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Combined Antibacterial Activity of Ethanol Extracts of *Psidium guajava* and *Persea americana* Leaves on MRSA

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ABSTRACT

Methicillin Resistance Staphylococcus aureus strains have prompted this study to be able to opt in for an alternative. This study aims to evaluate the combined antibacterial effect of ethanol extracts of *Persea americana* and *Psidium guajava* leaves against Methicillin *Resistance Staphylococcus* aureus isolates. The powdered plant material was extracted using a cold maceration process using 80% v/v ethanol. Standard operating protocols were employed to analyze the phytochemical findings. The antibacterial sensitivity of the extracts was tested using the agar diffusion method. Ethanol extracts of *Persea americana* and *Psidium guajava* were subjected to an antibacterial test separately and combined antibacterial effect against isolates of Methicillin Resistant *Staphylococcus aureus*, (MRSA), by determining their zones of inhibition using the agar cup diffusion method. Results show varying zones of inhibition and minimum inhibitory concentration, MIC for Ethanol extracts for *Persea americana* and *Psidium guajava* leaves when tested against isolates of MRSA. Zones of inhibition ranging from 4-12 mm and MIC ranged from 50 mg/ml to 200 mg/ml, while that of ethanol extract of *Psidium guajava* zones of inhibition ranged from 4 mm to 12 mm, and minimum inhibitory concentration, MIC ranged from 12.5 mg/ml to 200 mg/ml. The combined antibacterial effect of the ethanol extracts of *Persea americana* and *Psidium guajava* leaves revealed a synergistic effect with the zones of inhibition ranging from 20-30 mm. In conclusion, the combined antibacterial effect of both plants showed a synergistic antibacterial effect with very high antibacterial activity and this could be a good candidate to combat MRSA and also prevent drug resistance.

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

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INTRODUCTION

Psidium guajava and *Persea americana* are known to be an excellent source of drugs. The incumbent global challenges of the increase in resistance of infectious agents to the already known synthetic drugs have paved the way for the search for newer sources of antibiotics in all institutions [1]. Every particular plant in nature has medicinal value responsible for its uniqueness. The plant *P. guajava*, commonly known as guava is a tropical plant widely grown for fruit. It belongs to the family; Myrtaceae and Class; Magnoliopsida. All parts of *P. guajava* are used for therapeutic purposes [2].

The leaves of *P. guajava* are opposite, oblong, 3 to 7 inches (7.6-18 cm) in length, with serrated margins having prominent veins on the lower side. All Guava trees are known to be well adapted to warm subtropical to tropical climatic conditions. The temperature range for its growth

and production ranges from 73 F to 83 F [3].

Active ingredients in P. guajava are thought to aid in the treatment and management of several illnesses. The aqueous extract of the root bark and leaves is effective in the management of gastrointestinal ulceration, diarrhea, and toothache among others [4]. The aqueous extract of the root has also been found to be effective in indigestion, stomach ache, constipation, and antitussives [5]. In addition, the extract of the *P. guajava* leaves has also been reported to be effective in the treatment of pulmonary diseases and in relieving episodes of asthma attacks [6].

The *P. guajava* extracts have been examined to determine whether the leaves and roots contain any bioactive elements [7]. The phytochemical analysis has given evidence that the aqueous and ethanol leaf extract of *P. guajava* contained different classes of bioactive constituents such as saponins, alkaloids, tannins, sterols cardiac-glycosides, terpenes, and flavonoids. The results showed that saponins, tannins, and alkaloids were present in high concentrations, while terpenes, cardiac glycosides, flavonoids, and sterols were present in small concentrations [8]. According to a report, flavonoids extracted from guava leaves are believed to be responsible for antibacterial activity [9].

Persea americana also known as 'avocado pear' belongs to the family 'Lauraceae'. To a large extent, they are cultivated amidst the tropics, including the subtropics of the world majorly for edible fruits and most importantly for their therapeutic and prescient uses [10]. The aqueous extracts of the leaves, fruits, and course the bark of the avocados have been effective as an anti-inflammatory, hypertension, and antibacterial [11-13].

Staphylococcus aureus, a Gram-positive bacterium, non-motile, is found in the nasal mucosa of humans in commensalism relationships [14]. The disruption in the cutaneous and the mucosa layer, as found in soft tissue infection could allow the penetration of the *Staphylococcus aureus* into the bloodstream to cause infection. Patients with compromised immune systems are more vulnerable [15].

Penicillin discovery by Alexander Flemming was seen as a huge effort to wage war against infection caused by *Staphylococcus aureus* [16]. However, due to the survival ability of this pathogen that paved the way for the emergence of strains that are resistant to penicillins, some exertions lead to the development of newer Beta-lactam (the anti-staphylococcus class), which include the Methicillin, flucloxacillin, Oxacillin, and dicloxacillin [17]. In the early 1960s, the emergence of the MRSA was documented [18]. The strain Methicillin S*taphylococcus aureus* (MRSA), has posed the greatest challenge in the healthcare system and community settings. This in a few cases leads to prolonged infection, an increase in direct and indirect costs, an increase in the number of stays in the hospital, and many cases increase in mortality [19].

The resistance of MRSA to the anti-staphylococcal penicillin subclass of the beta-lactam class of antibiotics and the emergence of accumulating resistance to obtainable synthetic antibacterial antibiotics have implications for current and future treatment options for this particular pathogen [20]. This has called for the investigation into plants as sources for novel drug compounds. Plants as known provide a very reliable source of lead compounds which are effective in mitigating the spread of infection globally. These plants can also be improved through modifications to render them less toxic. Consequently, the objective of this research is to evaluate the combined effect of the ethanol extract of *P. guajava* leaves and the ethanol extract of *Persea americana* leaves on Methicillin Resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS

Collection and identification of plant materials

The leaves of *P. guajava* and *Persea americana* were collected from Umuno in Abraka, Delta State, Nigeria in December 2022. The identification and authentication of the plants were done by Dr. Emmanuel Ikpefan, a botanist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmacy, Delta State University, Abraka, Nigeria. A sample was kept at the herbarium, Department of Pharmacognosy & Traditional Medicine, Delta State University, Abraka, Nigeria. The plant samples collected were air-dried and comminuted, and then both powders were stored at room temperature.

Extraction of plant materials

About 420 g of the powdered sample of *Persea americana* leaves and 280 g of the powdered sample of *Persea americana* were collected. A

420 g of the powdered sample of *Persea americana* was divided into three portions in a separate bucket. A 280 g of the powdered sample of *P. guajava* was divided into two portions. Each portion contains 140 g of powdered plant material. They were macerated with 80% ethanol, with each 140 g of the different plant material soaked in 560 ml of ethanol separately in an airtight bucket for 72 hours. The extracts of the three portions of *P. guajava* were filtered using the muslin cloth. Also, the extracts of the three portions of *Persea americana* were filtered using muslin cloth. The filtrates were collected in separate beakers and were concentrated to dryness in a water bath. The resulting brown concentrate was then reconstituted using distilled water for a final weight per volume of 100 mg/ml and stored in a refrigerator at 4 °C until when it was required for use in the experiment.

Phytochemical screening

Secondary metabolites such as terpenes, alkaloids, flavonoids, tannins, and saponins were screened for using the standard approaches according to Evans and Trease [21].

Cell cultures

Stock cultures of 30 *Staphylococcus aureus* previously isolated were kept on Nutrient agar slants at room temperature in the Pharmaceutical Microbiology laboratory, Delta State University, Abraka for further use in this study. For re-identification, the stock cultures were further subcultured on Mannitol Salt agar and incubated for 24 hours at 37 °C. Stock cultures were further sub-cultured on Mannitol Salt agar and incubated at 37 °C for a duration of 24 h. Those that produced yellow color indicated *Staphylococcus aureus* and these were the ones further subjected to a Methicillin-resistant test. These bacterial isolates were isolated into a single colony, streaked on a brand-new nutrient agar plate, cultured for an entire night, and then preserved at 4 °C pending additional research.

Test for methicillin resistance

The agar diffusion approaches were used for the evaluation of the 30 *Staphylococcus aureus* isolates for Methicillin resistance [22]. Flucloxacillin cap. (500 mg) (Ernest Chemists Limited, Accra, Ghana) was used for this study. A pure colony of each *Staphylococcus aureus* isolate was picked using a wire loop and then inoculated into an already sterilized nutrient broth covered with aluminum foil and left overnight. Each nutrient broth culture's turbidity was adjusted to meet McFarland turbidity requirements. The surface of the thirty Mueller Hinton Agar plates that had already solidified and been meticulously labeled was then swapped out using each of the modified broth cultures. After discarding the leftover nutrient broths into a jar for disinfection, each Mueller Hinton agar plate was allowed time to dry on its surface. Using a sterile surgical blade, the antibiotic discs were placed aseptically in duplicate on each plate rightly on the surface of the already dried inoculated Mueller Hinton agar plates. The plates were incubated at 37 °C for a duration of 24 h. After the incubation, all plates were carefully examined for inhibition zones around the two paper discs on each plate. The zones of inhibitions on each plate were measured using a meter rule in diameters and they were recorded accordingly. Means of inhibition zone diameter were calculated and recorded to the nearest whole millimeter. Thereafter each of the organism isolates was classified as Methicillin Resistant *Staphylococcus aureus* strain, (MRSA), or not using a guideline given by the CLSI (2022).

Antimicrobial testing

Determination of the Zones of inhibition of Ethanol extract of *Persea americana* and *P. guajava* leaves.

Sensitivity: Agar well diffusion method

Ethanol extracts of *Persea americana* leaves were screened for their effect on Methicillin Resistant *Staphylococcus aureus* (MRSA).

Mueller Hinton agar was prepared for 15 Petri dishes according to the manufacturer's specifications and autoclaved at 121 °C for 15 minutes. The media was allowed to cool before pouring 20 ml into each petri dish and they were allowed to solidify. The petri dish was each labeled according to the numbered strain of MRSA previously identified. Each of the Petri dishes was also labeled accordingly with different concentrations (200, 100, 50, 25, 12.5, and 6.25) mg/ml of the ethanol plant extract previously prepared, and ciprofloxacin was used as a positive control. The agar plates were swabbed with the test organisms as labeled

aseptically. Using a 6 mm cork borer, a duplicate well was pouched in the agar plates. Two drops of each concentration of the ethanol extract of *Persea americana* leaves were placed into the corresponding well using a Pasteur pipette. Ciprofloxacin which served as the positive control of the experiment was placed in the well at the centre of the agar plate using a 2ml syringe. Ethanol extract of *P. guajava* leaves was also evaluated using the same procedures as above. The plates were incubated for 24 hours at 37 °C. After incubation, zones of inhibition were examined using a hand lens for proper magnifications, and zones were measured. A metric rule was placed across zones of inhibition, and measured from one edge of the zone to the other edge. We looked for inhibitory zones surrounding the wells on the plates. Using a meter ruler, the zone diameters were measured to the closest whole millimeter. Three trials of each test were conducted, and the mean IZD was recorded to the closest whole millimeter.

Determination of minimum inhibitory concentration (MIC) of plant extracts

The MIC was evaluated using the agar dilution method as specified in the procedures of CLSI (2022). Mueller Hinton agar was prepared according to the manufacturer's instructions. 19ml of molten nutrient agar was mixed with 1ml of the dilution extract of *Persea americana*, thoroughly poured into a sterile petri dish, and allowed to solidify. Each petri dish contained different concentrations of the dilution of ethanol extract of *Persea amaricana* leaves, (200, 100, 50, 25, 12.5, and 6.5 mg/ml). The agar plates were divided into 15 parts and labeled for each test strain of MRSA. The plates were kept in the incubator overnight to check for their sterility. Using a sterile wire loop, an overnight broth culture of each of the test organisms was streaked on the surface of the agar plate on the part of the plate labeled for the highest concentration of the dilution of ethanol extract of *Persea americana* leaves (200 mg/ml). The same procedure was repeated for the other five different concentrations. A nutrient agar without an extract was as well streaked and this served as a negative control. The plates were then incubated for 24 hours at 37 °C and they were observed for any visible growth of each MRSA. The least concentrations that inhibited the growth of the test organisms were selected as the MIC.

The same procedures above were repeated to determine the minimum inhibitory concentrations of the different concentrations of the dilution of ethanol extract of *P. guajava* leaves, (200, 100, 50, 25, 12.5, and 6.5 mg/ml). The least concentrations that inhibited the growth of the test organisms were selected as the MIC.

Determination of combined zone of inhibitions of ethanol extract of Persea americana and P. guajava leaves.

Agar plates with a subculture colony of Methicillin-Resistant Staphylococcus aureus labeled MRSA 1, MRSA 4, MRSA 6, MRSA 7, and MRSA 10 were selected to prepare the overnight broth.

Mueller Hinton agar was prepared according to the manufacturer's specification and autoclaved at 121 °C for 15 minutes. The media was allowed to cool before pouring 20 ml into each petri dish and they were allowed to solidify. The agar plate surface was swabbed using a swab stick with the first test organism (standardized overnight nutrient broth of MRSA 1). The petri dishes were labeled according to the test organisms being used.

Using a 6mm cork borer, two wells were pouched close to each other with a distance of about 3mm in each agar plate. A 1 ml of each concentration with the least minimum inhibitory concentration, MIC on both plant extracts of this test organism was individually placed into the corresponding well with the aid of a Pasteur pipette.

This was also done for other test organisms (MRSA 4, MRSA 6, MRSA 7, and MRSA 10) using both extracts as well.

The plates were incubated for 24 hours at 37 °C. The combined antibacterial assay was evaluated in duplicate. After incubation, the combined zones of inhibition were carefully examined using a hand lens for proper magnification, and the zones were measured. A metric rule was placed across combined zones of inhibitions and measured from one edge of the zone to the other edge, both vertically and horizontally, and was averaged. The combined Inhibition zone diameter (IZD) was reported in millimeters.

RESULTS AND DISCUSSION

The combined use of these extracts could be efficient in the treatment of complicated infections as compared to single plant extracts. It would also reduce resistance, reduce the high cost of drugs, increase effectiveness, and reduce toxicity. However, the process could be tedious, and clinical trials could be required to ascertain safety and efficacy.

Persea americana has previously been documented to possess many antimicrobial activities [13]. Research has also been previously carried out and reveals *P. guajava* Linn. to have promising medicinal properties in combating and managing resistant bacteria like MRSA [23].

In this study, a combined antibacterial effect of ethanol extract of *P. guajava* and *Persea americana* leaves on Methicillin-resistant Staphylococcus aureus was done.

The preparative phytochemical constituents of ethanol extract of *P. guajava* and *Persea americana* leaves are presented in **Tables 1 and 2**, respectively. **Table 1** indicates that ethanol extract of *P. guajava* leaves contained alkaloids, saponins, and tannins in high concentration while terpenes, flavonoids, and cardiac glycosides were in moderate concentration. **Table 2** indicates that ethanol extract of *Persea americana* leaves contained Alkaloids in high concentration while Saponins, terpenes, flavonoids, and tannins were in moderate concentration. Tannins had previously been reported to be responsible for the antibacterial actions 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 *Persea americana* leaves.

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

++: moderate concentration
+++: high concentration

The results from this study revealed that the combined antibacterial effect of ethanol extract of *P. guajava* and *Persea americana* produced a synergistic antibacterial effect against Methicillin Resistant *Staphylococcus aureus* isolates, (MRSA). The antibacterial activity of ethanol extract of *P. guajava* leaves was evaluated by comparing the zone of inhibition of each MRSA isolate with that of the standard antibiotic (control) Ciprofloxacin using the agar well diffusion method. Ten clinical isolates of MRSA were subjected to the agar well diffusion test, and the findings of the MRSA screening test using *Persea americana* leaf extract are reported in **Table 3**, similarly, that of the screening test of the leaf extract of *P. guajava* against MRSA isolates are presented in **Table 4**. The Minimum Inhibitory concentrations were also carried out. The MIC of tested MRSA isolates varied in their sensitivities to different concentrations of the extracts.

Table 3. Sensitivity of MRSA isolates to ethanol extract of *Persea 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 *Persea americana* leaves was active against the ten MRSA isolates tested with a mean inhibition zone diameter between the range from 4 to 13 mm.

The Ethanol extract of *P. guajava* leaves was active against the ten MRSA isolates tested with a mean inhibition zone diameter within the range of 4 to 12 mm. The cleared zones that appeared around the well after incubation showed the degree of inhibition/antibacterial effect possessed by each concentration of the individual plant extracts against the test MRSA isolates, while those with cloudy appearance around the wells with no clear zones indicated that isolates were not inhibited by the extracts or they were resistant to the extracts.

The Minimum Inhibitory Concentration, MIC findings in **Table 5** show that the ten MRSA isolates tested were inhibited by the Ethanol extracts of *Persea americana* leaves with activities ranging from 50 to 200 mg/ml while the nine isolates were inhibited by the ethanol extract of *P. guajava* leaves varied between 12.5 and 200 mg/ml. The result of the MIC of Psidium gujava plant extract confirmed the antibacterial actions of Methicillin-Resistant *Staphylococcus aureus* as previously published by other co-workers [25]. Also, the result of the MIC of *Persea americana* plant extract ascertained the antibacterial action of Methicillin Staphylococcus aureus as previously published by other co-workers [13].

Table 5. Minimum inhibitory concentration for the MRSA isolates

MRSA Isolates	Ethanol Extract from *Persea 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 combined antibacterial activity of ethanol extract of *P. guajava* and *Persea americana* leaves was evaluated by comparing the combined Inhibition zone diameter, (CIZD), of each MRSA isolate with the least minimum inhibitory concentration (MIC), combined on both extracts. Also, the CIZD was compared with that of the standard antibiotic (control) Ciprofloxacin using the agar well diffusion method. Five clinical MRSA isolates were subjected to the agar well diffusion test, and **Table 6** further presents the findings of the extracts' screening test against the tested MRSA isolates.

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 1 with the least MIC of 50mg/ml and 25 mg/ml for the ethanol extract of *Persea americana* and *P. guajava* leaves, respectively, gave a mean combined inhibition zone diameter of 23 mm. MRSA 4 with the least MIC of 50 mg/ml and 25 mg/ml for the ethanol extract of *Persea americana* and *P. guajava* leaves respectively, gave a mean combined inhibition zone diameter of 22.25 mm. MRSA 6 with the least MIC of 50 mg/ml and 25 mg/ml for the ethanol extract of *Persea americana* and *P. guajava* leaves respectively, gave a mean combined inhibition zone diameter of 20 mm. MRSA 7 with the least MIC of 100 mg/ml and 12.5 mg/ml for the ethanol extract of *Persea americana* and *P. guajava* leaf, respectively, gave a mean combined inhibition zone diameter of 20.75 mm. MRSA 8 with the least MIC of 100 mg/ml and 12.5 mg/ml for the ethanol extract of *Persea americana* and *P. guajava* leaves respectively, gave a mean combined inhibition zone diameter of 29.5 mm.

The mean of the combined inhibition zone diameter varied between 20 to 30 mm. This result signifies a synergism in the antibacterial effects of both plant extracts against Methicillin-resistant *Staphylococcus aureus*. The zone of inhibition of the test Isolate MRSA 1 when conducted as a single extract using *Persea americana* produced 8mm with the least MIC (50 mg/ml), while 7 mm was observed with the least MIC (25 mg/ml) using *P. guajava*. The combined zone of inhibition using this same isolate with the same MICs when conducted gave 23 mm which signifies a synergistic effect. The same observation was carefully noted with significant improvement on other isolates. In addition, in **Figure 1**, this combined effect showed a significant improvement in the antibacterial action, when compared with the antibacterial effect of a sole plant extract and in addition with the standard antibiotic. Thus, the interesting synergistic effect of ethanol extract of *Persea americana* and *P. guajava* leaves extracts will be of good alternative to combat multidrug resistance organisms, and to the best of our knowledge and literature review, this is the first report on the combined effects of *Persea americana* and *P. guajava* leaves on Methicillin Resistant *Staphylococcus aureus* (MRSA). This could be of significance in health care as it could be used as an alternative to conventional drugs in the treatment of diseases caused by Methicillin-Resistant *Staphylococcus aureus* (MRSA). This could also be used in the case of blind treatment where the case of infection is not known. Since time immemorial, early man has been said to use plants in the treatment of various ailments. Herbal medicine is still practiced in many parts of the world for the treatment and prevention of diseases especially in local regions with a variety of vegetation.

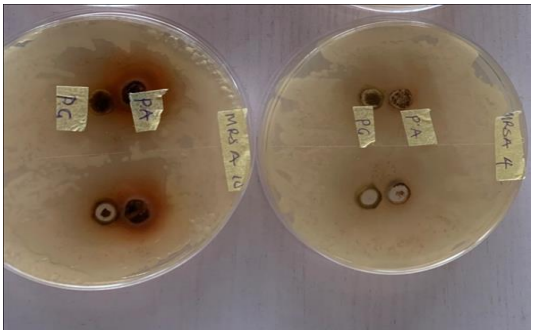

Figure 1. Combined zones of inhibition of ethanolic extract of *Persea americana* and *P. guajava* leaves.

CONCLUSION

The results of this research indicated that the combined antibacterial effect of both plant extracts has significant antibacterial activity in isolation and a significant synergistic effect against Methicillin Resistant *Staphylococcus aureus*, (MRSA) when combined. Subsequently from the results, it could serve as a good candidate for those strains which have developed resistance or as an alternative to conventional drugs in the treatment of these strains of Staphylococcus aureus. This combined effect would not only produce a synergistic effect but also reduce drug toxicity and also reduce the emergence of drug resistance.

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