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Antibacterial Potential of Ethanol Extracts from *Psidium guajava* and *Persea Americana* Leaves Against MRSA

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ABSTRACT

The emergence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains has motivated this research to explore alternative treatment options. This study focuses on evaluating the combined antibacterial activity of ethanol extracts from *Persea americana* and *Psidium guajava* leaves against MRSA isolates. The plant materials were powdered and extracted by cold maceration method using 80% v/v ethanol. Phytochemical analysis followed standard protocols. The antibacterial properties of the individual extracts, as well as their combined effect, were evaluated using the agar diffusion method. The ethanol extracts of *P. americana* and *P. guajava* were tested separately and their combined antibacterial activity was evaluated against MRSA isolates based on the inhibition zones measured using the agar cup diffusion technique. The results showed diverse inhibition zones and minimum inhibitory concentrations (MIC) for the ethanol extracts of both plants. *P. americana* extracts showed inhibition zones between 4 mm and 12 mm, with a MIC ranging from 50 mg/ml to 200 mg/ml. *P. guajava* extracts showed inhibition zones ranging from 4 mm to 12 mm, with a MIC ranging from 12.5 mg/ml to 200 mg/ml. The combined ethanol extracts of both plants showed a synergistic antibacterial effect with inhibition zones ranging from 20 millimeters to 30 millimeters. In conclusion, the combined extracts exhibited a potent antibacterial activity, indicating their potential as effective candidates to combat MRSA and help prevent drug resistance.

Keywords: MRSA, *Persea americana*, Antibacterial, *Psidium guajava*, Synergistic

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Introduction

Persea americana and *Psidium guajava* are recognized as rich sources of medicinal compounds. The rising global issue of infectious agents developing resistance to conventional synthetic antibiotics has intensified the search for new antibiotic sources across various institutions [1]. Each plant in nature offers unique medicinal properties. *P. guajava*, known as guava, is a tropical plant primarily cultivated for its fruit. It is classified under the family Myrtaceae and the class Magnoliopsida. Virtually all parts of the guava plant are utilized for therapeutic purposes [2].

The leaves of *P. guajava* are opposite, oblong, and measure three to seven inches (7.6-18 centimeters) in length, with serrated edges and noticeable veins on the underside. Guava trees thrive in warm subtropical to tropical climates, with optimal temperature ranges between 73 °F and 83 °F for growth and fruit production [3].

The bioactive compounds in *P. guajava* are believed to play a significant role in managing various health conditions. The aqueous extracts from the root bark and leaves are known to help treat gastrointestinal ulcers, diarrhea, and toothaches [4]. Additionally, the root extract has been found beneficial for treating indigestion, stomach pain, and constipation, and as an antitussive [5]. Furthermore, guava leaf extracts have been shown to assist in treating respiratory issues and alleviating asthma symptoms [6].

The bioactive components of *P. guajava* extracts, particularly from the roots and leaves, have been studied to assess their medicinal properties [7]. Phytochemical analysis indicated that both aqueous and ethanol extracts from the leaves of *P. guajava* contain various bioactive compounds, including saponins, tannins, alkaloids, sterols, cardiac glycosides, terpenes, and flavonoids. Among these, saponins, tannins, and alkaloids were found in higher concentrations, while terpenes, cardiac glycosides, flavonoids, and sterols were present in lower amounts [8]. According to some studies, the antibacterial effects of guava leaves are attributed to the presence of flavonoids [9].

P. americana, commonly referred to as "avocado pear," belongs to the Lauraceae family. It is predominantly grown in tropical and subtropical regions around the world, mainly for its edible fruit and for its significant therapeutic and medicinal applications [10]. Aqueous extracts from the leaves, fruit, and bark of avocados have demonstrated effectiveness in treating conditions such as inflammation, hypertension, and bacterial infections [11-13].

Staphylococcus aureus is a gram-positive, non-motile bacterium typically found in the nasal mucosa of humans, where it coexists in a commensal relationship [14]. However, when the skin or mucosal barrier is compromised, as seen in soft tissue infections, *S. aureus* can enter the bloodstream, leading to potential infections. Individuals with weakened immune systems are particularly susceptible to such infections [15].

The discovery of penicillin by Alexander Fleming was considered a monumental step in combating infections caused by Staphylococcus aureus [16]. However, the pathogen's ability to survive led to the emergence of strains resistant to penicillin, prompting the development of newer beta-lactam antibiotics, such as methicillin, oxacillin, flucloxacillin, and dicloxacillin [17]. By the early 1960s, Methicillin-resistant *Staphylococcus aureus* (MRSA) was identified [18]. This strain has become one of the most significant challenges in both healthcare and community settings, often leading to prolonged infections, higher healthcare costs, extended hospital stays, and increased mortality rates [19].

The resistance of MRSA to the anti-staphylococcal penicillin subclass of beta-lactam antibiotics, along with the rising resistance to available synthetic antibacterial agents, raises concerns for the future treatment of this pathogen [20]. As a result, there has been growing interest in plants as potential sources of novel therapeutic compounds. Plants are recognized as a reliable source of bioactive compounds that can help combat the global spread of infections. Moreover, these plant-based compounds can be modified to reduce toxicity. Therefore, this study aims to assess the combined antibacterial effect of ethanol extracts from *P. guajava* leaves and *P. americana* leaves on MRSA.

Materials and Methods

Collecting and identifying plant materials

The leaves of *P. guajava* and *P. americana* were gathered from Umuno in Abraka, Delta State, Nigeria, in December 2022. Doctor Emmanuel Ikpefan, a botanist from the Department of Pharmacognosy and Traditional Medicine at the Faculty of Pharmacy, Delta State University, Abraka, Nigeria, identified and authenticated the plants. A specimen was preserved at the herbarium in the Department of Pharmacognosy and Traditional Medicine, Delta State University, Abraka, Nigeria. The collected plant material was air-dried, ground into powder, and stored at room temperature for further use.

Extracting plant materials

A total of 420 grams (g) of powdered *P. americana* leaves and 280 g of powdered *P. guajava* leaves were collected. The 420 g of *P. americana* powder was divided into 3 equal portions, while the 280 g of *P. guajava* powder was split into 2 portions, each weighing 140 g. Each portion was macerated separately with eighty percent

ethanol, with 560 milliliters of ethanol added to each 140 g of plant material. The mixtures were left to soak in airtight containers for 72 hours. Afterward, the extracts from the 3 portions of *P. guajava* and *P. americana* were filtered through muslin cloth. The filtrates were collected in separate beakers and concentrated to dryness in a water bath. The resulting brown residue was then reconstituted with distilled water to achieve a final concentration of 100 milligrams per milliliter, and the solution was stored in a refrigerator at 4 °C until needed for the experiment.

Phytochemical screening

The presence of secondary metabolites, including terpenes, flavonoids, alkaloids, tannins, and saponins, was examined using standard methods outlined by Evans and Trease [21].

Cell cultures

Thirty stock cultures of Staphylococcus aureus, previously isolated, were stored on Nutrient agar slants at room temperature in the Pharmaceutical Microbiology laboratory at Delta State University, Abraka, for future use in this research. To confirm their identity, the stock cultures were subcultured on Mannitol Salt agar and incubated for 24 hours at 37 °C. After sub-culturing on Mannitol Salt agar, the plates were again incubated at 37 °C for 24 hours. Colonies that exhibited a yellow color were identified as Staphylococcus aureus and selected for the Methicillin-resistance test. These isolates were transferred to fresh nutrient agar plates, incubated overnight, and then stored at 4 °C for further analysis.

Test for methicillin resistance

The agar diffusion method was employed to assess Methicillin resistance in 30 isolates of *Staphylococcus aureus* [22]. This study utilized flucloxacillin capsules (500 milligrams) manufactured by Ernest Chemists Limited, Accra, Ghana. A single pure colony from each *Staphylococcus aureus* isolate was collected using a wire loop and inoculated into a sterilized nutrient broth, which was then covered with aluminum foil and incubated overnight. The turbidity of each culture was adjusted to align with McFarland turbidity standards.

Following this, thirty pre-solidified and accurately labeled Mueller-Hinton Agar plates were inoculated with the prepared broth cultures. After disposing of the remaining nutrient broths into a disinfection jar, the plates were allowed to dry before proceeding. Using a sterile surgical blade, antibiotic discs were aseptically placed in duplicate onto each dried inoculated plate. The plates were then incubated at 37 °C for 24 hours.

After incubation, the inhibition zones surrounding each antibiotic disc were observed. These zones were measured in diameter using a meter rule and recorded appropriately. The mean inhibition zone diameter was calculated and rounded to the nearest whole millimeter. Based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (2022), each isolate was classified as either MRSA or not.

Antimicrobial testing

Measurement of the inhibition zones for the ethanol extracts of *P. americana* and *P. guajava* Leaves.

Sensitivity: agar well diffusion method

The ethanol extracts of *P. americana* leaves were assessed for their antimicrobial activity against MRSA. Mueller Hinton agar was prepared following the manufacturer's guidelines and sterilized in an autoclave at 121 °C for 15 minutes. After cooling, 20 milliliters of the medium were poured into each of the 15 Petri dishes, which were then left to solidify. Each dish was labeled according to the corresponding MRSA strain previously identified. Labels were also assigned to indicate the varying concentrations of the ethanol plant extract (200, 100, 50, 25, 12.5, and 6.25 milligrams per milliliter), while ciprofloxacin served as the positive control.

Aseptic techniques were used to swab the test organisms onto the agar plates. A sterile cork borer (6 millimeters) was employed to create duplicate wells in each agar plate. Using a Pasteur pipette, 2 drops of each ethanol extract concentration from *P. americana* leaves were introduced into their respective wells. Ciprofloxacin, the positive control, was dispensed into a central well using a 2-milliliter syringe.

The ethanol extract of *P. guajava* leaves underwent evaluation following the same methodology. The inoculated plates were incubated at 37 °C for 24 hours. Post-incubation, inhibition zones were observed under a hand lens for enhanced visibility. A metric ruler was used to measure the diameter of each inhibition zone from edge to

edge, recorded to the nearest whole millimeter. Each test was conducted in triplicate, and the mean inhibition zone diameter (IZD) was documented to the nearest whole millimeter.

Determining MIC of plant extracts

The MIC was determined using the agar dilution technique following CLSI (2022) guidelines. Mueller Hinton agar was prepared following the manufacturer's instructions. A total of 19 milliliters of molten nutrient agar was combined with 1 milliliter of the ethanol extract dilution of *P. americana* and thoroughly mixed before being poured into sterile Petri dishes to solidify. Each dish contained varying concentrations of the ethanol extract dilution (200, 100, 50, 25, 12.5, and 6.5 milligrams per milliliter). The agar plates were segmented into 15 sections and labeled according to the specific MRSA test strain. To ensure sterility, the plates were incubated overnight before use.

A sterile wire loop was used to streak an overnight broth culture of each test organism onto the section of the agar plate designated for the highest concentration (200 milligrams per milliliter). The procedure was repeated for the remaining concentrations. Additionally, a control plate containing nutrient agar without extract was inoculated to serve as a negative control. The plates were incubated at 37 °C for 24 hours, after which they were examined for visible bacterial growth. The lowest concentration that successfully inhibited bacterial growth was recorded as the minimum inhibitory concentration.

The same protocol was applied to determine the MIC for the ethanol extract dilution of *P. guajava* leaves at the same concentration levels (200, 100, 50, 25, 12.5, and 6.5 milligrams per milliliter). The minimum concentration at which bacterial growth was inhibited was identified as the MIC.

Determining the combined zone of inhibitions of ethanol extract of P. Americana and P. guajava leaves

Agar plates containing subcultured colonies of Methicillin-Resistant *Staphylococcus aureus* strains labeled MRSA 1, MRSA 4, MRSA 6, MRSA 7, and MRSA 10 were chosen for overnight broth preparation.

Mueller Hinton agar was prepared following the manufacturer's guidelines and sterilized in an autoclave at 121 °C for 15 minutes. After cooling, 20 milliliters of the medium was dispensed into each Petri dish and left to solidify. The surface of the agar plates was inoculated by swabbing with a standardized overnight nutrient broth culture of MRSA 1 using a sterile swab stick. Each Petri dish was labeled accordingly to correspond with the test organisms.

A 6-millimeter cork borer was used to create two wells in each agar plate, positioned approximately 3 millimeters apart. One milliliter of each plant extract at the lowest minimum inhibitory concentration was dispensed into the designated well using a Pasteur pipette for the respective test organism. The same procedure was repeated for the other test strains (MRSA 4, MRSA 6, MRSA 7, and MRSA 10) with both plant extracts.

All plates were incubated at 37 °C for 24 hours. The combined antibacterial activity was assessed in duplicate. Following incubation, inhibition zones were carefully examined under a hand lens for better magnification. A metric ruler was used to measure the inhibition zones from one edge to the other, both horizontally and vertically, and the values were averaged. The final combined inhibition zone diameter (IZD) was recorded in millimeters.

Results and Discussion

The combination of these plant extracts may offer a more effective approach to managing complex infections compared to using individual extracts. This strategy could help mitigate drug resistance, lower treatment costs, enhance therapeutic efficacy, and reduce toxicity. However, the process might be labor-intensive, and further clinical trials may be necessary to confirm both safety and effectiveness.

Previous studies have documented the antimicrobial properties of *P. americana* [13]. Additionally, research has demonstrated that *P. guajava* exhibits promising medicinal potential in combating resistant bacterial strains such as MRSA [23].

This study focused on evaluating the combined antibacterial activity of ethanol extracts from *P. guajava* and *P. americana* leaves against Methicillin-resistant *Staphylococcus aureus*.

The preparative phytochemical composition of the ethanol extracts from *P. guajava* and *P. americana* leaves is detailed in **Tables 1 and 2**, respectively. **Table 1** shows that the ethanol extract of *P. guajava* leaves had a high concentration of alkaloids, saponins, and tannins, while terpenes, flavonoids, and cardiac glycosides were present in moderate amounts. **Table 2** reveals that the ethanol extract of *P. americana* leaves contained alkaloids in high

concentration, whereas saponins, terpenes, flavonoids, and tannins were moderately abundant. Tannins have been previously identified as a key factor in the antibacterial effects against *Staphylococcus aureus* [24].

Table 1. Phytochemical constituents of *P. guajava* leaves.

SN	Test Plant Compound	Result
1	Alkaloid	+++
2	Terpenes	++
3	Flavonoids	++
4	Saponins	+++
5	Tannins	+++
6	Cardiac glycosides	++

++: moderate concentration
+++: high concentration

Table 2. Phytochemical constituents of *P. americana* leaves.

SN	Test Plant Compound	Result
1	Alkaloid	++
2	Terpenes	++
3	Flavonoids	+++
4	Saponins	++
5	Tannins	++

++: moderate concentration
+++: high concentration

The findings of this study demonstrated that the combined antibacterial activity of ethanol extracts from *P. guajava* and *P. americana* exhibited a synergistic effect against Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. The antibacterial efficacy of the ethanol extract of *P. guajava* leaves was assessed by comparing the inhibition zones of each MRSA isolate with those of the standard antibiotic control, ciprofloxacin, using the agar well diffusion method.

A total of ten clinical MRSA isolates underwent testing through the agar well diffusion assay. The results of the MRSA screening using the ethanol extract of *P. americana* leaves are summarized in **Table 3**, while the screening outcomes for *P. guajava* leaf extract against MRSA isolates are presented in **Table 4**. Additionally, the MIC was determined, revealing variations in the sensitivity of the MRSA isolates to different extract concentrations.

Table 3. Sensitivity of MRSA isolates to ethanol extract of *P. americana* leaves.

	MRSA isolates diameters of the inhibitory zones (in mm)									
Extract concentration in mg/ml	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
200 mg/ml	11	13	10.5	7.5	7	8.5	8.5	8.5	8.5	10.5
100 mg/ml	9	11	9	7	6	8	7	7	7	10
50 mg/ml	8	8	8	6	6	7	6	6	7	9
25 mg/ml	6	7	7	5	5	6	6	6	6	9
12.5 mg/ml	6	5	5	5	5	6	5	5	5	7
6.25 mg/ml	5	5	5	4	4	5	5	5	4	7

Table 4. Sensitivity of MRSA isolates to ethanol extract of *P. guajava* leaves.

	MRSA isolates diameters of the inhibitory zones (in mm)									
Extract concentration in mg/ml	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
200 mg/ml	12	11	11	11	10	10	9	10	11	11
100 mg/ml	9	9	8	8	8	7	7	8	8	7
50 mg/ml	8	7	7	6	7	6	6	7	6	6

25 mg/ml	7	6	7	6	6	5	6	5	5	6
12.5 mg/ml	6	5	5	6	6	6	5	6	5	5
6.25 mg/ml	5	4	4	5	5	6	5	5	5	4

The ethanol extract of *P. americana* leaves exhibited antibacterial activity against all ten tested MRSA isolates, with mean inhibition zone diameters ranging from 4 mm to 13 mm. Similarly, the ethanol extract of *P. guajava* leaves was effective against the ten MRSA isolates, displaying inhibition zones between 4 mm and 12 mm. The presence of clear zones surrounding the wells after incubation indicated the extent of antibacterial activity exerted by each concentration of the plant extracts against the MRSA isolates. In contrast, wells with a turbid appearance and no distinct inhibition zones suggested either resistance of the isolates or a lack of inhibitory effect by the extracts.

The MIC results, presented in **Table 5**, revealed that MRSA isolates were inhibited by the ethanol extract of *P. americana* at concentrations ranging from 50 mg/ml to 200 mg/ml, whereas the ethanol extract of *P. guajava* exhibited inhibitory effects on nine isolates at concentrations between 12.5 mg/ml and two hundred mg/ml. The MIC findings for *P. guajava* extract support previously documented antibacterial activity against MRSA, as reported in earlier studies [25]. Similarly, the MIC results for *P. americana* extract validate its antibacterial properties against MRSA, following prior research findings [13].

Table 5. Minimum inhibitory concentration for the MRSA isolates

MRSA isolates	**Ethanol extract from *P. americana* leaves**	**Ethanol extract from *P. guajava* leaves**
	MIC (mg/ml)	**MIC(mg/ml)**
1	50	25
2	50	12.5
3	50	25
4	50	25
5	100	12.5
6	50	25
7	100	12.5
8	100	12.5
9	50	200
10	200	-

The synergistic antibacterial activity of the ethanol extracts from *P. guajava* and *P. americana* leaves was assessed by analyzing the combined inhibition zone diameter for each MRSA isolate about the lowest MIC observed when both extracts were used together. Additionally, the combined inhibition zone diameter was compared to the inhibition zone of Ciprofloxacin, the standard antibiotic control, utilizing the agar well diffusion method. Five clinical MRSA isolates underwent testing through this method, and the results of the extract screening against these isolates are detailed in **Table 6**.

Table 6. Combined zone of inhibitions of selected MRSA isolates with minimum inhibitory concentration for both plant extracts.

MRSA isolates diameters of the combined inhibitory zones (in mm)					
Combined MIC on both extracts	**1**	**4**	**6**	**7**	**8**
50 mg/ml + 25 mg/ml	23				
50 mg/ml +25 mg/ml		22.5			
50 mg/ml + 25 mg/ml			20		
100 mg/ml +12.5 mg/ml				20.75	
100 mg/ml + 12.5 mg/ml					29

MRSA isolates 1, with the lowest MIC values of 50 mg/ml for *P. americana* and 25 mg/ml for *P. guajava*, exhibited a mean combined inhibition zone diameter of 23 mm. For MRSA isolate 4, the lowest MIC values of 50 mg/ml for *P. americana* and 25 mg/ml for *P. guajava* resulted in a combined inhibition zone of 22.25 millimeters. MRSA isolates 6, with the same MIC values, showed a combined inhibition zone of twenty mm. MRSA isolates 7, with MIC values of 100 mg/ml for *P. americana* and 12.5 milligrams per milliliter for *P. guajava*, produced a combined zone of 20.75 mm, while MRSA isolates 8, with 100 mg/ml for *P. americana* and 12.5 milligrams per milliliter for *P. guajava*, had a combined inhibition zone of 29.5 millimeters. The overall combined inhibition zone diameters ranged from twenty to thirty mm, indicating a synergistic antibacterial effect of both plant extracts against MRSA.

When tested separately, MRSA 1 showed an inhibition zone of 8 mm with *P. americana* (MIC 50 mg/ml) and 7 mm with *P. guajava* (MIC 25 mg/ml). However, when both extracts were combined at their respective MICs, the zone of inhibition increased to 23 mm, highlighting the synergistic interaction between the two extracts. This trend of enhanced antibacterial activity was observed across all isolates. Additionally, **Figure 1** illustrates the significant improvement in antibacterial effectiveness when the combined extracts were used, in comparison to the individual extracts and the standard antibiotic.

The promising synergistic effect of *P. americana* and *P. guajava* leaf extracts provides a valuable alternative for combating multidrug-resistant organisms. This study is the first to report on the combined effects of these plant extracts against MRSA. This discovery could have significant implications in healthcare, offering a potential alternative to conventional treatments for MRSA infections. Moreover, it may be useful in cases of empirical treatment where the specific infection is unknown. Herbal medicine, an age-old practice, continues to play a crucial role in the treatment and prevention of diseases, especially in regions with diverse plant life.

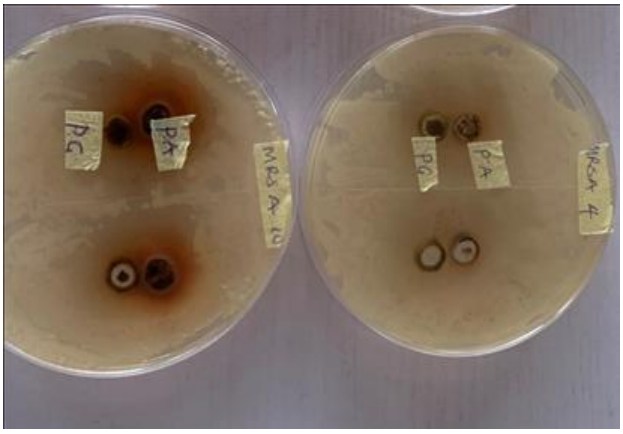

Figure 1. Combined zones of inhibition of ethanolic extract of *P. americana* and *P. guajava* leaves.

Conclusion

The findings of this study demonstrated that the combined antibacterial properties of the two plant extracts exhibited notable antibacterial effects both individually and even more so when used together, showing a significant synergistic action against MRSA. Based on these results, the combination of these extracts could serve as an effective alternative treatment for MRSA strains that have become resistant to conventional antibiotics. In addition to enhancing antibacterial efficacy, this combined approach may also help minimize drug toxicity and reduce the likelihood of further resistance development.

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