percent\ inhibition = \frac{{}^{F}neg\ ctrl - {}^{F}extract}{{}^{F}neg\ ctrl} \times 100\%$$

Equation 1: Equation for calculating average percent inhibition.

The dose-response assay was performed based on this protocol, after optimizing the concentration ranges (seven 2-fold dilutions) for each crude extract and fraction. IC_{50} was determined using GraphPad PRISM 7 software.

Purification of fraction 5231

The crude chloroform extract was loaded into a glass column (27.6 cm × 4.8 cm) packed with silica then eluted using the following gradient: 100% hexane, 1:1 hexane-chloroform, 100% chloroform, 1:49 methanol-chloroform, 1:39 methanol-chloroform, 1:29 methanol-chloroform, 1:19 methanol-chloroform, 1:14 methanol-chloroform, 1:9 methanol-chloroform, 1:4 methanol-chloroform, 1:2 methanol-chloroform, 1:1 methanol-chloroform, 100% methanol. The eluents were collected every 15 mL and pooled using 1:19 methanol-chloroform and 1:10 methanol-chloroform. From this, fraction 5 was collected and then purified further using another glass column (27.6 cm × 4.8 cm) packed with silica, which was eluted using 100% hexane, 1:1 hexane-chloroform, 100% chloroform, 1:49 methanol-chloroform, 1:44 methanol-chloroform, 1:39 methanol-chloroform, 1:34 methanol-chloroform, 1:29 methanol-chloroform, 1:24 methanol-chloroform, 1:19 methanol-chloroform, 1:14 methanol-chloroform, 1:9 methanol-chloroform, 1:4 methanol-chloroform, 1:1 methanol-chloroform, 100% methanol. The eluents were collected every 15 mL and pooled using 1:19 methanol-chloroform and 1:10 methanol-chloroform. This time, fraction 2 was collected for final purification using MPLC (CombiFlash Rf 200® [Teledyne, ISCO]). Here, a 4 g column and 18 mL/min flow rate used. The solvent gradient was: 0% ethyl acetate (5 min), 0% ethyl acetate-30% ethyl acetate (10 min), 30% ethyl acetate (5 min), 30% ethyl acetate-50% ethyl acetate (5 min), 50% ethyl acetate (5 min), 50%–75% ethyl acetate (10 min), 75% ethyl acetate (5 min), 75%–100% ethyl acetate (10 min), change solvent ethyl acetate to chloroform (5 min), 100% chloroform (5 min), 100%–70% chloroform-methanol (3 min), 70% chloroform-methanol (5 min). From here, fraction 1 was collected (yield = 0.5 mg) and labeled Fraction 5231.

Purification of fraction 52335A

For the purification of Fraction 52335A, the same protocol was followed for the purification of Fraction 5231, until the MPLC step. Here, after the purification run, fraction 3 was collected and then purified further using gravity column using the following solvent gradient: 100% hexane, 5% ethyl acetate, 7.5% ethyl acetate, 10% ethyl acetate, 12.5% ethyl acetate, 15% ethyl acetate, 17.5% ethyl acetate, 20% ethyl acetate, 100% ethyl acetate, 10% methanol, 30% methanol, 100% methanol. Eluates were collected every 5 mL and then pooled using 1:19 $MeOH-CHCl_3$ solvent system. From here, fraction 5 was collected. Solvent-solvent extraction using equal parts hexane and acetonitrile was used to obtain Fraction 52335A (yield = 0.5 mg).

Purification of fraction 543W

For the purification of Fraction 543W, the fourth fraction of the second purification step in Fraction 5231 was collected instead. Then, purification by medium-phase column chromatography: 0% ethyl acetate (5 min), 0% ethyl acetate-25% ethyl acetate (5 min), 25% ethyl acetate (5 min), 25%–50% ethyl acetate (5 min), 50% ethyl acetate (5 min), 50%–75% ethyl acetate (5 min), 75% ethyl acetate (5 min), 75%–100% ethyl acetate (5 min), change solvent ethyl acetate-chloroform (5 min), 100% chloroform (5 min), 100%–85% chloroform-methanol (5 min), 85%–70% chloroform-methanol (2 min), 70% chloroform-methanol (2 min), 70%–60% chloroform-methanol (2 min), 60% chloroform-methanol (2 min), 60%–50% chloroform-methanol (5 min), 50% chloroform-methanol (5 min), 50%–0% chloroform-methanol (3 min), 100% methanol (10 min); pooled using 1:19 $MeOH-CHCl_3$. From this, the third fraction was collected and labeled Fraction 543W (yield = 37.9 mg).

Selectivity assay compared to human beta-glucuronidases

The selectivity assay was based on the *E. coli* β-GUS inhibitory activity screening assay, but with modifications. Five microliters of the active fractions and crude extract (prepared at the highest possible concentration in DMSO) and negative control (DMSO) were added to 75 μL HEPES buffer and 10 μL enzyme (10 μg/mL). These were incubated for 15 min; after which, 10 μL 4-MUG (10 mM) were added. These were further incubated for 20 min, and then 100 μL stop buffer was added. The fluorescence intensity was measured using Excitation: 365 nm and Emission: 445 nm. Average percent inhibition of the fraction against the human GUS was calculated using Equation 1. In a parallel experiment, the same fractions were tested against the *E. coli* B-GUS. The average percent inhibitions against either β-GUS were compared and analyzed using GraphPad PRISM 7 (Student's *t*-test, α =0.05).

Antibacterial assay against *Escherichia coli*

Antimicrobial activity screening was done using 96-well plate time-kill assay. *E. coli* cells were first inoculated into LB broth overnight (37°C, 150 RPM). Afterward, the cells were diluted until it made OD_{600} = 0.5 and total volume of 200 mL (LB broth). This was then plated in 96 well-plates (190 μL/well). Active fractions were added into the wells at 10 μL each (four replicates, 0.5 mg/mL) to make a total concentration of 25 μg/mL, in addition to the broth only (background), untreated cells, vehicle control (DMSO), and positive control ampicillin (0.1 mg/mL or 5 μg/mL total concentration). The plates were first read for OD600 at time 0 for a baseline measurement. Then, it was incubated at 37°C (150 RPM) and taken out to be read after 10, 30, 60, 120, 300, 420, 600, 720, and 1440 min. A growth curve was plotted for each treatment and then compared against the vehicle control using One-way analysis of variance and Dunnett's *post hoc* test.

Gas chromatography-mass spectrometry identification of compounds in fractions 5231, 52335A, and 543W

Fractions collected were analyzed at the Metabolomics Core Facility of the Agricultural Biotechnology Research Center, Academia Sinica. To the dried sample, 20 μL of 20 mg/mL methoxyamine was added and then incubated at 30°C for 90 min. Afterward, 100 μL of BSTFA was added and then incubated again at 70°C for 120 min. These derivatized samples (1 μL) were injected in triplicates to an Rtx-5MS (30 m × 0.25 mm × 0.25 μm) column at 1 mL/min flow rate in helium and injector temperature of 250°C. The temperature program was set

as follows: 40°C for 1 min, 10°C/min to 310°C, and finally 310°C for 8 min. The ion source temperature was 200°C and detector voltage used was 1800 V. Data were acquired at 10 spectra/s, targeting the mass range of 50–600 *m/z*. Data processing and acquisition was performed using LECO Chroma TOF software (4.43.3.0). Compounds were identified by mass spectra matching against the NIST, LECO/Fiehn, and Wiley Registry 9th Edition MS libraries.

In silico docking analysis of compounds in fractions 5231, 52335A, and 543W in *Escherichia coli* beta-glucuronidases

From the Protein Databank,[35] the three-dimensional (3D) structure of *E. coli* β-GUS bound with a novel potent inhibitor (1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl)methyl)-1-(2-hydroxyethyl)-3-(4 hydroxyphenyl) thiourea) (PDB ID: 5czk; resolution = 2.39 Å)[33] was obtained. Using Chimera,[36] the solvents and other ligands were removed, and the inhibitor saved as a separate pdb file. To the protein structure, it was prepared for docking using the "Dock Prep" function of Chimera. Here, the modified residues were corrected (specifically, selenomethionine to methionine), hydrogens and charges were added, protonation states were corrected and the whole structure minimized. After preparing for docking, the *E. coli* β-GUS protein structure was imported as a receptor for docking into the PyRx platform.[37] Subsequently, the inhibitor (1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl) methyl)-1-(2-hydroxyethyl)-3-(4-hydroxyphenyl)thiourea) was also imported as a ligand for docking protocol validation with AutoDock Vina.[38] The binding region (*i.e.,* gridbox) was selected to cover the region wherein the inhibitor was also bound. After optimizing the gridbox's center (−11.9250, −32.2327, 58.2878) and dimensions (8.5115 Å, 9.8500 Å, 11.9868 Å), the RMSD of the docked ligand (comparison to the experimental pdb file) was calculated and found to be satisfactory (RMSD = 0.444, 28–28 atoms). The analysis of the two-dimensional (2D)-interaction diagram of the redocked ligand shows similar amino acid interactions compared to the experimental pdb file. To analyze the binding affinities of the compounds identified by GC-MS, the compounds were first drawn, then 3D conformations were predicted using PyRx software, which were subsequently imported to be used as AutoDock ligands. The gridbox optimized in the docking validation step was used for the *in silico* docking analyses. After docking, the binding affinities of each compound (reported as-kcal/mol) as well as docking conformations were exported. 2D- and 3D-interaction diagrams between the top inhibitors and *E. coli* β-GUS were subsequently generated using Discovery Studio Visualizer.[39]

RESULTS AND DISCUSSION

Plectranthus amboinicus chloroform extract and fractions inhibit *Escherichia coli* beta-glucuronidases

Prior to fractionation, preliminary experiments were performed wherein different extracts of *P. amboinicus* – hexane, ethyl acetate, dichloromethane, chloroform, ethanol, and water – were prepared and the IC_{50} of each compared (data not shown). From these preliminary assays, we found that the chloroform extract showed the highest inhibitory activity with an IC_{50} of 57.8 μg/mL (43.05–77.84 μg/mL). The chloroform extract was subjected to further bioassay-guided fractionation to yield three fractions with significant increase in bioactivity relative to the crude extract [Table 1].

Natural product compounds as a source of β-GUS inhibitors are unexplored, with only few reported inhibitors. Silymarin components were found to inhibit *E. coli* β-GUS with the component silybin having IC_{50} value of 120 μg/mL.[40] Four fractions from *Chondria crassicualis* crude methanol extract were found to inhibit *E. coli* β-GUS with IC_{50} values of 7.26–18.9 μg/mL.[41] Twelve plant species screened by Molan and Saleh Mahdy[42] showed *Mentha piperita* to maximally inhibit *E. coli* β-GUS with an IC_{50} of 140 μg/mL. Clearly, the *P. amboinicus* fractions isolated in this study showed higher activity than those natural products previously reported.

Table 1: 50% inhibitory concentration values of *Plectranthus amboinicus* crude extract and fractions against *Escherichia coli* β-glucuronidases

Extract and fraction	IC_{50} (95% CI)
Crude $CHCl_3$ extract (μg/mL)	57.8 (43.05-77.84)
Fraction 543W	16.24 (10.24-25.75)
Fraction 5231	3.087 (2.273-4.193)
Fraction 52335A	12.93 (7.236-23.11)

CI: Confidence interval; IC_{50}: 50% inhibitory concentration

Plectranthus amboinicus chloroform extract and fractions selectively inhibit *Escherichia coli* beta-glucuronidases over human beta-glucuronidases

The crude extract and fractions also had their β-GUS inhibitory activity compared against the human, in order to test their selectivity against the human enzyme [Figure 1]. A specific bacterial β-GUS inhibitor should have high activity against the bacterial enzyme without affecting human β-GUS activity.[43] In humans, the role of β-GUS is in degrading glucuronate-containing glycosaminoglycans.[44] Previous reports show increased human β-GUS expression in the tumor microenvironment.[45,46] Furthermore, cancer cells with increased human β-GUS expression was shown to be more sensitive to SN-38G (CPT-11 therapy).[47,48] This suggests that nonselective inhibition of human β-GUS by a bacterial β-GUS inhibitor could have detrimental effect on anticancer therapy.

All of the purified fractions had significantly higher activity against the *E. coli* enzyme compared to the human β-GUS, with Fraction 5231 having the highest difference in percent inhibition. This result demonstrates the selectivity of the *P. amboinicus* extract and fractions for *E. coli* β-GUS, compared to the human β-GUS. The high selectivity of the bacterial β-GUS inhibitor could be due to the structural differences between the *E. coli* and human β-GUS, wherein a unique bacterial loop present in the bacterial β-GUS (and absent in the human β-GUS) could provide additional interactions with the inhibitor, resulting in tighter binding and inhibition.[49,50]

Plectranthus amboinicus chloroform extract and fractions are not toxic against gut microbiota (*Escherichia coli*)

In addition to being active against the *E. coli* β-GUS and relatively inactive against the human β-GUS, an appropriate bacterial β-GUS inhibitor must also be non-toxic to *E. coli* cells, *i.e.,* it must have low bactericidal effect. The reason being that bacterial β-GUS inhibitors with bactericidal activities also eliminate gut microbiota, which are essential in the metabolism and processing of carbohydrates, vitamins, bile acids, sterols and xenobiotics.[51,52] Furthermore, unnecessary intake of bactericidal compounds could do more harm, by increasing risk of infections (by suppression of indigenous microflora) or contributing to antibiotic resistance accumulation.[53,54]

To demonstrate non-toxicity of the *P. amboinicus* compounds against *E. coli*, we did a time-kill assay of the crude extract and fractions,

compared against a positive (0.5 µg/mL ampicillin), negative (DMSO) and untreated control [Figure 2]. As expected, the DMSO solvent exhibited a small bactericidal effect, with lower OD_{600} absorbance values compared to the untreated cells. Thus, the effects of the fractions were compared instead against the vehicle control. At all the time points, the OD_{600} absorbance values of the positive control was lower than that of the vehicle control ($P < 0.05$), while none of the *P. amboinicus* extract nor fractions had any significant bactericidal effect. This suggests that at the concentration of 25 µg/mL, which is at least 2-fold higher than the IC_{50} values did not exhibit significant bactericidal activity. Thus, the active fractions and isolates purified in the study satisfy the criteria of selectivity (compared to the human β-GUS) and non-toxicity (to *E. coli* cells).

Gas chromatography-mass spectrometry identified the compounds present in each *Plectranthus amboinicus* fraction and *in silico* docking predicted binding affinity to *Escherichia coli* β-GUS

GC coupled with MS was performed to determine the compounds present in each *P. amboinicus* fraction [Tables 2-4]. Fraction 543W contained at least 29 compounds, with 3 compounds exhibiting high abundance (i.e., Phthalic acid, 2-ethylhexyl isohexyl ester, Dodecanoic acid, methyl ester, 1,2-Benzenedicarboxylic acid, dihexyl ester). Fraction 5231 contained at least 35 compounds, with 2 compounds exhibiting high abundance (i.e., 1,2-Benzenedicarboxylic acid, diisooctyl ester and 2-Undecyltetrahydrofuran). Fraction 52335A contained at least 36 compounds, with 4 compounds exhibiting high abundance (i.e., 2-Methyl-4-(2-thienyl) quinoline, 1,2-Benzenedicarboxylic acid, diisooctyl ester, hexadecanoic acid, and Decanedioic acid, bis (2-ethylhexyl) ester). GC-MS analysis showed high relative abundances of volatile components in each *P. amboinicus* fraction, which were similar to the compounds present in leaf essential oil extracts.[55-58]

In silico docking using the AutoDock Vina algorithm through the PyRx platform, was able to rank the compounds identified by GC-MS in the *P. amboinicus* fractions according to their predicted binding affinities against *E. coli* β-GUS [Tables 2-4]. For the docking simulations, the crystal structure by Wallace *et al.*[33] (PDB ID: 5czk) was used, particularly due to the presence of an inhibitor. Using the inhibitor as a template, the region surrounding, it was selected as the binding grid for the docking studies. Based on the analyses, the compounds with the highest binding affinities from each fraction were: Phthalic acid, cyclobutyl tridecyl ester (-7.5 kcal/mol) from Fraction 543W, N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine (-8.0 kcal/mol) from Fraction 5231, and Dehydroabietic Acid (-7.9 kcal/mol) from Fraction 52335A. The compounds with high abundance are not the same compounds with the highest predicted binding affinities against *E. coli* β-GUS although some ranked second third or fourth in the list [Tables 2-4].

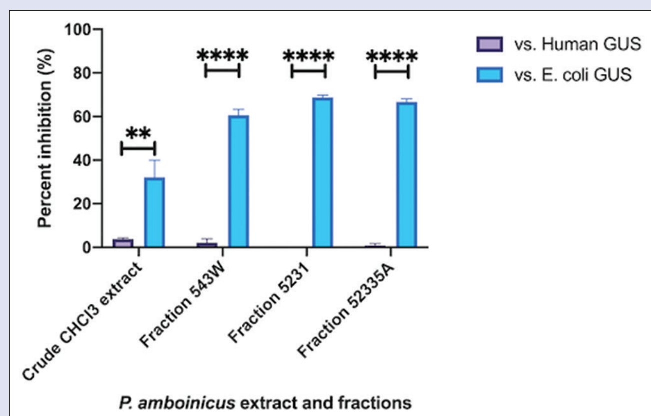

Figure 1: Significant differences between the inhibitory activities of *Plectranthus amboinicus* extract and fractions against *Escherichia coli* and human beta-glucuronidases

In silico analysis shows possible binding modes of predicted *Escherichia coli* beta-glucuronidases inhibitors from *Plectranthus amboinicus*

From the top-binding compounds in each fraction-Phthalic acid, cyclobutyl tridecyl ester from Fraction 543W, N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine from Fraction 5231, and Dehydroabietic Acid from Fraction 52335A–the 2D interaction were constructed to show the *E. coli* β-GUS amino acid residues binding to these compounds [Figure 3]. Roberts *et al.*[32] previously identified inhibitors that bind to the amino acid residues in the active site cleft of *E. coli* β-GUS– Asp163, Val446, Phe448, Tyr472, Arg562, the catalytic residue Glu413, and Leu361 from the bacterial loop (residues 360–376)–by protein crystallization. The inhibitor used in the docking validation, (1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl) methyl)-1-(2-hydroxy ethyl)-3-(4-hydroxyphenyl) thiourea) was designed by Wallace *et al.*[33] to specifically inhibit *E. coli* β-GUS and was found to interact with the residues Tyr472, Tyr 469, Phe448, Ile363, and Glu413. A computational-guided screening approach by Cheng *et al.* identified several *E. coli* β-GUS inhibitors that also interact with active site residues Tyr472 and Tyr469, as well as bacterial loop residues Leu361 and Ile363, among others.[50]

In our models, we found that the top-binding compounds from each fraction also interact with the active site and bacterial residues. Phthalic acid, cyclobutyl tridecyl ester from Fraction 543W [Figure 3a, b] formed alkyl interactions with Leu361, Ile363, and Ile560, and Pi-Pi stacked interactions with Tyr 469 and Tyr 472. On the other hand, N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine from Fraction 5231 [Figure 3c, d], also formed alkyl interaction with Leu361, contacts with Phe448 and Met447, Pi-alkyl interactions with Ile363, as well as Pi-Pi stacked interactions with Tyr 469 and Tyr 472. Finally, Dehydroabietic Acid from Fraction 52335A [Figure 3e, f] formed mostly Pi-Pi stacked interactions with Tyr 469 and Tyr 472, and Pi-alkyl interactions with Ile363 and Trp471. These three compounds formed interactions with

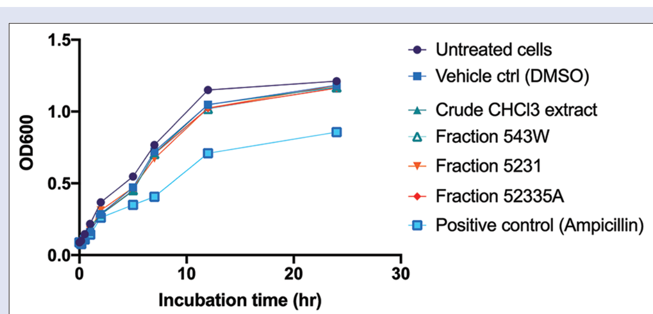

Figure 2: Time-kill antibacterial assays of *Plectranthus amboinicus* extract and fractions against *Escherichia coli*

Table 2: Compounds identified in Fraction 543W by gas chromatography-mass spectrometry, ranked by predicted binding affinity against *Escherichia coli* β-glucuronidases by AutoDock Vina

Compound name	RT (min)	Relative abundance (%)	Similarity score	Library	Predicted binding affinity (kcal/mol)
Phthalic acid, cyclobutyl tridecyl ester	26.3217	1.9668	809	NIST	−7.5
4, 7-methanol-1H-indene, 3A, 4, 7, 7A-tetrahydro-	8.46667	3.3216	771	Wiley registry 9th Ed	−7.2
3,9-Diphenyl-1,6-dioxaspiro[4.4]non-3-en-2-one	21.1367	0.6130	786	Wiley registry 9th Ed	−7.1
Phthalic acid, 2-ethylhexyl isohexyl ester	26.7483	14.2333	792	NIST	−7.1
1, 2-Benzenedicarboxylic acid, dihexyl ester	19.7917	7.1950	840	NIST	−6.8
1, 2-Benzenedicarboxylic acid, dipentyl ester	27.0633	0.6448	773	NIST	−6.7
9-Octadecenoic acid (Z)-	24.2717	1.3525	787	Wiley registry 9th Ed	−6.7
2, 6, 10-trimethyl-tridecane	11.1467	5.2854	869	Wiley registry 9th Ed	−6.6
3, 3-Difluoro-1- (1, 3-dioxolan-2-yl)-1-iodo-4-octanone	14.8083	2.7652	776	Wiley registry 9th Ed	−6.3
Docosane	13.32	3.7617	908	Wiley registry 9th Ed	−6.2
Phthalic acid, heptyl tridec-2-yn-1-yl ester	26.9533	1.7254	791	NIST	−6.2
2-methoxy-2-i-propyloxypropane	14.7083	2.2387	737	Wiley registry 9th Ed	−6.1
Butyl hydroxy toluene	14.87	0.8430	788	Wiley registry 9th Ed	−6.1
Pentadecylamine	19.2583	1.7341	729	NIST	−6
3,3-dimethyl-1-dodecen-4-ol	22.4633	1.0747	727	Wiley registry 9th Ed	−5.7
Dodecanoic acid, methyl ester	19.28	17.6145	767	Wiley registry 9th Ed	−5.7
Hexanedioic acid, mono (2-ethylhexyl) ester	23.4883	1.0011	758	Wiley registry 9th Ed	−5.6
Benzene, (nitromethyl)-	7.57333	6.2089	762	Wiley registry 9th Ed	−5.5
Oxalic acid, allyl hexyl ester	15.8683	2.5481	776	NIST	−5.5
Heptane, 2,4-dimethyl-	10.7933	2.5671	863	Wiley registry 9th Ed	−5.4
3,4-Dihydroxyphenemethylamine	8.34167	5.2504	830	Wiley registry 9th Ed	−5.3
Butyl citrate	22.3783	0.4293	812	NIST	−5.3
1-Heptanol, 6-methyl-	11.15	1.1435	737	NIST	−5
2-methoxy-2-i-propyloxypropane	14.7083	2.2387	737	Wiley registry 9th Ed	−4
5-Iodopentan-2-one	10.2917	2.1848	740	Wiley registry 9th Ed	−4
2,2'-Bi-1,3-dioxolane	12.4067	3.2089	779	Wiley registry 9th Ed	−3.9
4-Chloro-3-hydroxy-1-butene	32.3933	4.6574	727	Wiley registry 9th Ed	−3.4
Propane, 2-ethoxy-	13.7167	1.6331	745	NIST	−3.4
Acetic acid, ethyl ester	8.19333	0.5588	769	Wiley registry 9th Ed	−3.1

NIST: National institutes of standards and technology, RT: Retention time

Table 3: Compounds identified in fraction 5231 by gas chromatography-mass spectrometry, ranked by predicted binding affinity against *Escherichia coli* β-glucuronidases by AutoDock Vina

Compound name	RT (min)	Relative abundance (%)	Similarity score	Library	Predicted binding affinity (kcal/mol)
N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine	7.575	4.5350	876[2]	Wiley registry 9th Ed	−8
1,2-Benzenedicarboxylic acid, bis (1-methylheptyl) ester	26.755	0.6418	775[2]	Wiley registry 9th Ed	−7
1,2-Benzenedicarboxylic acid, diisooctyl ester	26.1517	35.8964	762[1]	NIST	−6.9
2-Undecyltetrahydrofuran	15.8417	9.2009	873[2]	Wiley registry 9th Ed	−6.4
Benzene, 1-ethyl-2-methyl-	7.66167	6.2530	873[1]	NIST	−6.3
2-(1-methyl-cyclopentyloxy)-1-oxacyclohexane	16.4967	0.2725	735[2]	Wiley registry 9th Ed	−6.2
13-Docosenoic acid, methyl ester, (Z)-	20.96	0.5957	822[2]	Wiley registry 9th Ed	−6.2
n-cetyl thiocyanate	19.8867	0.8342	813[2]	Wiley registry 9th Ed	−6.2
Dodecyl acrylate	21.96	0.3819	715[1]	NIST	−6
Dodecanenitrile	19.1167	0.8994	742[2]	Wiley registry 9th Ed	−5.8
Oxalic acid, isobutyl hexyl ester	11.2517	6.6615	835[1]	NIST	−5.8
3-Decene, 2,2-dimethyl-, (E)-	17.8817	1.7469	824[1]	NIST	−5.7
(4Z)-3,3,6-trimethylhepta-4,6-diene-2,4-diol	8.82833	0.6920	871[2]	Wiley registry 9th Ed	−5.6
(3R,4R,6R)-3-(1,3-Dioxolane-2-yl)-6-isopropenyl -3,4-dimethyl-2-oxocyclohexanecarbonitrile	12.9417	1.9225	891[2]	Wiley registry 9th Ed	−5.5
3-Heptene, 2,6-dimethyl-	8.41333	0.9765	829[2]	Wiley registry 9th Ed	−5.5
Di(2-ethylbutyl) ether	10.2883	1.8848	723[2]	Wiley registry 9th Ed	−5.5
Tyramine	24.095	0.3489	835[2]	Wiley registry 9th Ed	−5.4
Methoxyacetic acid, 2-ethylhexyl ester	14.4617	0.6516	847[1]	NIST	−5.2
Pentane, 2-cyclopropyl-	8.54167	0.5198	721[1]	NIST	−5
Tributyl acetylcitrate	22.3883	0.4663	826[1]	NIST	−5
(1R)-cyclooct-5-ene-1,2-diol	8.96833	0.4396	851[2]	Wiley registry 9th Ed	−4.9
Butane, 2,2'-[methylenebis (oxy)]bis[2-methyl-	7.68333	0.5447	703[1]	NIST	−4.8
2-Hydroxypyridine	8.48167	1.1876	779[2]	Wiley registry 9th Ed	−4.5
6-methyl-4,5,8,9-tetrathia-1,11-dodeca-diene	9.775	0.5560	804[2]	Wiley registry 9th Ed	−4.4
1,5-Hexadien-3-ol	32.4017	6.2497	735[1]	NIST	−4.2

Contd...

Table 3: Contd...

Compound name	RT (min)	Relative abundance (%)	Similarity score	Library	Predicted binding affinity (kcal/mol)
3-Pentanol, 2,4-dimethyl-	20.2483	1.0571	748[2]	Wiley registry 9th Ed	−4.2
3,6-Dioxa-2,7-disilaoctane, 2,2,4,4,5,5,7,7-octamethyl-	10.6817	0.5029	737[1]	NIST	−4
4-methylpent-1-ene-2,4-diol	9.205	1.0882	794[1]	NIST	−4
Pentanoic acid	11.6867	0.2811	775[2]	Wiley registry 9th Ed	−3.9
4-Aminopentanoic acid	8.34167	3.0099	793[2]	Wiley registry 9th Ed	−3.9
2-Propanol, 1-(1-methylethoxy)-	9.885	0.9632	731[2]	Wiley registry 9th Ed	−3.8
2-methyl-1,4-butanediol	10.5517	1.2406	823[2]	Wiley registry 9th Ed	−3.5
D-Lactic acid	8.76833	5.1012	764[2]	Wiley registry 9th Ed	−3.2
1,3-Butanediol	18.0083	0.3568	792[2]	Wiley registry 9th Ed	−3.2
Methane, trimethoxy-	15.4083	2.0396	700[2]	Wiley registry 9th Ed	−2.7

NIST: National institutes of standards and technology, RT: Retention time

Table 4: Compounds identified in fraction 52335A by gas chromatography-mass spectrometry, ranked by predicted binding affinity against *Escherichia coli* β-glucuronidases by AutoDock Vina

Compound name	RT (min)	Relative abundance (%)	Similarity score	Library	Predicted binding affinity (kcal/mol)
Dehydroabietic Acid	23.5917	0.2075	735	LECO-Fiehn Rt×5	−7.9
2-Methyl-4-(2-thienyl) quinolone	18.575	25.7304	865	Wiley registry 9th Ed	−7.8
1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	18.8667	0.3519	820	Wiley registry 9th Ed	−7.4
2,4-Dimethyl-4-phenyl-1-pyrroline	20.28	1.0123	754	Wiley registry 9th Ed	−7.1
1,2-Benzenedicarboxylic acid, diisooctyl ester	24.7117	8.5286	899	NIST	−7
2,5-Pyrrolidinedione, 1-[(3-pyridinylcarbonyl) oxy]-	12.8367	3.1925	759	NIST	−7
9,12-Octadecadiynoic acid	18.805	0.2974	718	NIST	−6.8
α-Linolenic acid	18.7317	0.3182	731	NIST	−6.8
(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19- hexaenoic acid	17.9883	1.7725	724	NIST	−6.7
oleic acid	21.9517	1.0133	804	NIST	−6.7
hexadecanoic acid	20.4167	26.0686	894	Wiley registry 9th Ed	−6.5
2-Undecyltetrahydrofuran	15.8617	0.4408	921	Wiley registry 9th Ed	−6.5
10,12-Tricosadiynoic acid	18.9567	1.7493	729	NIST	−6.2
Hexadecanoic acid, 2,3-bis[(trimethylsilyl) oxy] propyl ester	24.9483	0.4664	795	Wiley registry 9th Ed	−6.2
Decanedioic acid, bis (2-ethylhexyl) ester	26.515	7.4306	906	NIST	−6
Oxalic acid, isobutyl hexyl ester	7.80667	3.3364	855	NIST	−5.8
tetradecan-1-ol	17.6983	0.5181	753	Wiley registry 9th Ed	−5.8
Decanal, O-methyloxime	21.0917	0.2649	921	NIST	−5.6
(3R,4R,6R)-3-(1,3-Dioxolane-2-yl)-6-isopropenyl-3,4-dimethyl-2-oxocyclohexanecarbonitrile	16.1867	0.9336	733	Wiley registry 9th Ed	−5.5
Oxalic acid, hexyl neopentyl ester	11.1217	0.1700	802	NIST	−5.3
N-(α-Hydroxyethyl)-4-(cis-hydroxypropyl) piperidine	19.3483	0.7469	705	NIST	−5.2
3,5-dioxooctanedioic acid	20.025	0.2549	706	Wiley registry 9th Ed	−5
3-Pyridinecarboxylic acid	11.7133	5.9573	888	NIST	−5
7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-	18.8267	0.9986	787	Wiley registry 9th Ed	−4.9
Hexa (methoxymethyl) melamine	24.2417	0.2513	726	NIST	−4.6
Methyl 4,4-dimethyl-3-methoxy pentanoate	8.61167	0.5861	765	Wiley registry 9th Ed	−4.4
pyridin-3-ol	9.78833	1.3458	831	NIST	−4.1
Propanoic acid, ethenyl ester	28.1917	1.6484	846	Wiley registry 9th Ed	−3.6
2,3-Butanediol	10.245	0.4278	722	Wiley registry 9th Ed	−3.4
2-hydroxypropanoic acid	8.6	0.2275	854	Wiley registry 9th Ed	−3.3
1-Butanamine	10.505	0.3818	795	Wiley registry 9th Ed	−3.2
Propane, 1,1-dimethoxy-	23.045	1.4510	700	NIST	−3.2
Dimethoxypropane	9.095	0.9204	740	Wiley registry 9th Ed	−2.9

NIST: National institutes of standards and technology, RT: Retention time

active site amino acids Tyr 469 and Tyr472, as well as with bacterial loop residues such as Leu361 and/or Ile363. Roberts *et al.*[32] deduced that active bacterial β-GUS inhibitors could bind effectively to the bacterial β-GUS by interacting with the inner loop unique to bacterial β-GUS.

The assay-guided purification of the *P. amboinicus* crude chloroform extract yielded fractions with β-GUS inhibitory activity and high selectivity for *E. coli* β-GUS compared to human β-GUS with no toxic effects on *E. coli*. The GC-MS profiling of the active fractions identified compounds that are similar to essential oil extracts of *P. amboinicus* reported in literature. The *in silico* docking identified the compounds: Phthalic acid, cyclobutyl tridecyl ester, N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine, and Dehydroabietic Acid, to be highly binding with modes suggesting interaction with active site residues Tyr 469 and Tyr 472, as well as with several residues in the β-GUS unique bacterial loop. The GC-MS and *in silico* modeling enabled elucidation of the β-GUS inhibitory activity of *P. amboinicus* semi-purified fractions and may provide an alternative approach to support drug discovery initiative.

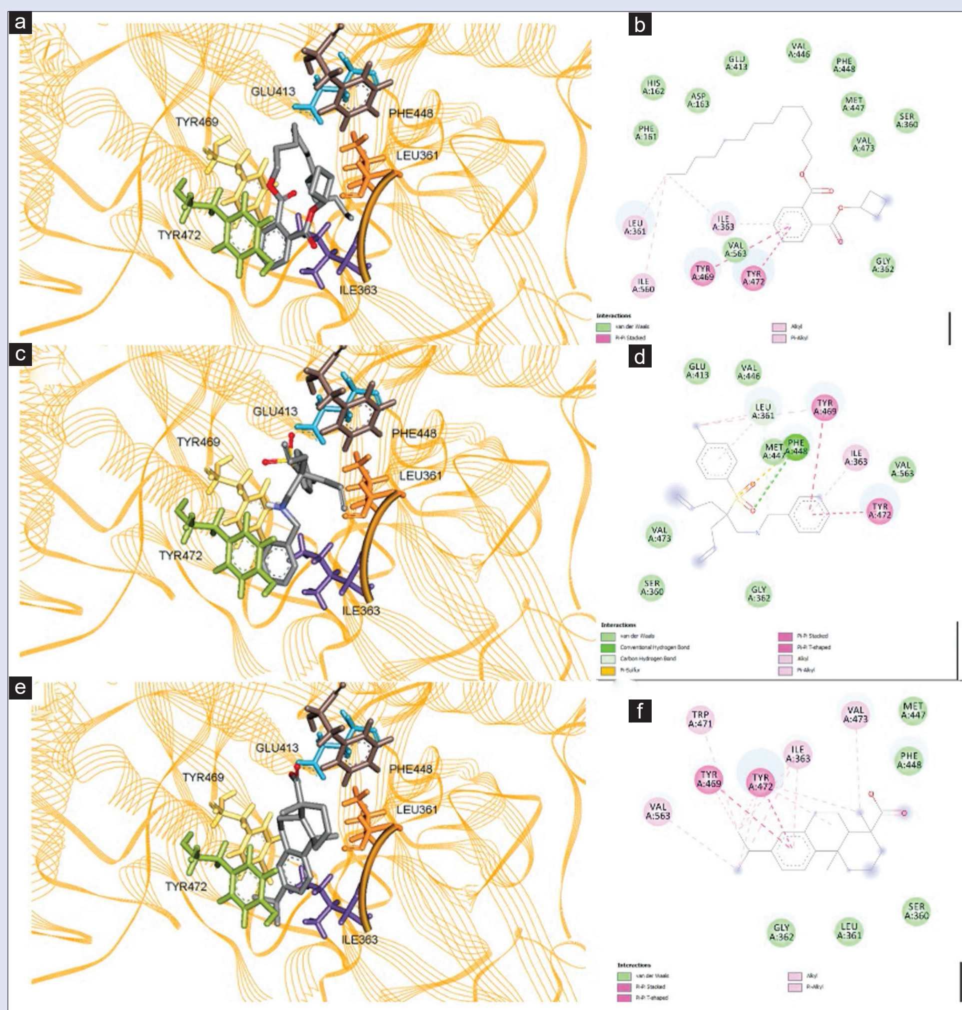

Figure 3: Predicted binding modes and amino acid interactions in the *Escherichia coli* beta-glucuronidases active site cleft of Phthalic acid, cyclobutyl tridecyl ester from Fraction 543W (a and b), N-Benzyl-2-allyl-2-tosyl-4-penten-1-amine from Fraction 5231 (c and d), and Dehydroabietic Acid from Fraction 52335A (e and f)

CONCLUSION

In this study, we identified several highly-active and highly-selective E. coli β-GUS inhibitors from the partially-purified fractions of the *P. amboinicus* leaf chloroform extracts, utilizing a bioassay-guided purification coupled by metabolomics and *in silico* docking approach. Furthermore, these compounds were shown to be highly selective against *E. coli* β-GUS compared to human β-GUS. Using antibacterial assays, we have shown that these are non-toxic to *E. coli* bacteria, preserving gut microbiota. These compounds were identified using GC-MS metabolomics, and subsequently modelled for binding affinities against E. coli β-GUS to study their interactions with active site residues. This is the first report on the potential of *P. amboinicus* as selective inhibitor of E. coli β-GUSs.

Acknowledgements

The authors would like to acknowledge the Metabolomics Core Facility of the Agricultural Biotechnology Research Center (ABRC), Academia Sinica, Taipei, Taiwan, for the use of their GC-MS facility in identifying the compounds. The authors would like to thank Nitish Verma, Punsaldulam Dashnyam, and Nadia Morales for their valuable input during experimentation and data collection.

Financial support and sponsorship

This project was supported by the Philippine Department of Science and Technology's ASTHRDP research grant.

Conflicts of interest

There are no conflicts of interest.

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