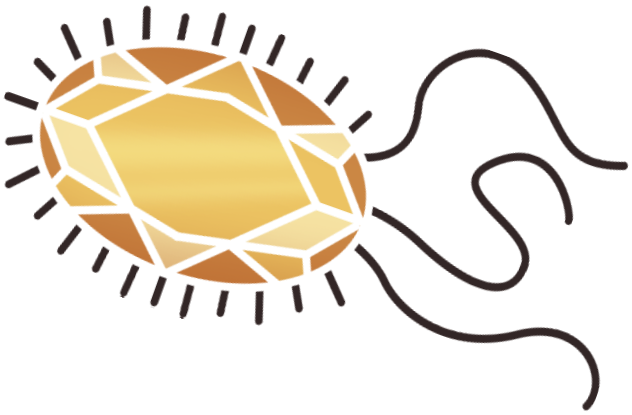

CLUSTER THREE

MICROBIOLOGY AND BIOCHEMISTRY

Yellow is associated with both caution and sickness, commonly linked with the advent of antibiotic-resistant pathogens. The gem depicts a quintessential microbe for this reason. However, the brighter shades of yellow are associated with hope and positivity. The following research studies, tackling the above-mentioned issues and other related topics through various means both in the laboratory and virtually, hope to emulate and be symbols of such positive traits.

The microbiology studies fall under the scope of the Health Research and Development Agenda, primarily due to their potential contributions to drug discovery and antibacterial research in the face of pressures of current health problems and drug-resistant bacteria. The biochemistry studies fall under the scope of the Health Research and Development Agenda and the Aquatic, Agriculture, and Natural Resources (AANR) Research and Development Agenda as they provide substantial findings which contribute to knowledge-gathering in their respective fields and form bases for future practical applications.

BASED ON: Harmonized National Research and Development Agenda (HNRDA)

Formulation and evaluation of antibacterial gel incorporated with *Stachytarpheta jamaicensis* crude ethanolic leaf extract against *Staphylococcus aureus*

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Abstract

Stachytarpheta jamaicensis (sentimento) is utilized by locals from Maasin, Iloilo as an open wound poultice for its antibacterial properties. This study aimed to formulate and evaluate an antibacterial gel incorporated with *S. jamaicensis* leaf extract against *Staphylococcus aureus*. The gel's antibacterial activity was compared to that of a negative control (gel base) and a positive control (hand sanitizer) using the agar well diffusion method. The gel was found to be stable in all physicochemical parameters evaluated (pH, viscosity, spreadability, centrifugation, and mechanical vibration) except for viscosity. The hand sanitizer exhibited the highest zone of inhibition (6.17 ± 0.29 mm) followed by the gel (4.67 ± 0.29 mm). Although not comparable to the positive control, the gel exhibited antibacterial activity. Thus, the drug delivery system effectively delivered the extract's active ingredient. However, it can not be used as a hand sanitizer with its current extract concentration.

Introduction. - Plant species traditionally used as an alternative medicine to address various illnesses and diseases have been widely investigated through phytochemical screenings. Those proven to contain phytochemical constituents whose functions coincide with their intended use are then subjected to antibacterial and anti-inflammatory tests, among others [1]. Once their efficacy has been tested in the laboratories, they can now be incorporated into medicinal preparations such as syrups, tablets, capsules, and topical formulations for mass production and commercial sale [1].

One such plant is the *Stachytarpheta jamaicensis*, locally known as kandikandilaan or sentimento, a flowering plant that belongs to the family of *Verbenaceae*. This plant can be found thriving in the tropical forests of the Americas, and the subtropical forests of Asia and Africa. It has numerous medicinal benefits in infectious and chronic health systems [2]. In the Philippines, there is an abundance of *S. jamaicensis* where locals use its leaves as a poultice in treating open wounds, for it is known to have antibacterial properties [3,4].

Since Abadilla et al. [5] have already developed an ointment using *S. jamaicensis* leaf extracts, this study formulated and evaluated a gel. Gel formulations are generally preferred over other topical semisolid preparations because they stay longer on the skin, have a higher viscosity, are more bioadhesive, and cause less irritation [6]. In addition, gel formulations are moisturizing, water-dependent, have a smooth application, and release active ingredients more effectively [6,7].

According to Taylor and Unakal [8], *Staphylococcus aureus* is a common bacteria usually found in the skin of most healthy humans, since *S. aureus* is one of the standard components of the human's environment and normal flora. According to the study of Jacopin et al. [9], a significant number of community-acquired and hospital-acquired diseases are triggered by commensal bacteria such as *Escherichia coli*, *Staphylococcus aureus*, or *Streptococcus pneumoniae* which can also be opportunistic pathogens.

With this, an antibacterial gel incorporated with *S. jamaicensis* crude ethanolic leaf extract was formulated and evaluated. If proven effective, the antibacterial gel may be commercialized to produce a sanitizer affordable for the masses and address the necessity of discovering new drug delivery systems for herbal medicine.

Thus, the study aimed to formulate and evaluate an antibacterial gel incorporated with *Stachytarpheta jamaicensis* (sentimento) crude ethanolic leaf extract against *Staphylococcus aureus.* The specific objectives of this study were to:

(i) Formulate an antibacterial gel incorporated with *S. jamaicensis* crude ethanolic leaf extract against *S. aureus*;

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For supplementary data, contact: publiscience@wvc.pshs.edu.ph.

(ii) Evaluate and compare the results of the physicochemical tests of the formulated gel before and after accelerated stability testing;

(iii) Evaluate the antibacterial activity of the formulated gel against *S. aureus* by measuring its zone of inhibition using the agar well diffusion method; and

(iv) Determine if there is a significant difference between the antibacterial activity of the formulated gel with the gel base without the extract as the negative control and a commercially available hand sanitizer as the positive control.

Methods. - The methodology is divided into four (4) parts: extraction, gel formulation, physicochemical evaluation, and antibacterial evaluation. The formulated gel's physicochemical properties and antibacterial activity against *Staphylococcus aureus* alongside the gel base (negative control) and a commercially available sanitizer (positive control) were evaluated.

Collection and Identification of Samples. A random sampling method was employed in the leaf collection in a lot located at Brgy. Daja, Maasin, Iloilo, at 10°53'45.3"N, 122°24'43.5"E on November 27, 2020. A 36-sq. meter (6 m by 6 m) main plot was established that was further divided into 36 1-square meter subplots with dimensions of 1 m by 1 m. Each subplot was labeled with numbers from 1 to 36, and 9 subplots were randomly chosen as sampling sites. Sentimento plants with green leaves and bright even colors were uprooted and then verified as *S. jamaicensis* by the Department of Agriculture in Sta. Barbara, Iloilo.

Extract Acquisition. The collected *S. jamaicensis* leaves were washed under running tap water and rinsed with distilled water. The leaves were oven-dried for 48 hours [4], pulverized using a blender, and sifted with sieves mesh numbers 5 and 10 [10]. Fifty (50) grams of the leaf powder was mixed with 500 mL of 70% ethanol [11,12] and was sonicated using an ultrasonic cleaner (42 kHz, 135 W; Branson Ultrasonic Corporation, USA) for 60 minutes. The mixture was filtered twice [4] using a vacuum pump and was subjected to a rotary evaporator (Biobase IKA RV8-S099) at 40 °C with 150 revolutions per minute (rpm) for 10 hours [13]. The aqueous extract with a concentration of 100 mg/mL was then used for the antibacterial gel formulation for better solubility with the gel base.

Antibacterial Gel Formulation. To formulate the gel, propylene glycol, an antifreeze and anti-melting preservative, was added to enhance its stability [14]. Glycerin was also added to help the gel stay on the skin for a prolonged period [15]. Five (5) grams of carbomer 934 (1%), 35 mL of propylene glycol (7%), and 35 mL of glycerin (7%) were dispersed using a hot plate with a magnetic stirrer in 410 mL of distilled water. The mixture was allowed to rest for 60 minutes for the carbomer to hydrate and swell [16].

The initial mixture was neutralized with 2 mL of triethanolamine to attain the desired pH of 8.0 [16]. Forty (40) milliliters of the formulation was then set aside in a beaker at room temperature until use, while 460 mL was incorporated with the leaf extract. Five (5) mL of the leaf extract [4] was diluted with 5 mL of polysorbate 20, which also improves the gel's stability [17]. The leaf extract and polysorbate 20 mixture was then added to the carbomer mixture. The final concentration of the extract in the carbomer mixture was 106 mg/mL.

Physicochemical Evaluation of Gel. The tests suggested by the Food and Drug Administration (FDA), the United States Pharmacopeia (USP), and the Brazilian Health Surveillance Agency (ANVISA) were conducted with the formulated antibacterial gel [18].

pH. The pH of the formulated antibacterial gel was measured using a digital pH meter. The electrode was dipped into the antibacterial gel and left for 10 minutes at room temperature before pH reading [19,20]. The measurement was carried out in triplicates and the average of the three readings was recorded to ensure accuracy.

Viscosity. The viscosity of the antibacterial gel was determined using a viscometer at 25 °C with a spindle speed of 12 rpm [21]. The measurement was carried out in triplicates and the average of the three readings was recorded to ensure accuracy.

Spreadability. The parallel-plate method was used to measure the spreadability of the formulated gel [22]. Spreadability was calculated using the formula:

$$S = \frac{M \cdot L}{T}$$

Where:
S = Spreadability (g · cm/s)
M = Weight (g) tied to the upper slide
L = Length (cm) moved by the glass slide
T = Time (s) it took to separate the upper and lower slides

The measurement was carried out in triplicates and the average of the three readings was recorded to ensure accuracy.

Centrifugation Test. Five (5) grams of the antibacterial gel were subjected to a centrifuge at a cycle of 3000 rpm for 30 minutes at room temperature [23] to observe the occurrence phase separation.

Mechanical Vibration Test. Five (5) grams of the antibacterial gel were transferred to a test tube and subjected to a vortex shaker for 10 seconds to observe the occurrence of phase separation [24].

Stability Test. The formulated antibacterial gel underwent a hot and cold temperature cycling adopted from Krongrawa et al. [22]. It was placed alternately at 4 ± 1 °C and 45 ± 1 °C for 24 hours each for 6 cycles. The pH, viscosity, and spreadability were measured, and centrifugation and vibration testing were conducted in the post-stability test antibacterial gel.

Antibacterial Evaluation of Gel. Samples obtained from the *S. aureus* Tryptic Soy Broth (TSB) subculture from the Philippine Biobank Facility in University of the Philippines Los Baños were inoculated to the surface of the Mannitol Salt Agar (MSA) using the quadrant streaking method. The plate was then incubated for 24 hours at 35 °C. Large, bright yellow, and opaque isolated colonies of *S. aureus* were inoculated to 15 mL of TSB and incubated [23]. Tryptic Soy Broth (TSB) was then added to reduce and achieve the turbidity of 0.5 McFarland standard [23].

Using a sterile blue micropipette tip, three Mueller Hinton Agar (MHA) plates were each punctured to create three uniformly sized wells. Pure colonies of *S. aureus* from the TSB were then inoculated and swabbed to the three MHA plates [23]. Treatments were then dispensed using a micropipette into the wells according to their labels with a uniform amount of 1 mL. The plates were then incubated for 24 hours at 37 °C [23]. The antibacterial activity of the formulated gel, gel base without leaf extract (negative control), and a commercially available hand sanitizer (positive control) were evaluated by measuring the zone of inhibition [23].

Data Analysis. For calculations, p-values were calculated using R (v4.04, GNU GPL v2). Paired t-test was then performed to determine if there is a significant difference between the mean pH, viscosity, and spreadability of the formulated gel obtained before and after accelerated stability testing. One-way ANOVA test with the statistical significance set at 5% was then used to determine if there is a significant difference between the antibacterial activity of the gel base, formulated gel, and commercially available hand sanitizer against *S. aureus* based on their generated zones of inhibition, and post-hoc analysis was evaluated using Tukey HSD test.

Safety Procedure. Proper protective equipment was worn throughout the conduct of the data gathering to avoid sample and bacterial contamination. Working areas were disinfected with 70% ethanol. All chemical wastes were handled according to their respective safety data sheet, placed inside empty water bottles, and were disposed of by the personnel of the school. Biological materials such as cultures and contaminated glassware were autoclaved before disposal.

Results and Discussion. - The data from the agar well diffusion assay were statistically analyzed using one-way ANOVA to determine if there is a significant difference between the zones of inhibition generated. Paired t-test was used to determine if there is a significant difference among the pH, viscosity, and spreadability values acquired.

Physicochemical Evaluation. For the physicochemical evaluation, three parameters, namely the pH, viscosity, and spreadability of the gel, were assessed. Each parameter was then statistically analyzed through paired t-tests set at 0.05 alpha with n=3 trials. With this, the p-values of the pH, viscosity, and spreadability are 0.11, 5.17 x 10^{-2}, and 0.23, respectively. The paired t-test showed that there is no significant difference in the formulated gel before and after accelerated stability testing (AST) in terms of pH and spreadability, indicating stability and good quality of the formulated gel in these parameters. Meanwhile, a significant difference in the viscosity of the formulated gel was established before and after stability testing, indicating that the formulated gel is not of good quality in terms of this parameter. No phase separation was observed in the formulated gel following the centrifugation and mechanical vibration tests before and after stability testing, indicating stability and retained homogeneity of the formulated gel.

The formulated gel is slightly runny, immediately dries after spreading on the skin, has a chartreuse color, and has a smooth and somewhat heavy feel. The chartreuse color of the gel is due to the dark green color of the extract used.

Stability studies on pharmaceutical gels are done to determine if a formulation stored in a specific container is capable of retaining its physical, chemical, and microbiological properties, as well as evaluate the effect of the environmental factors on the formulation [24].

Topical treatments usually have an acidic pH, since an acidic environment improves the release of oxygen in wounded or affected tissues, hence aiding in the healing of the wounds [25]. The formulated gel had an acidic pH due to its main component being carbomer, which is an acid-based polymer [26]. Although the pH of the formulated gel is slightly lower than that of the skin which is 4.1 to 5.8 [27], it was not acidic enough to cause skin irritation, therefore safe to use [28].

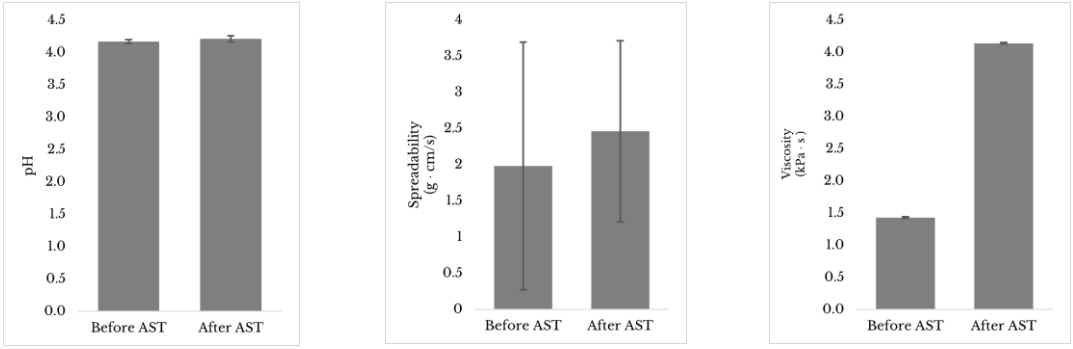


Figure 1. Results of the physicochemical evaluation. Data are expressed in terms of mean ± standard deviation.

Viscosity is important in evaluating a gel formulation because it affects the spreadability and release of the active ingredient. Spreadability aids the ability of the gel to be uniformly applied to the skin [16]. The spreadability of the formulated gel before stability testing was low therefore not ideal [29,30]. The low pH attained by the formulated gel caused a decrease in its viscosity [30]. The viscosity of a gel is highest at its gelling point [32], which in the case of the gelling agent used, carbomer, is 8 [16]. With this, it could be inferred that the low pH of the gel affected its viscosity. The decrease in viscosity then caused an increase in the gel's spreadability [16]. The formulated gel is therefore favorable for wound healing in terms of its pH [28]. However, the formulated gel still requires a lower viscosity and, consequently, a higher spreadability in order to improve in these parameters [16,29,30].

Antibacterial Evaluation. The One-way ANOVA test conducted on the results of the agar well diffusion assay showed that there is a significant difference between the formulated gel and the positive control. It is significantly different in favor of the positive control (refer to Figure 2).

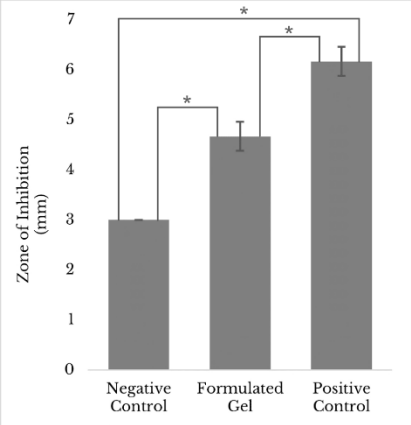


Figure 2. Results of the antibacterial evaluation. Data are expressed in terms of mean ± standard deviation.

A higher concentration of the extract may have achieved the same effectivity as the commercially available hand sanitizer which corresponds to the findings of Ruma and Zipagang [33], which states that higher concentration extract results in better effectivity in bacterial growth inhibition.

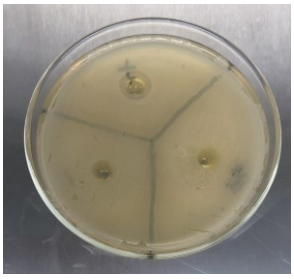
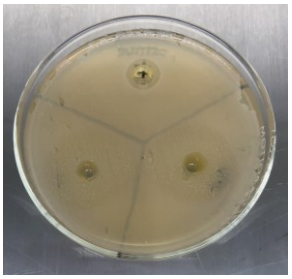
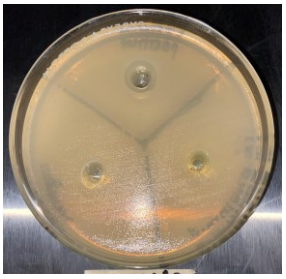
Although the formulated gel is effective as an antibacterial gel, it still has areas of improvement and the findings of this study may be different to future research. Therefore it is prescribed that more studies be conducted that would improve the formulated gel's antibacterial activity and physicochemical properties.

This indicates that the positive control is a more effective antibacterial formulation in comparison to the formulated gel. The commercially available hand sanitizer used for the positive control is alcohol-based therefore is more effective compared to the formulated gel which is water-based. This difference in formulation provides the commercially available hand sanitizer with more efficacy attributed to its alcohol content. The formulated gel is not as effective as the commercially available hand sanitizer because of the concentration of the crude ethanolic leaf extract of *S. jamaicensis*, which was based on the minimum inhibitory concentration (MIC) test of Idu et al. conducted in 2007 [4].

Limitations. The data gathering was conducted for two months. Within those two months, the period between the acquisition of the extract and gel formulation was a month. Hence, the quality of the extract may also have been compromised, particularly the antibacterial activity. Despite the setback, the findings of this research may help future studies in improving the formulation and discover the most effective concentration for antibacterial inhibition. Furthermore, the commercially available hand sanitizer used as a positive control has a different formulation as to that of the formulated gel, which may have affected its diffusion to the agar in the antibacterial evaluation.

Conclusion. - The formulated gel with *S. jamaicensis* crude ethanolic leaf extract has antibacterial activity against *S. aureus*. However, due to it having a significantly smaller zone of inhibition, it is not comparable to that of the commercially available sanitizer.

Table 1. Zones of inhibition generated on the MHA plates after incubation.

	Replicate No. 1	Replicate No. 2	Replicate No. 3
MHA Plates			

Recommendation. - To further improve the results of the study, it is recommended to perform the MIC test before proceeding with gel formulation since the literature where the MIC was based may be outdated. It is also recommended to use a higher concentration of *S. jamaicensis* extract or incorporate the extract of another plant that exhibits antibacterial properties to investigate synergistic effects to attain a higher antibacterial activity. Isolation of known phytochemicals associated with the antibacterial activity of *S. jamaicensis*, such as tannins and saponins [33], can be done to further improve its bactericidal effect. With this, it is recommended that the positive control would be the gel base incorporated with a known antibiotic with the same concentration as the extract. Furthermore, it is recommended to perform a microbial load count on the gel to determine its degree of microbial contamination. It is also recommended to use a paddle attachment in formulating the gel base in addition to the overhead stirrer to thoroughly mix the gel and reduce the formation of bubbles. Moreover, it is recommended to measure the physicochemical properties in regular intervals during AST to be able to plot a trend line that monitors the state of the gel throughout the stability testing. Lastly, it is recommended to perform the accelerated stability testing for a longer period to identify the limit of the gel and to determine its expiration date.

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Molecular docking of selected phytochemicals from malunggay (*Moringa oleifera*) against the chromosomal trehalose-6-phosphate phosphatase (PDB ID: 6CJ0) enzyme of *Pseudomonas aeruginosa*

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Abstract

The trehalose-6-phosphate phosphatase (TPP) enzyme, a common enzyme that is important for organism survival under stress, is utilized by some strains of *Pseudomonas aeruginosa*. Its presence in such pathogens but absence in hosts such as humans makes it a viable anti-pathogenic target. As for potential inhibitors, phytochemicals are known to possess medicinal properties and offer holistic drug action. Using molecular docking, the study screened selected phytochemicals from *Moringa oleifera* against the TPP enzyme of *P. aeruginosa*. The 13 initial phytochemicals were tested for drug-likeness using the Lipinski test, of which 11 passed and were used in the docking procedure done in AutoDock Vina. Analysis of the generated docks has shown that seven phytochemicals bind in close proximity to the active site, two bound elsewhere on the surface of the TPP enzyme, and one has both attributes. The docked phytochemicals were determined to act as either possible competitive or noncompetitive inhibitors.

Introduction. - The efficacy of antibiotics is at risk due to rapidly emerging resistant bacteria [1]. Antibiotic resistance in clinically relevant microorganisms such as *Pseudomonas aeruginosa* has been associated with an increase in hospitalization and mortality rates [2]. Antibiotic resistance is observed in microorganisms that develop the ability to survive medicine targeted against it [3]. Multidrug resistance patterns in both Gram-positive and Gram-negative bacteria are difficult to treat, if not untreatable. The increasing numbers of bacterial strains acquiring resistance to a wide range of antibiotics in recent decades is also alarming [4].

Trehalose is a disaccharide that plays an important role in the survival of some pathogens. Recent studies have highlighted its role in desiccation resistance, osmoprotection, and resistance to heat or cold [5,6,7]. One of the most studied pathways of trehalose synthesis in bacteria is the trehalose-6-phosphate phosphatase (TPP) pathway due to its conserved biosynthesis route [8]. The TPP enzyme is a member of the haloacid dehydrogenase (HAD) superfamily, a group of enzymes that facilitates the hydrolysis of a diverse range of organic phosphate substrates [9].

P. aeruginosa, a Gram-negative, opportunistic pathogen, is known to infect many organisms, including humans [10]. Surveys of genomic databases have shown that *P. aeruginosa* strains possess two different TPP coding genes which are chromosomal and extrachromosomal [11]. Trehalose can be utilized by *P. aeruginosa* as a carbon and energy source for its growth and survival [12]. An increasing number of occurrences of drug-resistant *P. aeruginosa* strains have been observed in recent years [12]; a solution to this problem must be urgently identified.

Studies have shown the potential of phytochemicals in antibiotic resistance research [13]. Phytochemicals are known to affect specific molecular targets both directly and indirectly through affecting metabolic pathways as stabilized conjugates [14]. They are also known to have a wide range of medicinal properties and offer holistic drug action against pathogens without having many side effects [15].

One important factor to consider in the invention of new drugs is the risk that such drugs would target additional or multiple receptors [16]. According to the study of Umesh et al. [13], the TPP enzyme could become a viable anti-pathogenic target due to its important role in pathogen stress tolerance while being completely absent in animal hosts. However, there has only been limited research done utilizing the chromosomal TPP enzyme of *P. aeruginosa* as an

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anti-pathogenic target.

In recent years, the incorporation of computer-based methods in medicinal chemistry has brought with it advantages in rational drug design [17]. Methods such as molecular docking are now available for the *in silico* study of biological systems and drug discovery [17].

The present study proposed to virtually screen the selected phytochemicals from *Moringa oleifera* against the chromosomal trehalose-6-phosphate phosphatase (TPP) enzyme of *P. aeruginosa*. Phytochemicals from *M. oleifera* were used as they were the most abundant types of phytochemical [18], and others have already been used in previous molecular docking studies [19]. Specifically, the study aimed to:

(i) evaluate the drug-likeness of the phytochemicals using the Lipinski rule through SwissADME;

(ii) identify the predicted binding sites of the selected phytochemicals to the TPP enzyme of *P. aeruginosa* through AutoDock Vina;

(iii) predict the interactions between the TPP enzyme and each phytochemical through LigPlot+; and

(iv) provide proof-of-concept for the mechanism of binding between each phytochemical and the TPP enzyme.

Methods. - A preliminary Lipinski test was conducted to test the drug-likeness of 13 selected phytochemicals from *M. oleifera*. The phytochemicals which passed the test were then subjected to molecular docking with the chromosomal TPP enzyme of *P. aeruginosa* using AutoDock Vina [20]. Data analysis on the predicted binding sites and interactions was done using LigPlot+ [21], PyMOL [22], and UCSF Chimera [23]. The phytochemicals' potential to be possible inhibitors of the TPP enzyme was contextualized using existing literature.

Selection of Phytochemicals. Thirteen phytochemicals involved in the studies of Lin et al. [18] and Zainab et al. [19] from *M. oleifera* were selected to be docked with the TPP enzyme. These phytochemicals and their respective PubChem Compound ID numbers are: (1) alpha-carotene (4369188), (2) anthraquinone (6780), (3) apigenin (439726), (4) excoecariatoxin (5281400), (5) flavylium (145858), (6) hemlock tannin (15559687), (7) isorhamnetin (5281654), (8) kaempferol (5280863), (9) laurifolin (102301875), (10) phenolic steroid (439726), (11) quercetin (5280343), (12) serpentine (73391), and (13) sitogluside (5742590). The three-dimensional structures of trehalose-6-phosphate (T6P)-substrate (positive control), carbon tetrachloride (negative control), and the phytochemicals in structured data format (SDF) were retrieved from the National Center for Biotechnology Information (NCBI) PubChem.

Lipinski Test for Drug-likeness. To evaluate and assess compounds during drug discovery and optimization, the Lipinski rule of five is used [24]. The phytochemicals were evaluated for their drug-likeness using the Lipinski rule by uploading their SDF files to SwissADME, a web-based application [25]. The phytochemicals that passed the rule with one or no violations were the only ligands to be tested with TPP.

Preparation of Molecular Models. The three-dimensional structure of TPP (PDB ID: 6CJ0) in PDB format was retrieved from Protein Data Bank (PDB). The natural ligands (CO_3 and Mg^{+2}) of the TPP enzyme were deleted using UCSF Chimera before the file was saved in PDB format. This file was then opened in AutoDock Tools to remove water molecules to avoid distortion in the search for possible binding sites [26]. Afterward, polar hydrogen atoms were added to establish the hydrogen bonds that may be involved in the binding of the protein and ligand. The whole macromolecule was enclosed by the grid box. The offset numbers and the number of points in the x, y, and z dimensions were noted down to define the search space for ligand binding in AutoDock Vina . The TPP was saved as a PDBQT file. Moreover, the SDF files of each phytochemical were converted in PDB format using UCSF Chimera. The substrate and each phytochemical were then opened in AutoDock Tools to detect its root to assign rotatable torsion angles of the ligand. After that, the controls and each phytochemical were saved as a PDBQT file.

Molecular Docking Proper. The config file was written in Python programming language. The input placed were the receptor (TPP enzyme) and the ligand (phytochemicals and controls). The filenames of the output of AutoDock Vina in PDBQT and TXT format were then stated. After, the offset values and grid box size were also stated. The exhaustiveness was then set to 24. The MS/DOS command prompt was opened to run AutoDock Vina. The directory was changed to the file path of the folder where the PDBQT and config files were saved. To dock the ligand and the receptor, the file path of the .exe file of AutoDock Vina was pasted on the command prompt. This was then followed by two dashes and the word 'config' and its TXT file extension.

Data Analysis. AutoDock Vina automatically generates the top nine conformations per ligand. Each conformation of the docked ligand and receptor was individually saved as a PDB file using PyMOL The PDB files of the conformations were analyzed using UCSF Chimera and LigPlot+. LigPlot+ was utilized to analyze the two-dimensional (2D) structure of the conformations and to generate schematic 2D diagrams of ligand-protein interactions. The amino acids involved in hydrogen bonding and hydrophobic interactions were noted down and verified using UCSF Chimera, which was also used to generate three-dimensional (3D) structures of the conformations.

Safety Procedure. Since the study was done *in silico*, the researchers took frequent breaks and practiced the 20-20-20 rule; every 20 minutes, watch an object 20 feet away for 20 seconds. This was done to ensure that the researcher's eyes were not strained from long exposure to digital screens during the data gathering procedure.

Results and Discussion. - The study aimed to virtually screen the selected phytochemicals from *M. oleifera* against the chromosomal trehalose-6-phosphate phosphatase (TPP) enzyme of *P. aeruginosa*. The 13 selected phytochemicals were subjected to the Lipinski rule. Those that passed were docked with the TPP enzyme using AutoDock Vina. Each generated conformation was then analyzed using UCSF Chimera and LigPlot+.

Lipinski Drug-likeness Test. According to the Lipinski rule, the requirements for a compound to be considered drug-like state that an orally active drug must not violate more than one of the following criteria: the molecular weight should not exceed 500 grams/mole, the MlogP [27] should not exceed 4.15, there must not be more than five hydrogen bond donors, and there must not be more than ten hydrogen bond acceptors [28]. The 13 phytochemicals were subjected to Lipinski drug-likeness test using SwissADME. Among the 13 phytochemicals, 11 passed the Lipinski drug-likeness test. These can be referred to in Table 1.

Table 1. Drug-likeness of selected *M. oleifera* phytochemicals based on Lipinski rule.

a	*b*	*c*	*d*	*e*	*f*
Alpha-carotene*	536.87	12.46	0	0	2
Anthraquinone	208.21	1.86	0	2	0
Apigenin	270.24	0.52	5	3	0
Excoecariatoxin	528.63	1.47	3	8	1
Flavylium	207.25	3.28	0	1	0
Hemlock tannin*	578.52	-0.26	10	12	3
Isorhamnetin	316.26	-0.31	4	7	0
Kaempferol	286.24	-0.03	4	6	0
Laurifolin	356.37	1.09	3	6	0
Phenolic steroid	256.38	4.46	1	1	1
Quercetin	302.24	-0.56	5	7	0
Serpentine	349.40	2.21	0	4	0
Sitogluside	576.85	3.96	4	6	1

a Phytochemical; *b* Molecular weight (g/mol); *c* MlogP; *d* No. of hydrogen bond donors; *e* No. of hydrogen bond acceptors; *f* No. of violations; *Phytochemicals that did not pass the Lipinski test.

Alpha-carotene and hemlock tannin did not meet two and three out of four criteria, respectively; they were not included in the list of phytochemicals that were used in the molecular docking process.

Analysis of Predicted Binding Sites and Interactions. After generating the top nine conformations in AutoDock Vina, the top two poses of each docked ligand (phytochemical or control) were selected to be analyzed further. For ligands which are bound restrictively to one chain, the two poses correspond to the top-ranked conformations for Chain A and Chain B, respectively. For ligands that interacted with both chains, the first and second-ranked conformations were selected.

The chromosomal TPP enzyme of *P. aeruginosa* is a member of the haloacid dehydrogenase (HAD) superfamily [11], which comprises enzymes such as phosphatases, ATPases, phosphomutases, phosphonatases, and dehalogenases [29]. TPP possesses a core phosphatase domain with α/β-hydrolase fold, which is common among the hydrolase family, as well as a cap domain [30]. While the fold of the core domain, which functions as base and side walls of the active site, is well conserved among the HAD superfamily, the cap domain, which functions as the cover, can vary in size and structure [30]. Through comparisons to other bacterial TPPs, the structure of the TPP enzyme from *P. aeruginosa* is also revealed to have four HAD conserved motifs located in the core domain [11].

The study found that all of the top predicted conformations of the docked phytochemicals interacted with amino acid residues located in the core domain. The top predicted conformations (for both chain A and B) of seven phytochemicals were in close proximity to the active site of the TPP enzyme; this is because they bound to one or more motifs within the core domain [11]. The exception is the top predicted conformation of sitogluside in chain A since it is bound near the β12 sheet. The other two phytochemicals (flavylium and serpentine) that did not bind near the active site were also bound to amino acid residue/s near the β12 sheet, the hydrophobic interface that links the two monomers.

The active site of an enzyme is defined as the region that binds the substrate (a ligand that becomes the starting material of an enzymatic reaction) and converts it into a product [31]. It is formed by amino acid residues; the properties and spatial arrangement of these determine which molecules can bind to and become substrates for the enzyme. The forces which bind the substrate are multiple weak forces such as hydrogen bonds, hydrophobic interactions, electrostatic interactions, and van der Waals bonds [31]. This study only observed the hydrogen and hydrophobic bonds that each phytochemical had with the TPP enzyme.

The phytochemicals anthraquinone, apigenin, isorhamnetin, kaempferol, laurifolin, phenolic steroid, and quercetin may be possible competitive inhibitors since these phytochemicals bind in close proximity to the active site of the TPP enzyme – all of these phytochemicals bound to one or more motifs – through steric hindrance. Steric hindrance prevents the further interaction of the natural substrate to the receptor when a competitive inhibitor is bound to the active site [32]. This effect of steric hindrance implies that if the phytochemicals bound to the active site of the TPP enzyme, T6P (the natural substrate) could not be catalyzed by the TPP enzyme into trehalose.

As for flavylium and serpentine, they could be considered as allosteric modulators or possible noncompetitive inhibitors due to their binding site being quite different from the active site. Allosteric

modulators bind elsewhere on the protein surface other than the active site and induce an allosteric conformational change of the active site of the receptor by shifting the free energy landscape [33,34] However, while noncompetitive inhibitors may or may not affect the structure of the protein, it is less certain what effect they may have on the binding affinity of the natural substrate since noncompetitive inhibitors do not compete with the substrate for active site binding [35].

As for sitogluside, its different conformation in each chain may suggest that it may possibly act as an allosteric modulator (in the case of Chain A) or a competitive inhibitor (in the case of Chain B). This is illustrated in Figures 1 and 2.

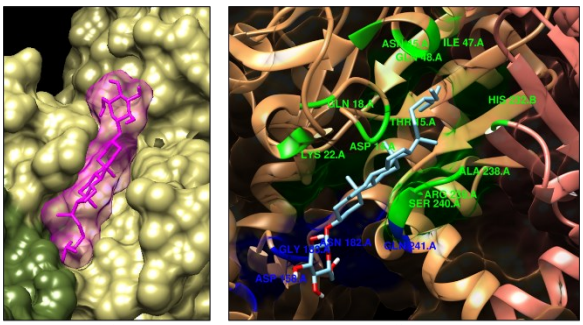

Figure 1. *(a)* Third-ranked predicted binding site of sitogluside in the TPP enzyme; *(b)* Top predicted binding site of sitogluside in chain A of the TPP enzyme with corresponding amino acid residues with *(green)* hydrogen bonds or *(blue)* hydrophobic bonds.

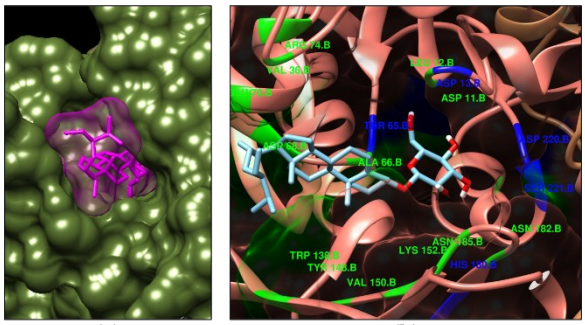

Figure 2. *(a)* Top-ranked predicted binding site of sitogluside in the TPP enzyme; *(b)* Top predicted binding site of sitogluside in chain B of the TPP enzyme with corresponding amino acid residues with *(green)* hydrogen bonds or *(blue)* hydrophobic bonds.

Inhibiting the T6P-substrate from binding to the active site would eventually lead to its accumulation. The intracellular accumulation of T6P is toxic to host organisms [36]. Previous studies determined that T6P accumulation can be lethal to *Caenorhabditis elegans* and *Mycobacterium tuberculosis* [36,37]. This adverse effect is due to the inhibition of metabolic enzymes such as phosphotransferases caused by sugar-phosphatases acting as antimetabolites [38]. The effects of the accumulation of T6P in *P. aeruginosa* are still unknown. However, the widespread toxicity of T6P and the presence of a glycolytic enzyme - known to be inhibited by T6P in other organisms - in *P. aeruginosa* all suggest that T6P accumulation may have adverse effects on the bacteria [11].

Antibacterial Properties of Phytochemicals. Several of the phytochemicals are already known to exhibit antibacterial properties against bacteria. Likewise, *M. oleifera* is also known to exhibit antibacterial properties against both Gram-positive and Gram-negative bacteria [39]. As for the phytochemicals, four of the seven possible competitive inhibitors are known to exhibit antibacterial activity against *P. aeruginosa* [40,41,42,43]. There is a lack of studies on the antibacterial activity of isorhamnetin, and the noncompetitive inhibitors flavylium and serpentine, against *P. aeruginosa*. Sitogluside exhibited low activity against *P. aeruginosa* in a study testing the antibacterial activity of daucosterol isolated from the roots of *Cissus populnea* [44].

Limitations. The study was not able to analyze the top rankings of excoecariatoxin since the generated conformation files could not be opened in LigPlot+ for further data analysis. This affected the third objective because the study was not able to predict the interactions between the TPP enzyme and the excoecariatoxin.

Conclusion. - The study concluded that after virtually screening the selected phytochemicals *of M. oleifera* against the chromosomal trehalose-6-phosphate phosphatase (TPP) enzyme of *P. aeruginosa*, all of the 11 docked phytochemicals that were analyzed are either possible competitive inhibitors or allosteric modulators of the enzyme.

Recommendations. - The researchers would recommend the utilization of software programs which could process the docked excoecariatoxin conformations. *In situ* analysis via nuclear magnetic resonance spectroscopy is also encouraged to examine the possible allosteric sites. Isothermal titration calorimetry to confirm the intended binding target of the TPP enzyme and each phytochemical is also recommended.

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