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Elucidating the Differences in the Biofilm Suppression Mechanism of 6-Gingerol and 6-Shogaol in *Pseudomonas aeruginosa* through Molecular Docking

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Abstract

The virulence of *Pseudomonas aeruginosa* (*P. aeruginosa*) is associated with its biofilm formation via quorum sensing. Compounds present in *Zingiber officinale* (*Z. officinale*) such as 6-gingerol and 6-shogaol show antibacterial property against different bacteria. However, the mechanism of action of these compounds is underexplored. Hence, we compared the biofilm reduction of these compounds and visualized their binding interactions to different pathways to hypothesize a possible mechanism of action associated with the biofilm reduction. In this study, the biofilm formation of *P. aeruginosa* treated with varying concentrations of either 6-shogaol or 6-gingerol was determined through biofilm accumulation assay. The binding affinity of 6-gingerol and 6-shogaol with various enzymes involved in the membrane integrity, lipopolysaccharide formation, motility, and fatty acid synthesis were ranked through molecular docking. The crystal structures of the compounds docked to the top 3 enzymes with the most negative docking score were evaluated. Results show that 6-gingerol suppressed the biofilm formation of *P. aeruginosa* significantly higher ($p<0.05$) than 6-shogaol. Besides, 6-gingerol has a strong binding affinity to an enzyme associated with membrane integrity while 6-shogaol to a motility-related enzyme. The top enzymes were associated with membrane integrity, lipopolysaccharide formation, and motility. The differences in the binding affinity of 6-gingerol and 6-shogaol may be attributed to the varying substructures involved during the non-covalent interactions. Moreover, we speculate that there are other factors involved that resulted in a higher binding affinity of 6-shogaol despite having fewer interactions. These factors may be independent or complementary with the non-covalent bonding, which may be essential to their biofilm suppression property.

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Introduction

Epidemiological studies show that *P. aeruginosa* is one of the most commonly acquired hospital infections (Bodey *et al.*, 1983). However, the hospital setting does not represent the permanent habitat of *P. aeruginosa*. One study shows different *P. aeruginosa* strains found in various fruits and vegetables (Schroth *et al.*, 2018). Interestingly, this same study shows the association of *P. aeruginosa* strain from a clinically diagnosed infected individual cause's bacterial rot in tomato, potato, cucumber, and lettuce (Schroth *et al.*, 2018). Additionally, there were reported cases that *P. aeruginosa* infection in various farm animals (Al Bayssari *et al.*, 2015). Further, studies also show that it causes mastitis in the dairy cow (Sela *et al.*, 2007). These studies reveal the importance of addressing *P. aeruginosa* infections as it affects the clinical environment and agricultural production.

One significant measure in mitigating *P. aeruginosa* infection is through biofilm inhibition. The biofilm of *P. aeruginosa* is associated with its antibiotic resistance and regulation of environmental nutrients (O'Toole and Kolter, 2002). The bacterial membrane stability is the framework for the biofilm architecture (Kostakioti *et al.*, 2013). LPS is one of the major players in the outer network of gram-negative bacteria, which studies associate with biofilm formation (Nakao *et al.*, 2012). Several studies also consider the participation of extracellular machinery in bacterial movement also affects biofilm formation (O'Toole and Kolter, 2002; Li *et al.*, 2007; Wolfe *et al.*, 2004; and Shi *et al.*, 2004). Lastly, fatty acid synthesis in bacteria contributes to the membrane fatty acids, which are also associated with the biofilm lifestyle of the bacteria (Dubois-Brissonett *et al.*, 2016).

Several studies demonstrated that ginger extract inhibits biofilm formation in *P. aeruginosa* (Kim *et al.*, 2013; Sasidharan *et al.*, 2010; Nikolic *et al.*, 2014). Besides, compounds found in *Z. officinale* like 6-gingerol and 6-shogaol gain attention due to their potential antibacterial properties (Suekawa *et al.*, 1984; Ha *et al.*, 2012; Weng *et al.*, 2010). However, the mechanism of action of 6-gingerol and 6-shogaol needs extensive exploration.

To hypothesize a possible mechanism of action involved during the biofilm suppression of 6-gingerol and 6-shogaol in *P. aeruginosa*, the effects of 6-gingerol and 6-shogaol in the biofilm attenuation in *P. aeruginosa* were determined; the highest binding enzymes of 6-gingerol and 6-shogaol were identified; and the crystal structures of these compounds with the top 3 highest binding enzymes were evaluated.

Materials and methods

Preparation and storage of the compounds

The compounds 6-gingerol (CAS number: 23513-14-6) and 6-shogaol (CAS number: 555-66-8) were obtained from Biopurify (Chengdu Biopurify Phytochemicals Ltd., Sichuan, China). To prepare the solution with the desired concentration, we reconstituted the compounds with 1% Dimethyl sulfoxide in sterile deionized water. We stored the compounds at 0 to 4 °C until used.

Maintenance and Preparation of P. aeruginosa

The *P. aeruginosa* (ATCC 10145) strain was purchased from Fil-Anaserve, INC. (Manila, Philippines). For the maintenance and preparation of the nutrient broth, the protocol from a previous study was followed (Nas *et al.*, 2019). The bacteria were at 37 °C to allow optimal bacterial growth. The 0.5% McFarland was the standard for the concentration of the bacterial suspension and diluted with sterile deionized water.

Biofilm accumulation assay

In this assay, the protocol suggested by Wilson and peers (2017) with some modifications was followed but minor modifications. About 200 µL of bacterial suspensions mixed with either 6-gingerol or 6-shogaol into final concentrations of 0, 2.5, 5, and 10 µM were prepared and incubated at 37 °C for three days. The biofilm aggregation on the plate was examined daily for three days by harvesting the biofilm through the removal of the nutrient broth and

the planktonic cells. The plates were washed with sterile deionized water and added 1% crystal violet. The suspension was added 95% ethanol before incubating for another 30 minutes. The suspension was transferred to a clean 96 well plate for quantification using Varioskan Flash (Thermo Fischer Scientific, MA, USA) to measure the absorbance of the solution at 600 nm.

Virtual molecular docking

The data for 6-gingerol (CID: 44559528) and 6-shogaol (CID: 5281794) were from PubChem (pubchem.ncbi.nlm.nih.gov). Meanwhile, the crystal structure of the all the proteins such as Anhydro-N-acetylmuramic acid kinase (PDB ID: 3qbw), Glucose-1-phosphate thymidylyltransferase (PDB ID: 1g2v), Fucose-binding lectin PA-IIL (PDB ID: 3dcq), Alkaline metalloproteinase (PDB ID: 1kap), UDP-3-O-[3-hydroxymyristoyl] N-acetyl glucosamine deacetylase (PDB ID: 2ves), GDP-mannose 6-dehydrogenase (PDB ID: 1mfz), Phosphoheptose isomerase (PDB ID: 1x92), WbpP (PDB ID: 1sb8), WbpB (PDB ID: 3oa2), Phosphomannomutase/phosphoglucomutase (PDB ID: 1p5d), SAM-dependent uroporphyrinogen III methyltransferase NirE (PDB ID: 2ybo), FimX (PDB ID: 3hv8), PilT (PDB ID: 3jvv), Rhamnolipids biosynthesis 3-oxoacyl-[acyl-carrier-protein] reductase (PDB ID: 2b4q), and Biotin carboxylase (PDB ID:2vqd) were downloaded from Protein Data Bank (www.rcsb.org/pdb).

The identified active sites for each enzyme were prepared using Autodock tools version 4.2.6 (The Scripps Research Institute, CA, USA) and Mcule (Mcule Inc., USA). These ligands docked to the identified binding center of the enzymes. The ligand poses of 6-shogaol and 6-gingerol with the most negative docking score was considered as it represents the highest binding affinity (Mobley *et al.*, 2017).

Characterization of the ligand docked pose on the enzyme with the highest binding affinity

The docked crystal structures of 6-shogaol and 6-gingerol with the enzymes with the highest binding affinity were visualized. The binding interactions of the ligand and the enzyme was validated by re-docking the known inhibitor of the ligand with the enzyme. The crystal structures of the re-docked ligand-enzyme were superimposed with the original crystal structure through Superpose v.1.0. (Wishartlab, University of Alberta, Canada). The root means square deviation (RMSD) of the superimposed crystal structures was the basis for the similarity of the re-docked ligand from the original. The RMSD value should be <1.2 Å to be considered similar (Kufareva and Abagyan, 2011). After the validation of the similarity of the re-docked structures, the interactions of the amino acid residues and the substructures of 6-gingerol and 6-shogaol were identified and compared with the inhibitory ligand.

Statistical analysis

The absorbance was normalized against the blank and presented as mean ± SD. The difference within treatment groups was computed using analysis of variance through GraphPad Prism version 7 (GraphPad Software, CA USA). The significance was set at $p \leq 0.05$ and $p \leq 0.01$.

Results and discussion

6-gingerol reduces biofilm formation in P. aeruginosa better than 6-shogaol

In the biofilm accumulation assay, the absorbance of the biofilm in the bacterial suspension treated with the varying concentrations of 6-gingerol is significantly lower ($p<0.05$ and $p<0.01$) than the untreated in the three-day observation period, as shown in Figure 2. Meanwhile, only the 5 µM of 6-shogaol exhibited a significant reduction ($p<0.05$) in the biofilm formation only on the third day.

In this study, 6-gingerol reduced the biofilm accumulation in *P. aeruginosa* consistently in contrast with 6-shogaol. Kim and peers (2015) demonstrated that as low as 0.1 µM 6-gingerol showed biofilm inhibition in *P. aeruginosa*. Besides, this is the first-time biofilm inhibition in *P. aeruginosa* observed in 6-shogaol. Our result shows

that about 5 µM of 6-shogaol exhibited modest biofilm reduction in *P. aeruginosa*.

This observation suggests that a higher concentration of 6-shogaol may eventually increase biofilm inhibition in *P. aeruginosa*. Notable studies done on the antibacterial activity of 6-gingerol in other microorganisms suggest the reduction in the cholera toxin and virulence factor in cholera (Saha *et al.*, 2013), hyphal inhibition in *Candida albicans* (Lee *et al.*, 2018), and inhibition of quorum sensing through LasR in *P. aeruginosa* (Kim *et al.*, 2015). Aside, one study has shown that 6-shogaol demonstrated hyphal inhibition in *Candida albicans* (Lee *et al.*, 2018).

Table 1. Docking score of 6-Gingerol and 6-Shogaol to Different Enzymes Affecting Quorum Sensing in *Pseudomonas aeruginosa*

Association	Enzymes	6-Gingerol	6-Shogaol
Membrane Integrity	Anhydro-N-acetylmuramic acid kinase	-6.6	-6.6
	Glucose-1-phosphate thymidylyltransferase	-7.2*	-7.0
	Fucose-binding lectin PA-IIL	-4.9	-4.9
LPS production	Alkaline metalloproteinase	-5.6	-5.9
	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	-5.6	-6.1
	GDP-mannose 6-dehydrogenase	-6.8	-7.1
	Phosphoheptose isomerase	-5.4	-5.0
	Phosphomannomutase/phosphoglucomutase	-6.2	-6.3
	WbpP	-6.6	-6.7
	WbpB	-7.0	-7.0
Motility	SAM-dependent uroporphyrinogen III methyltransferase NirE	-6.6	-7.6*
	FimX	-6.5	-6.1
	PilT	-5.9	-4.9
Fatty acid synthesis	Rhamnolipids biosynthesis 3-oxoacyl-[acyl-carrier-protein] reductase	-6.5	-7.0
	Biotin carboxylase	-6.9	-6.5

*Highest Binding Affinity.

6-gingerol may affect membrane integrity while 6-shogaol may affect motility

With the knowledge that both 6-gingerol and 6-shogaol affect the biofilm formation in *P. aeruginosa*, we docked 6-gingerol and 6-shogaol to enzymes associated with the membrane integrity, LPS production, motility, and fatty acid synthesis. We found out that the binding affinity of 6-gingerol and 6-shogaol comparable with each other when bound to various enzymes associated with membrane integrity, LPS production, motility, and fatty acid synthesis. Overall, our findings reveal that 6-gingerol has the highest binding affinity to glucose-1-phosphate thymidylyltransferase, an enzyme required in maintaining the bacterial membrane. No study has associated 6-gingerol with glucose-1-phosphate thymidylyltransferase despite studies associating 6-gingerol with membrane biofouling in bacteria (Ham *et al.*, 2019; Ham *et al.*, 2018). We also observed that 6-shogaol has the highest binding affinity to SAM-dependent uroporphyrinogen III methyltransferase NirE.

This enzyme affects the movement of *P. aeruginosa*. However, there is no investigation done on the effects of 6-shogaol on SAM-dependent uroporphyrinogen III methyltransferase NirE nor the motility-related physiological aspect of the bacteria. To the best of our knowledge, no research has associated Z. officinale extract with these enzymes. The only literature we found associated ginger extract in the prevention of motility in Aeromonas septicaemia (Korni *et al.*, 2017). These findings or a lack thereof, suggest that the present knowledge on Z. officinal, primarily 6-gingerol and 6-shogaol, on this perspective remains elusive.

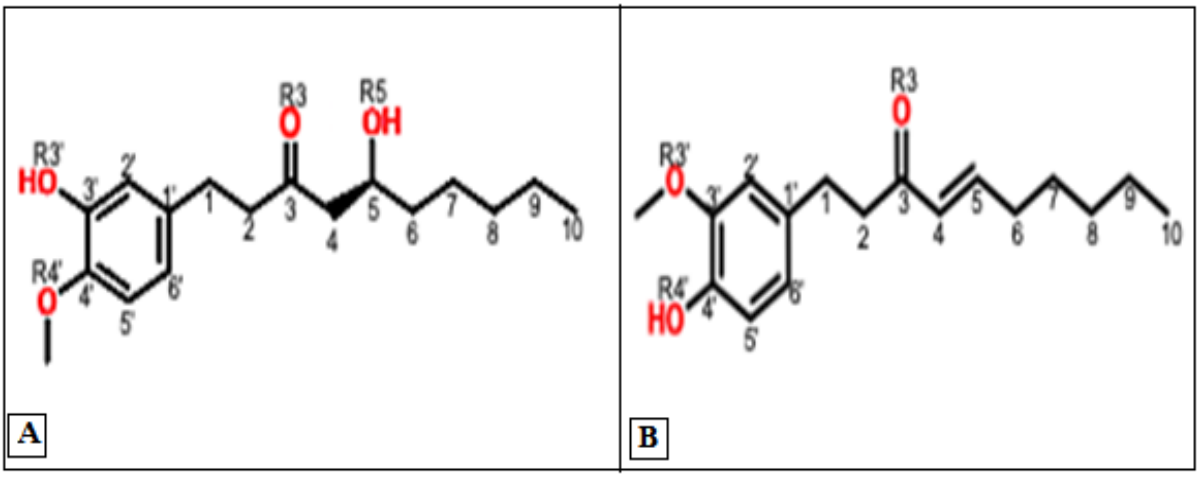

Fig. 1. Structures of 6-gingerol (A) and 6-shogaol (B).

Characterization of the crystal structure of 6-gingerol and 6-shogaol

The crystal structures of 6-gingerol and 6-shogaol on the top 3 enzymes with the highest binding affinity were visualized and compared with the inhibitory ligand. The three enzymes with the highest binding affinity with the compounds were glucose-1-phosphate thymidylyltransferase, WbpB, and SAM-dependent uroporphyrinogen III methyltransferase NirE.

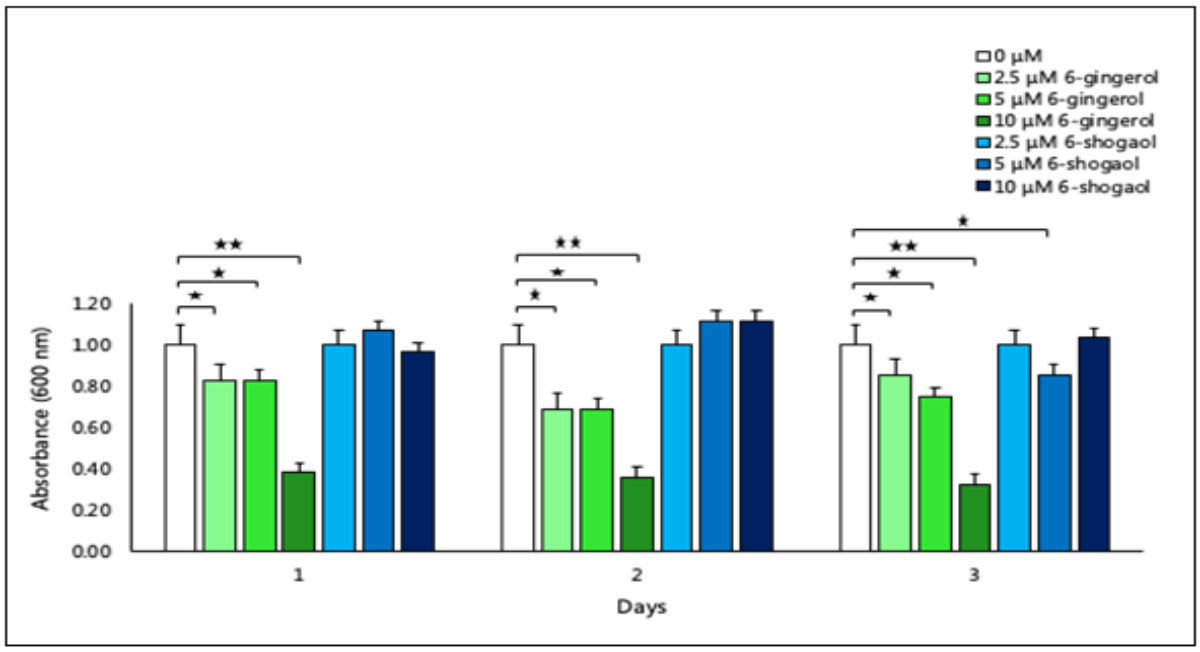

Fig. 2. Normalized absorbance of 6-gingerol and 6-shogaol treated bacterial suspension in crystal violet assay. We assessed the absorbance of the bacterial suspension treated with varying concentrations of 6-gingerol (0, 2.5, 5, and 10 µM) and 6-shogaol (0, 2.5, 5, and 10 µM) every day for three days, *significance at $p<0.05$, ** at $p<0.01$, replicates = 3 and trials = 3.

To validate the crystal structure of these enzymes, the inhibitory ligands for these enzymes were re-docked and compared with the original structure. The inhibitor of glucose-1-phosphate thymidylyltransferase is thymidine-5'-triphosphate (TTP) (CID: 64968). The RMSD values of its α-carbon are 0.12 Å, the backbone is 0.12 Å, heavy atoms are 0.12 Å, and overall is 0.12 Å. Meanwhile, the inhibitor of WbpB is nicotinamide-adenine-dinucleotide (NAD) (CID: 5892).

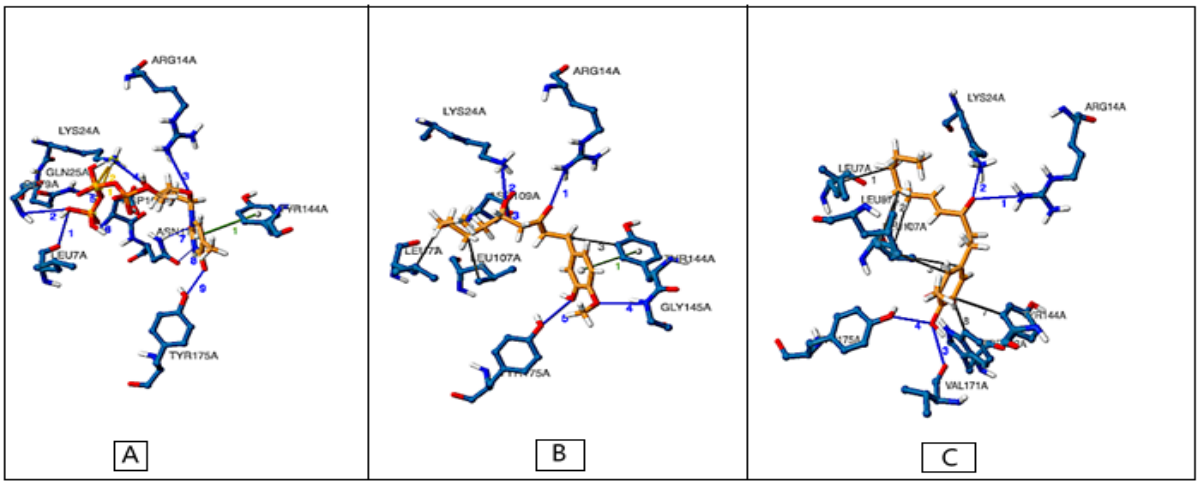

Fig. 3. Binding interactions of TTP (A), 6-gingerol (B), and 6-shogaol (C) with the amino acids of Glucose-1-phosphate thymidylyltransferase.

The RMSD values of NAD are 0.07 for α-carbon, 0.07 Å for backbone, 0.1 Å for heavy, and an overall RMSD of 0.1 Å. Lastly, the inhibitor of SAM-dependent uroporphyrinogen III methyltransferase NirE is s-adenosyl-l-homocysteine (SAH) (CID: 439155). SAH has RMSD values of 0.179 Å for α-carbon, 0.19 Å for backbone, 0.3 Å for heavy, and an overall RMSD of 0.3 Å. All of these enzymes have an overall RMSD value lower than 1.2 Å, which implies that the crystal structures of the re-docked ligand-enzyme are similar to the original (Kufareva and Abagyan, 2011). Hence, the binding affinities in the molecular docking experiment were valid. The ligands TTP, gingerol, and shogaol were docked on the chain A of glucose-1-phosphate thymidylyltransferase, as shown in Figure 3. TTP has hydrophobic interactions with leu7, gly9, arg14, lys24, gln25, asp109, asn110 (2), and tyr175. Meanwhile, it forms H bond with tyr144 and two salt bridges with lys24. Meanwhile, 6-gingerol forms hydrophobic interactions with leu7 (C9), leu107 (C7), and tyr144 (C1). It also forms H bond with arg14 (R3), lys24 (R5), asp109 (R5), gly145 (R4'), and tyr175 (R3'). Also, there is a pi-stacking in the aromatic ring of gingerol with tyr144.

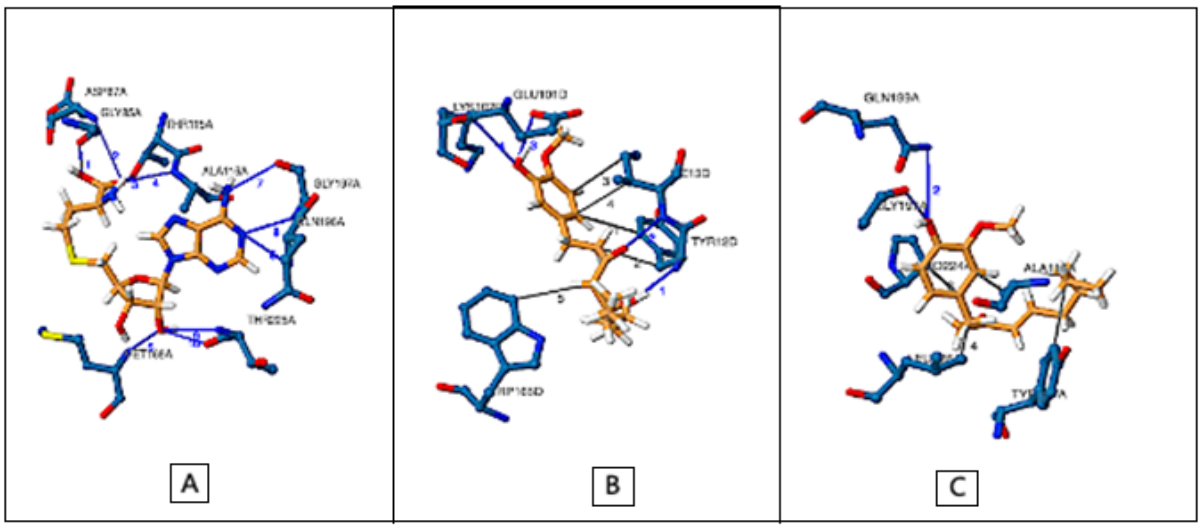

Fig. 4. Binding interactions of NAD (A), 6-gingerol (B), and 6-shogaol (C) with the amino acids of WbpB.

In 6-shogaol, the hydrophobic interactions were developed with leu7 (C8), leu87 (2: C7 and C2'), leu107 (C6), leu107 (2: C7 and C1'), tyr144 (C5'), and tyr222 (C5'). It also has H bonds with arg14 (R3), lys24 (R3), val171 (R4'), and tyr175 (R4'). TTP has a higher binding affinity which is -8.5 kcal/mol

compared to 6-gingerol and 6-shogaol. This binding affinity may be attributed to the high number of hydrophobic interactions, which may affect a large portion of the chain resulting in a decrease of movements in the enzyme (Myslinski *et al.*, 2013). 6-Gingerol and 6-shogaol have the same hydrophobic interactions in leu7, leu107, and tyr144, but the involved substructures in each ligand are different. Similarly, H bond formations with lys24 and tyr175 involve different substructures of the ligands. Conversely, H bond with arg14 interacted with R3, which acts as an H bond acceptor in 6-gingerol and 6-shogaol. Besides, the presence of pi-stacking may have little effect on the binding affinity of 6-gingerol.

NAD, 6-gingerol, and 6-shogaol were docked in the chain D of Wbp B, as shown in Figure 4. NAD develops hydrophobic interactions with ile34 (2), pro80, and leu83. Also, it forms an H bond with gly8, tyr12, ile13, tyr32, ile34, asn35, asp36 (2), glu55, asn81, lys102, rg157, lys166, and asn178 (2). Aside from those, it has two salt bridges in lys166. Meanwhile, 6-gingerol has hydrophobic interactions with tyr12 (2: C6' and C2), ile13 (2: C5' and C6'), and trp165 (C4). It forms H bond with tyr12 (R5), ile13 (R3), glu101 (R3'), and lys102 (R4'). Besides, 6-shogaol has hydrophobic interactions with tyr12 (2: C10 and C9), ile13 (2: C1' and C6'), glu101 (C5'), trp160 (C9), and lys102 (R3). NAD has a higher binding affinity, -10.5 kcal/mol, compared to 6-gingerol and 6-shogaol. This affinity may be due to the high number of H bonds formed in NAD (Chen *et al.*, 2016). Apparently, 6-gingerol and 6-shogaol have the same binding affinity with WbpB, which suggests that the extra H bonds in 6-shogaol have a modest effect on the binding affinity. In fact, both 6-shogaol and 6-gingerol have hydrophobic interactions with tyr12 and ile13, but only common in C6' with ile13. The H bond of 6-gingerol and 6-shogaol with lys102 involve different H-bond acceptors in their substructure.

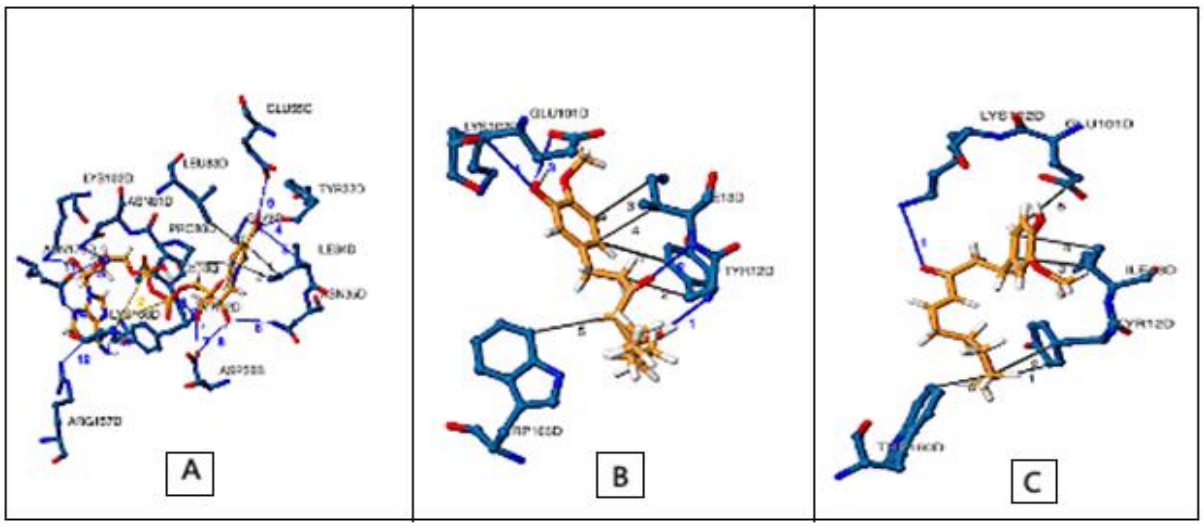

Fig. 5. Binding interactions of SAH (A), 6-gingerol (B), and 6-shogaol (C) with the amino acids of SAM-dependent uroporphyrinogen III methyltransferase NirE.

SAH, 6-gingerol, and 6-shogaol interacted with the amino acids of chain A in SAM-dependent uroporphyrinogen III methyltransferase NirE, as shown in Figure 5. SAH forms H bonds with gly85, asp87, thr115, ala116, met168, gln196, gly197 (2), and thr225 (2). Conversely, 6-gingerol has hydrophobic interactions with leu42 (C9), ala116 (C5'), tyr167 (C6'), pro224 (2: C10 and C9), and leu226 (C5'). It also forms H bonds with asp87 (R5), ile90 (R5), met168 (R3'), and thr225 (2: R3'). Meanwhile, 6-shogaol has hydrophobic interactions with ala116 (C2'), tyr167 (C9), pro224 (C1'), and leu226 (C1). Aside, it has H bonds in gly197 (R4') and gln199 (R4'). Both 6-shogaol and 6-gingerol form hydrophobic interactions with ala116, tyr167, pro224, and leu226 but in different substructures. Also, SAH has a higher binding affinity, -7.9 kcal/mol, compared to 6-gingerol and 6-shogaol, which may be attributed to

the high number of H-bond. Typically, hydrophobic interaction is stronger than the H bond, however, the strength of the H bond is enhanced if it involves strong acceptors and donors (Smith *et al*., 2009). Interestingly, 6-shogaol has a higher binding affinity in SAM-dependent uroporphyrinogen III methyltransferase NirE compared to 6-gingerol despite fewer non-covalent interactions. These findings only suggest that the binding affinity of 6-shogaol involves another mechanism, which may be independent or complementary to its non-covalent interactions. Overall, 6-gingerol and 6-shogaol mostly involve different substructures in their non-covalent interactions with the enzymes. In 6-gingerol, there is no common substructure involved with the hydrophobic interactions of three enzymes evaluated but has R5 and R3' involved in its H bond formation. In 6-shogaol, only C1' is the common atom included during the hydrophobic interactions with the three enzymes evaluated. Meanwhile, there is no common substructure involved during the development of the H bond with the three enzymes. These findings imply that there are different substructures involved during the non-covalent interactions of 6-gingerol and 6-shogaol, which may be the cause of the difference in their biofilm suppression efficiency.

Conclusion

Beneficial compounds found in *Z. officinale*, like 6-gingerol and 6-shogaol, diminishes biofilm accumulation in *P. aeruginosa* but in a different efficiency. The different efficiency may be attributed to different target enzymes. Overall, 6-gingerol has a high affinity to an enzyme involved in the maintenance of the membrane integrity, while 6-shogaol with enzyme associated with motility. Both of these enzymes influence biofilm formation in *P. aeruginosa*. Despite the similarity in their structure, the substructures involved in their non-covalent interactions vary. Particularly, R3' and R5 in 6-gingerol may be important in the H bond formation contrary to 6-shogaol, in which C1' may be essential for its hydrophobic interactions. These findings suggest that differences in the biofilm inhibition of 6-gingerol and 6-shogaol may be attributed to the dissimilar possible target enzymes and unalike substructures participating in the non-covalent interactions. Also, 6-shogaol may engage a distinct mechanism to enhance its binding affinity, which may be independent or complementary with its non-covalent interactions. It is highly recommended to conduct further investigations to determine this potential novel mechanism, which may enhance binding affinity.

Acknowledgements

None.

Authors contribution

Gellecanao, Malacad, Manugas, Mutia, Paguibitan, Quilala, Rodriguez, and Tee performed the biofilm accumulation assay and the statistics. Nas did the docking experiment. Nas wrote the paper. Everyone read and approved the paper.

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