Research Article
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Life Sciences, Medicine and Biomedicine

Metabolic perturbations in methicillin-resistant *Staphylococcus aureus* induced by *Psidium guajava* ethanolic leaf extract

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Received: 4 May 2023; **Revised:** 6 September 2023; **Accepted:** 17 September 2023; **Published:** 18 December 2023
DOI https://doi.org/10.28916/lsmb.7.1.2023.118

ABSTRACT

Psidium guajava ethanolic leaf extract (PGele) showed good antibacterial activity against *Staphylococcus aureus* in various in vitro and in vivo studies. It was also reported to be efficacious, safe, and well-tolerated in a phase 1/11a clinical trial of the *Psidium guajava* ointment among volunteer patients with persistent *Staphylococcus aureus* nasal colonizers. However, its mechanism of antibacterial action is yet to be elucidated. Changes in bacterial metabolism can contribute to the susceptibility of the organism to antibiotics, thus screening for metabolic perturbations induced by PGele can give insight into its mechanisms of antibacterial action. Minimum inhibitory concentration (MIC) of PGele against methicillin-resistant *Staphylococcus aureus* (MRSA) was determined using the agar dilution technique. The sole carbon utilization phenotype microarray was used to describe the metabolic perturbations. *Psidium guajava* ethanolic leaf extract showed antibacterial activity against MRSA with MIC at 1,250 µg/mL. The phenotypic profile of MRSA showed utilization of 51 carbon sources, however, when MRSA was treated with the plant extract at sub-MIC (625 µg /mL), the utilization of carbon sources belonging to carbohydrate group (51.43%), amino acid group (100%) and the group of esters, carboxylic acids and fatty acids (75%) were inhibited. The sub-MIC of PGele induced metabolic perturbations on carbon sources associated with Embden-Meyerhof-Parnas pathway (EMP), pentose phosphate pathway (PPP), and citric acid cycle (TCA) pathways of MRSA. This current study gave an insight into the mechanism of antibacterial action of the *Psidium guajava* ethanolic leaf extract, a promising plant-derived antibiotic.

Keywords: *Carbon utilization phenome; metabolic perturbation; methicillin-resistant Staphylococcus aureus; plant-derived antibacterial and sole carbon phenotype microarray*

INTRODUCTION

Diseases caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are a global health problem. This pathogen is one of the primary causes of hospital-acquired infections and its shift to an increasing trend of community-acquired infections and antibiotic resistance further aggravates the problem. It is associated with significant morbidity, mortality, length of hospital stays, and cost burden on treatment and management. In the Philippines, multiple studies have shown the marked prevalence of MRSA especially in the hospital setting. Based on the 2021 Antimicrobial Resistance Surveillance Program (ARSP), a total of 1,644 isolates of

Staphylococcus aureus have been reported. The report showed that thirty-two percent (32%) of the MRSA isolates were from wound specimens and were characterized as presumptive community-acquired infections (73.18%). Resistance of *Staphylococcus aureus* infections to commonly used antibiotics such as co-trimoxazole, erythromycin, clindamycin, tetracycline, rifampicin, vancomycin, and ciprofloxacin at rates ranging from less than 10%-47.8% were noted (Department of Health, Research Institute for Tropical Medicine, 2022).

Plants have been utilized for health and medicine for thousands of years. Likewise, chemical compounds derived from plants account for a substantial percentage of the pharmaceutical market (David et al., 2015; Brown et al., 2014). The wide array of plant secondary metabolites as potential drug leads is not yet fully explored. In order to understand the perturbations in bacterial cell functions caused by these plant bio-actives that inhibit bacterial growth, it is also necessary to elucidate how these secondary metabolites affect the metabolic pathways of the bacteria.

One of the most popular herbal medicines for a variety of illnesses is *Psidium guajava*. This plant's antibacterial properties against a variety of pathogenic and non-pathogenic gram-positive and gram-negative microorganisms have been the subject of extensive research (Adwan et al., 2009; Arya et al., 2012; Díaz-de-Cerio et al., 2017; Gutiérrez et al., 2008; Metwally et al., 2010; Sanches et al., 2005; Ukwueze et al., 2015*).* Various secondary metabolites of the leaves have all been implicated in its biological activity (Biswas et al., 2013; Díaz-de-Cerio et al., 2017; Naseer et al., 2018; Patel et al., 2019; Preston-Mafham et al., 2002); Raj et al., 2020; Oncho et al., 2021). Five flavonoids, tannins, gallic acid, and catechin were linked to their antimicrobial activity against some bacteria and fungi (de Araújo et al., 2014; Mailoa et al., 2014; Metwally et al., 2010; Miller & Rhoden, 1991). Cieneol and triterpenes, which have been shown to inhibit the growth of *Aeromonas hydrophila, Shigella, Vibrio, Sarcina lutea, Mycobacterium phlei,* and *Staphylococcus aureus*, are additional potential compounds identified from this plant (Gonçalves et al., 2008). Locally, in vitro and in vivo studies have been conducted on the native *Psidium guajava* L. grown and cultivated at the National Integrated Research Program on Medicinal Plants (NIRPROMP) farm at the University of the Philippines, Los Banos, Laguna. The in vitro studies of the PGele showed a minimum inhibitory concentration (MIC) of <5 mg/mL against methicillin-sensitive *S. aureus*, methicillin-resistant *S. aureus, Klebsiella pneumoniae, Proteus vulgaris,* and *H. influenzae* (Cavinta et al., 2010). Furthermore, an in vivo study using a non-fatal subcutaneous model to determine the antibacterial activity of *Psidium guajava* L. ointment (PG ointment) on Methicillin-susceptible *Staphylococcus aureus* (MSSA) showed good antibacterial activity in the subcutaneous infection model (Maramba-Lazarte et al., 2016). Subsequently, a phase 1/11a clinical trial among volunteer patients with persistent *Staphylococcus aureus* nasal colonizers showed that PG ointment was efficacious, safe, and well-tolerated (Maramba-Lazarte et al., 2021). Therefore, elucidating the mechanism/s of the antibacterial action of PGele will contribute to the development of this plant as a potential antibacterial agent, specifically for diseases caused by *Staphylococcus aureus*.

METHODOLOGY

Chemicals and reagents

Bone Biolog™ Gen III MicroStation System (Biolog™ Inc., Hayward, CA, USA), 800TS Microplate Reader (BioTek Instruments, Winooski, Vermont, USA), and Genesys 180 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used in this study. The Gen III Microplate™ pre-loaded with 71 carbon sources and inoculating fluid (IF-C) were from Biolog™. Vancomycin, a USP-grade antimicrobial standard was bought from Sigma-Aldrich Pte. Ltd. Singapore. All other chemicals, including ethanol, PBS, nutrient agar, and Mueller-Hinton agar were of analytical grade.

Test plant

A local variety of *Psidium guajava* L. leaves belonging to the Myrtaceae family, authenticated by the Institute of Biology, University of the Philippines, Diliman, was collected at the National Integrated Research Program on Medicinal Plants (NIRPROMP) farm at the University of the Philippines, Los Banos, Laguna, Philippines from July – August 2022. Quality control of the powdered leaves included the testing for ash content (total ash, acid-insoluble ash and water-soluble ash), extractable matter (ethanol-soluble extractive and water-soluble extractive) and moisture content. To ensure the absence of contamination with heavy metals and aflatoxin, the leaf powder was also tested for mercury by atomic absorption spectrometry (AAS), while for lead, cadmium, and arsenic by using inductively coupled plasma – optical emission spectrometer (ICP-OES). Aflatoxin level was also determined by RIDASCREEN® Manual. Phytochemical testing as well as metabolite profiling were also conducted.

Plant extraction and stock solutions and controls

Psidium guajava ethanolic and hydroalcoholic extracts can yield the highest presence of phytoconstituents (Thenmozhi et al., 2017). On this basis, absolute ethanol was used in the plant extraction following a standard procedure as described in previous study (Manglicmot-Yabes, 2020b). For MIC assay, three (3) concentration levels of *Psidium guajava* ethanolic leaf extract (PGele) and antibiotic control (vancomycin) were prepared by

serial dilution. Sub-MIC at 625 µg/mL of the plant extract and vancomycin at 0.625 µg/mL were also prepared and were used in the sole carbon utilization phenotype microarray.

Inoculum preparation

Stock cultures of Methicillin-resistant *Staphylococcus aureus* ATCC 43300 were obtained from the Medical Microbiology Laboratory, Department of Medical Microbiology, College of Public Health, UP Manila. MRSA subculture was first grown in nutrient agar for 16 hours at 35-37°C. For the MIC determination, the inoculum was first standardized by growing the MRSA with a turbidity equivalent to 0.5 McFarland standard, while for the sole carbon utilization phenotype microarray assay, MRSA was grown to 90-95 % Transmittance (%T) following the manufacturer's standard protocol. Freshly prepared inoculum was used within thirty (30) minutes in all assays.

MIC Determination by agar dilution technique

Following the standard method of CLSI (Cockerill, 2012), bacterial inoculum of 1.5×10^8 CFU/mL was placed in agar plates containing the different concentrations of PGele (312.5 µg/mL, 625 µg/mL and 1,250 µg/mL) as well as that of the positive control (0.625 µg/ml, 1.25 µg/ml and 2.5 µg/ml). Control agar plates i.e. plant extract, vehicle and negative control were prepared similarly and included in the experiment. All agar plates were run in triplicates. The inoculated plates were incubated at 35±2°C for 16 to 20 hours. The presence or absence of the growth of MRSA was evaluated visually to determine the minimum inhibitory concentration.

Sole carbon tilization phenotype microarray

The Sole Carbon Utilization Phenotype Microarray Biolog™ Gen III microplate was used to establish the metabolic profile or phenotypic profile of MRSA by assessing its ability to metabolize the 71 carbon sources. These biochemicals significant in bacterial metabolism which include 42 carbon sources from sugars and sugar derivatives, 11 from amino acids, 25 from esters, 25 from carboxylic acids, and 42 from amino acids were preloaded in the Gen III microplate. Following the Biolog™ standard protocol for inoculum preparation, 100 uL of the MRSA cell suspension was dispensed to the microplate wells. The microplates were incubated at 35±2°C from 0 hour to 24 hours During incubation, there was increased respiration in the wells reflecting the utilization of the carbon source, indicated by a purple color due to the reduction of the tetrazolium redox dye. Absorbance was read at 600nm at 1.5-hour intervals (Bochner et al., 2001; Bochner, 2003; Bochner, 2008).

Statistical analysis

The OD readings were automatically imported to Microsoft Excel to generate the corrected ODs. The use of corrected OD (>0.100) as basis for setting the threshold for "positive for carbon source utilization" was adapted from previous studies (Garland, 1996; Manglicmot-Yabes et al., 2020a; Manglicmot-Yabes et al., 2020b; Miller, 1991; Preston-Mafham, 2002; Vahjen, et al., 1995). Thus, only the wells visually comparable to the positive control and with corrected ODs (> 0.100) were interpreted as positive for carbon source utilization.

RESULTS

The preliminary quality control of the *Psidium guajava* leaf powder was all within the acceptable limits set by the Philippine Food and Drug Authority as stipulated in AO 84 s 2004 to ensure the quality of traditionally used herbal products. The powdered leaf contains carbohydrates, reducing sugars, flavonoids, alkaloids, tannins (gallic and catecholic tannins), glycosides, saponins, and phytosterols as shown in the phytochemical testing. Metabolic profiling using TLC in five different solvent systems showed that the ethyl acetate:methanol (95:5) solvent system yielded 9 spots under 366nm, 5 spots under 254nm, and 4 spots under white light. Ethyl acetate:n-Hexane (80:20) solvent system resulted in 8 spots under 366nm, 7 spots under 254nm, and 5 spots under visible light. There were total of 26 spots viewed under different visual techniques for toluene:ethyl acetate (75:25) solvent system; 16 spots under 366nm, 5 spots under 254nm, and 5 spots under normal light. The n-hexane:acetone (70:30) solvent system yielded 14 spots under 366nm, 9 spots under 254nm, and 5 spots under visible light. Solvent system containing n-Hexane:Ethyl acetate (95:5) yielded the least number of spots with no detectable spots under visible light and 254nm, and spots under 366nm.

The MIC of PGele against MRSA was 1,250 µg/ml. The positive control gave an MIC of 1.25 µg/mL. The metabolic profile or "phenotypic fingerprint" of MRSA was established during their aerobic metabolism. Out of the 71 carbon sources essential to bacterial metabolism, which were preloaded in Biolog™ Gen III microplate, 51 (71.83%) were utilized by MRSA. The metabolic profile of MRSA indicated that eighty-three percent (83%) of the carbon sources belonging to carbohydrate group (35/42), 72.73% of the carbon sources belonging to amino acids (8/11) and 44% of carbon sources belonging to esters, carboxylic acids and fatty acids (8/18) were utilized (Table 1-3). However, when MRSA was treated with PGele at sub-MIC (625 µg/mL), the utilization of

62.74 % (32/51) carbon sources were inhibited. These carbon sources belong to carbohydrate group (51.43%), amino acid group (100%) and methyl esters, carboxylic acid and fatty acids group (75%).

Table 1

Carbon sources (carbohydrate group) utilized by MRSA and inhibition induced by Psidium guajava ethanolic extract at sub-MIC (625 µg/mL).

Available carbon sources (Carbohydrate group)	Metabolic profile	
	MRSA without treatment	PGele-treated MRSA
Dextrin	+	(-)
D-Maltose	+	+
D-Trehalose	+	+
D-Cellobiose	+	+
Gentiobiose	+	(-)
Sucrose	+	+
D-Turanose	+	+
Stachyose	(-)	n/a
D-Raffinose	+	(-)
α-D-Lactose	+	(-)
D-Melibiose	+	(-)
β-Methyl-D-Glucoside	+	+
D-Salicin	(-)	n/a
N-Acetyl-D-Glucosamine	+	+
N-Acetyl-β-D-Mannosamine	+	(-)
N-Acetyl-D-Galactosamine	+	(-)
N-Acetyl Neuraminic Acid	+	(-)
α-D-Glucose	+	+
D-Mannose	+	(-)
D-Fructose	+	+
D-Galactose	+	(-)
3-Methyl Glucose	+	(-)
D-Fucose	+	(-)
L-Fucose	+	(-)
L-Rhamnose	+	(-)
Inosine	+	+
D-Sorbitol	(-)	n/a
D-Mannitol	+	+
D-Arabitol	+	(-)
Myo-Inositol	+	(-)
Glycerol	+	+
D-Glucose-6-PO4	+	+
D-Fructose-6-PO4	+	+
Pectin	+	+
D-Galacturonic Acid	+	(-)
L-Galactonic Acid Lactone	+	(-)
D-Gluconic Acid	+	+
D-Glucuronic Acid	(-)	n/a
Glucuronamide	(-)	n/a
Mucic Acid	(-)	n/a
Quinic Acid	(-)	n/a
D-Saccharic Acid	+	+

Note: + carbon source utilized by MRSA; (-) carbon source not utilized by MRSA; n/a – not applicable

Table 2

Carbon sources (amino acid group) utilized by MRSA and inhibition induced by Psidium guajava ethanolic extract at sub-MIC (625 µg/mL)

Available carbon sources (Amino acid group)	Metabolic profile	
	MRSA without treatment	PGele-treated MRSA
D-Aspartic Acid	(-)	n/a
D-Serine	(-)	n/a
Gelatin	+	(-)
Glycyl-L-Proline	(-)	n/a
L-Alanine	+	(-)
L-Arginine	+	(-)
L-Aspartic Acid	+	(-)
L-Glutamic Acid	+	(-)
L-Histidine	+	(-)
L-Pyroglutamic Acid	+	(-)
L-Serine	+	(-)

Note: + carbon source utilized by MRSA; (-) carbon source not utilized by MRSA; n/a – not applicable

Table 3

Carbon sources (carboxylic acid, esters, fatty acids and other carbons) utilized by MRSA and inhibition induced by Psidium guajava ethanolic extract at sub-MIC (625 µg/mL)

Available carbon sources (Carboxylic acid, esters, fatty acids and other carbons)	Metabolic profile	
	MRSA without treatment	PGele-treated MRSA
p-Hydroxy-Phenylacetic Acid	(-)	n/a
Methyl Pyruvate	+	(-)
D-Lactic Acid Methyl Ester	+	(-)
L-Lactic Acid	+	+
Citric Acid	(-)	n/a
α-Keto-Glutaric Acid	+	(-)
D-Malic Acid	(-)	n/a
L-Malic Acid	(-)	n/a
Bromo-Succinic Acid	(-)	n/a
Tween 40	(-)	n/a
γ-Amino-Butyric Acid	(-)	n/a
α-Hydroxy-Butyric Acid	+	(-)
β-Hydroxy-D, L-Butyric Acid	(-)	n/a
α-Keto-Butyric Acid	(-)	n/a
Acetoacetic Acid	+	+
Propionic Acid	(-)	+
Acetic Acid	+	(-)
Formic Acid	+	(-)

Note: + carbon source utilized by MRSA; (-) carbon source not utilized by MRSA; n/a – not applicable

DISCUSSION

Psidium guajava, commonly known as “bayabas” in the Philippines, is a widely used herbal product for a number of diseases. The plant's antibacterial activity on both pathogenic and non-pathogenic gram-positive and gram-negative bacteria has been thoroughly investigated (Adwan et al., 2009; Arya et al., 2012; Cavinta et al., 2010; Díaz-de-Cerio et al., 2017; Gutiérrez et al., 2008; Metwally et al., 2010; Sanches et al., 2005; Ukwueze et al., 2015;). An in vivo non-fatal subcutaneous model using *Psidium guajava* L. ointment on MSSA reported a good antibacterial activity (Maramba-Lazarte et al., 2016). This PG ointment was also found to be efficacious, safe and well-tolerated in a previously reported phase 1/11a clinical trial among volunteer patients with persistent *Staphylococcus aureus* nasal colonizers (Maramba-Lazarte et al., 2021). This current study gave an insight on the

mechanism of antibacterial action of the *Psidium guajava* ethanolic leaf extract used in the *Psidium guajava* L. ointment, a promising antibiotic, which are currently undergoing clinical trials.

The metabolic profile of MRSA obtained using the BiologTM Gen III is consistent with the metabolic pathways present in *Staphylococcus aureus* namely Embden-Meyerhof-Parnas pathway (EMP), pentose phosphate pathway (PPP), and citric acid cycle (TCA) pathway (Carvalho et al., 2017). In this study, the utilization of carbon sources belonging to carbohydrates is consistent with the preference of *Staphylococcus aureus* to catabolize carbohydrates via glycolytic and pentose phosphate pathways (Strasters & Winkler, 1963; Vitko et al., 2016). The utilization of amino acids, when glucose is absent, is also consistent with the ability of *Staphylococcus aureus* to encode the pathway for amino acid catabolism, including that which produces pyruvate, 2-oxoglutarate, and oxaloacetate (Halsey et al., 2017). This catabolic pathway for amino acid is used by MRSA to rapidly adapt to a variety of carbon and nitrogen sources in order to survive during invasion of a host and in a staphylococcal abscess (Patel et al., 2019). The utilization of α-keto-glutaric acid, α-hydroxy-butyric acid, acetic acid and formic acid by MRSA is consistent with the citric acid cycle (TCA) pathway present in *Staphylococcus aureus* (Carvalho et al., 2017; Collins & Lascelles, 1962).

The obtained MIC (1,250 µg/ml) of *Psidium guajava* ethanolic leaf extract against MRSA, based on the suggested cut-off points for botanicals or plant extracts to be considered to have an inhibitory effect, is considered moderate (Aligiannis et al., 2001; Duarte et al., 2005; Fabry et al., 1998; Garland, 1996). In this study, *Psidium guajava* ethanolic leaf extract at sub-MIC (625 µg/mL) showed metabolic perturbations which caused the inhibition of the utilization of MRSA to use the essential carbon sources belonging to carbohydrate group (51.43%), amino acid group (100%) and the group of esters, carboxylic acids and fatty acids (75%). *Staphylococcus aureus*, which primarily catabolized carbohydrates for it to survive via the glycolytic and pentose phosphate pathways were shown to be affected by PGele. Likewise, although *Staphylococcus aureus* survives through the catabolism of multiple amino acids, PGele also inhibits the ability of MRSA to utilize amino acids.

Bacterial metabolic pathways are now thought to contribute to the pathogenic organisms' inherent virulence, making them potential targets for antibacterial drugs. Thus, this study showed that one of the putative mechanisms of the antibacterial action of *Psidium guajava* ethanolic leaf extract on *Staphylococcus aureus* ATCC 43300 could be due to its metabolic perturbations on the glycolytic and pentose phosphate pathways as well as on amino acid catabolic pathway. The mechanism of antibacterial action of PGele on MRSA is significant since it induced perturbation in the amino acid catabolic pathway of MRSA, the alternative pathway during host invasion. This could be attributed to its secondary metabolites that affected the glycolytic, pentose phosphate and amino acid catabolic pathways. This study showed that PGele is not only singly targeting the metabolic pathway of MRSA, thus may circumvent the problems encountered in target-based resistance.

CONCLUSION

The metabolic perturbations induced on MRSA gave insight into the mechanism of antibacterial action of *Psidium guajava* ethanolic leaf extract. It was also noted that even the ability of *Staphylococcus aureus* to encode an alternative pathway that can catabolize amino acids during host invasion in order to survive was shown to be inhibited by the bioactives present in *Psidium guajava* ethanolic extract. Hence, *Psidium guajava* ethanolic extract could be a promising antibacterial agent as a therapeutic alternative for diseases caused by MRSA.

AUTHOR CONTRIBUTIONS

Both authors, Ailyn Manglicmot Yabes and Monica Angelique Orejas Ramos conceptualized, wrote the protocol, collected and analyzed data, and wrote the final manuscript. Monica Angelique Orejas Ramos collected and processed the data. Ailyn Manglicmot Yabes reviewed the protocol, data analysis and manuscript, as well as in charge of the fund acquisition and research management. Both authors, Ailyn Manglicmot Yabes and Monica Angelique Orejas Ramos approved the submitted version.

ETHICS APPROVAL

The Institutional Biosafety and Biosecurity Committee (IBBC 2020-001) and the Research Ethics Review Board (2020-089-EX) reviewed and approved this research and subsequently, the study was registered with the University of the Philippines-Manila Research Grant and Administration Office (RGAO-2019-1126). A university-based BSL-2 facility was used for all the microbiological assays.

FUNDING

This study was funded by the University of the Philippines Technology Transfer and Business Development Office (UP TTBDO) with fund code TTBDO IPO 20210101000.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

ACKNOWLEDGEMENTS

The authors express gratitude to the Department of Pharmacology and Toxicology, College of Medicine, University of the Philippines-Manila for allowing the researchers to work on plant preparation and extraction and to the Department of Medical Microbiology, College of Public Health, University of the Philippines-Manila for providing the MRSA and for allowing the researchers to conduct the experiments in its BSL-2 facility. The authors also acknowledge the University of the Philippines Technology Transfer and Business Development Office (TTBDO) for the research grant of the study (TTBDO IPO 20210101000).

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Citation:
Manglicmot Yabes, A., & Orejas Ramos, M. A. (2023). Metabolic perturbations in methicillin-resistant Staphylococcus aureus induced by Psidium guajava ethanolic leaf extract. *Life Sciences, Medicine and Biomedicine*, *7*(1).
https://doi.org/10.28916/lsmb.7.1.2023.118

Life Sciences, Medicine and Biomedicine
ISSN: 2600-7207