

RESEARCH ARTICLE

Novel Strategy for Optimizing the Antibacterial Activity of *Psidium guajava* Against Clinical Isolates of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and *Streptococcus* spp.

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Abstract:

The development of a new antibiotic is a challenging task, with an estimated failure rate of 95%. Minor changes in the chemical structure of a drug, such as stereochemistry, geometry, or functional group modifications, can significantly impact its medicinal activity. In this study, we aim to devise novel strategies for optimizing the antimicrobial properties of guava leaf extract through simple reactions, either by self-reaction or combination reactions with a reagent, drug, or different plant extract. Fourier transform infrared spectroscopy analysis revealed conjugation and formation of new functional groups in the prepared sample of Guava Guava (GG) and Guava Aspirin Guava (GAG), which were further confirmed by weight analysis. The results demonstrated that the antimicrobial activity of medicinal plants can be improved or optimized through simple reactions, such as combining the plant extract with a non-antimicrobial drug like aspirins or adding a small volume of concentrated sulfuric acid to the plant extract by heating at a temperature range of 100 – 110°C. Among the two combinatory methods used, GG exhibited better performance in inhibiting the growth of all tested bacterial strains at a concentration of 0.1 mg/mL compared to GAG at the same concentration, which inhibited the growth of only two bacterial strains: *Escherichia coli* and *Streptococcus* spp. These methods can be further explored and applied in various studies, including antifungal, anti-inflammatory, antiviral, and anticancer research, leveraging the availability and diverse range of natural products found in medicinal plants.

Keywords: Antimicrobials, Natural products, Functional groups, Bioactive components, Bacterial isolates, Infectious diseases

1. Introduction

The use of plant extracts and phytoproducts is gaining attention due to their availability, cost-effectiveness, proven specificity, biodegradability, low toxicity, and minimum residual toxicity in the ecosystem [1]. Guava (*Psidium guajava* Linn. family: Myrtaceae) fruits and leaves contain a high amount of vitamins and tannins, and polyphenols, and other compounds such as resin, sugars, triterpenes, essential oil, isoquercitrin, reynoutrin,

guajaverin, avicularin, kaempferol, guajanoic acid, saponin, carotenoids, lectins, leucocyanidin, ellagic acid, amritoside, β -sitosterol, uvaol, oleanolic acid, and ursolic acid [2,3]. Pharmacological research, both *in vitro* and *in vivo*, on guava leaves, has demonstrated their potential for the cotreatment of different ailments, including infectious and parasitic diseases, neoplasms, blood and immune system disorders, endocrine and metabolic diseases, circulatory system disorders, digestive system disorders, and skin-related issues. Furthermore,

several randomized and clinical trials conducted in the last two decades have explored the effects of guava leaf extract in treating various medical conditions [4,5].

One of the challenges associated with using medicinal plants as therapeutic agents is the isolation of the bioactive principles for further pharmacological research and the concentration of bioactive isolates to treat certain diseases. On the other hand, antimicrobial resistance has limited the direct use of medicinal plant extract for treating infections and diseases. However, medicinal plants boast a variety of simple and complex organic compounds that cannot be easily synthesized in the laboratory. With the emerging threats of antimicrobial resistance and the slowdown of new antibiotic development, medicinal plant extracts remain a viable solution to battling against multidrug-resistance in antimicrobial, antiviral, antifungal, anti-inflammatory, and anticancer drug development, given the natural diversity of plant species and their diverse pharmacological properties. Several studies suggest that aqueous plant extracts may effectively treat bacterial infections and could even demonstrate comparable or superior efficacy to certain antibiotics while potentially reducing the development of bacterial resistance [6-8].

The process of drug design, discovery, and development is a highly focused and challenging task for researchers. It involves synthesizing novel organic molecules with improved therapeutic properties, aiming for optimum medicinal activity, safety, and cost-effectiveness. In addition, the goal is to reduce preparation time for combinational molecules and create more effective treatment against life-threatening diseases such as malaria, tuberculosis, cancer, and others [9,10].

Natural products derived from nature stand as an infinite resource for drug development. They offer novel chemotypes, pharmacophores, and scaffolds that can be amplified into efficient drugs for various disease indications and other valuable bioactive agents. Moreover, natural products have been the backbone of the traditional system of healing since time immemorial [11-13].

Synthetic reactions can be utilized to modify or create novel compounds derived from medicinal plants, leading to improved or optimized antimicrobial

activities. Some approaches where synthetic reactions can be applied to enhance the antimicrobial properties of medicinal plant compounds include the following:

- (i) Structural modifications: Modifying the chemical structure of natural compounds through the introduction of functional groups, substitution patterns, or modifications to the core structure, aiming to improve potency, selectivity, or bioavailability [14].
- (ii) Semi-synthetic derivatives: Selectively modifying specific functional groups or substituents to improve antimicrobial activity in semi-synthetic derivatives [15].
- (iii) Prodrug synthesis: Designing prodrugs of natural antimicrobial compounds to improve their stability, solubility, or bioavailability, leading to enhanced antimicrobial activity [16].
- (iv) Combinatorial chemistry approach: Enabling the creation of libraries of diverse compounds that can be screened for enhanced antimicrobial activity [17].

Several studies have detailed synergistic combinations of antibiotics with medicinal plant extracts, using concentrated sulfuric acid and/or sodium hydroxide, in an effort to optimize the antimicrobial activities of medicinal plants against drug-resistant bacteria [18,19]. Minor changes in the chemical structure, such as changes in stereochemistry, geometry, functional group, removal of groups, derivative formation, oxidation, reduction, hydrogenation, chelation, and salt formation, may modify the medicinal activity of natural products. Consequently, there is a pressing need for improved methodologies and tremendous development efforts in combinatorial synthesis. This approach allows the generation of a series of miscellaneous molecular hybrids in drug discovery, employing well-organized synthetic and environmentally friendly methods [9,10].

The present study aimed to induce reactions in plant extracts without separating their bioactive components. In addition, we investigated the antimicrobial activities of a mixture of plant extract and aspirin against clinical isolates of multidrug-resistant *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and *Streptococcus* spp. Our objective was to strategically optimize antimicrobial effectiveness using low concentrations of the

combined species, aiming for minimal signs of bacterial growth inhibition at lower concentrations. Higher antimicrobial concentration might show signs of stronger bacterial inhibition, but an increase in drug concentration can indeed lead to adverse reactions or toxicity due to pharmacokinetic and pharmacodynamic factors [20,21].

2. Materials and methods

In this study, *P. guajava* was selected to evaluate its antimicrobial (antibacterial) activity. Fresh leaves at the fruit development stage were used. ANACIN (ASA 300 mg) from SKG-Pharmaceutical Limited Nigeria was purchased from pharmaceutical vendors within the Kaduna metropolis, Kaduna North, Kaduna, Nigeria. The leaves of *P. guajava* (guava) were harvested from a florist garden along Television Market, Kaduna South Kaduna State, Nigeria.

Clinical isolates of *E. coli*, *S. aureus*, *Salmonella* spp., and *Streptococcus* spp. were collected from the Chemical Pathology, Hematology and Microbiology Diagnostic Laboratory of Oxford Hospital Makera, Kakuri, Kaduna State, Nigeria.

2.2. Preparation of extracts, aspirin solution, and other solutions

The leaf sample was thoroughly washed and cut into small pieces. 5 g of the sample was weighed, mixed with 100 mL of distilled water, and then boiled for 10 min. After boiling, the mixture was filtered using filter paper and transferred into a conical flask for further analysis.

An aspirin solution of 2 mg/mL was prepared. Furthermore, a 5% of sodium hydroxide solution and concentrated sulfuric acid were also obtained.

2.3. Combinatorial process

The methods adopted by Mathew and Zakari [19] were used with a few modifications. In a 50 mL beaker labeled Guava Aspirin Guava (GAG), 4 mL of guava extract was added to 4 mL of aspirin solution and boiled in a water bath for a few minutes. Next, 0.4 mL of sodium hydroxide was added and the mixture continued boiling for 5 min. A fresh 2 mL portion of the prepared guava extract was added to the boiling mixture, followed by the addition of 0.3 mL of sulfuric acid. The mixture was allowed

to boil for an additional 10 min and then transferred into a centrifugal tube for centrifugation.

In a separate 50 mL beaker labeled Guava Guava (GG), 10 mL of guava extract was boiled in a water bath for a few minutes, 0.4 mL of sulfuric acid was added, and the mixture was boiled for 10 min before being transferred into a centrifugal tube for centrifugation.

In addition, 10 mL of guava extract was transferred into a centrifugal tube for centrifugation. All the samples were then dried in an oven at 48°C for 24 h.

The test tubes containing the prepared samples, as described in the methods above, as shown in **Figure 1**, which shows the differences in volume and coloration based on the reaction conditions.

2.4. Agilent attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR was used for functional group analysis of guava extract, aspirin, GAG, and GG. The spectroscopic parameters used were as follows: Sample scan of 30, background scan of 16, range of 4000 cm^{-1} to 650 cm^{-1} , resolution of 8, and system status set to good.

2.5. Antimicrobial screening

Antimicrobial susceptibility test was conducted on confirmed multidrug-resistant clinical isolates of *S. aureus* from high vaginal swabs (HVS), *E. coli* from urine, *Salmonella* spp. from stool, and *Streptococcus* spp. from sputum. The tests were performed using the prepared samples listed below.



Figure 1. Prepared samples of Guava Aspirin Guava, Guava, aspirin solution, and guava extract.

- (i). Sgu for guava extract, GU, 5.0 mg/mL (5 mg of GU was dissolved in 1 mL distilled water and was applied on the prepared antimicrobial disk)
- (ii). Tg1 for GAG, GAG, 0.1 mg/mL (1 mg of GAG was dissolved in 1 mL distilled water and was applied on the prepared antimicrobial disk)
- (iii). Tc2 for GG, GG, 0.1 mg/mL (0.1 mg of GG was dissolved in 1 mL distilled water and was applied on the prepared antimicrobial disk)
- (iv). Sg3 for GG, GG 0.2 mg/mL (0.2 mg of GG was dissolved in 1 mL distilled water and was applied on the prepared antimicrobial disk).

The Kirby-Bauer disk diffusion test using Mueller-Hinton Agar (MHA) was performed [20]. Area sterilization was carried out with disinfectant and an open burner. A sterile loop was used to pick a well-isolated, fresh bacterial colony from a pure culture plate and transfer it to the broth medium, while ensuring sterility throughout the process. Next, the entire surface of a MHA plate was inoculated by streaking the agar in three directions (north-south, east-west, and diagonally) using the sterile loop to ensure even distribution. The inoculated plate was allowed to dry for a few minutes to allow the bacteria to adhere to the agar surface. Prepared antimicrobial disks were then placed on the agar surface using sterile forceps and gently pressed to ensure proper contact. The inoculated plates were inverted and incubated for 24 h at 37°C. After incubation, the plates were examined and the diameter of the zone of inhibition around each disk was measured and recorded as susceptible or resistant.

3. Result and discussion

3.1. ATR-FTIR analysis of guava extract, aspirin, GAG, and GG

In **Figures 2-5**, characteristic peaks at 3693 cm^{-1} to 2545 cm^{-1} were attributed to single bonds of nitrogen-hydrogen (N-H) stretching, oxygen-hydrogen (O-H) stretching, and carbon-hydrogen (C-H) stretching. Peaks observed at 2091 cm^{-1} to 1416 cm^{-1} were attributed to double bonds of carbon-carbon (C=C), carbon-nitrogen (C=N), carbon-carbon-carbon (C=C=C), carbon-oxygen

(C=O) and cyclic carbon (C=C), and triple bonds of carbon-carbon (C≡C) and carbon-nitrogen (C≡N) stretching vibration. Peaks observed at 1367 cm^{-1} to 913 cm^{-1} were also attributed to C-O stretching, -CH₂- bending vibrations, and N-O stretch/nitro groups, while those observed at 838 cm^{-1} to 752 cm^{-1} were assigned to alkyl halides [21].

The observed disappearance of some peaks and changes in the wave number of certain peaks initially present in the unreacted guava extract and unreacted aspirin can be attributed to the conjugation of molecules, which is a factor affecting the location of peaks in infrared spectroscopy [22]. We also observed that the peaks at 2918 cm^{-1} , 1364 cm^{-1} , and 752 cm^{-1} in guava extract (**Figure 2**) and 1602 cm^{-1} , 1092 cm^{-1} , and 1013 cm^{-1} (**Figure 3**) in aspirin remained unchanged, indicating that they were not engaged in the reaction, and their linkages were unaffected by the mass, bond strength, or conjugation with the neighboring atom or molecules.

As for the ATR-FTIR analysis of GG, we observed the disappearance of some peaks and changes in the wave number of some peaks that were initially present in the unreacted guava extract (**Figure 4**). These changes were a result of molecular conjugation. In addition, a new peak at 1688 cm^{-1} emerged, indicating the formation of a new functional group (**Figure 5**). Meanwhile, the peaks at 1994 cm^{-1} and 1028 cm^{-1} remained unchanged, suggesting that they were not engaged in the reaction and their linkages were unaffected by the mass, bond strength, or conjugation by the neighboring atom or molecules.

Moreover, the decrease in percent transmittance after the reaction can be attributed to the presence of interfering functional groups or the formation of a new compound with a different or similar functional group(s) or molecular structure, providing important information about the chemical changes that have occurred in the sample [21-25].

3.2. Antimicrobial test for guava extract, GAG, and GG

The bacterial strains, including *E. coli*, *S. aureus*, *Salmonella* spp., and *Streptococcus* spp., showed resistance to the guava extract (GU) at a concentration of 5 mg/mL for 48 h, as shown in **Table 1** and **Figure 6**. This resistance can be

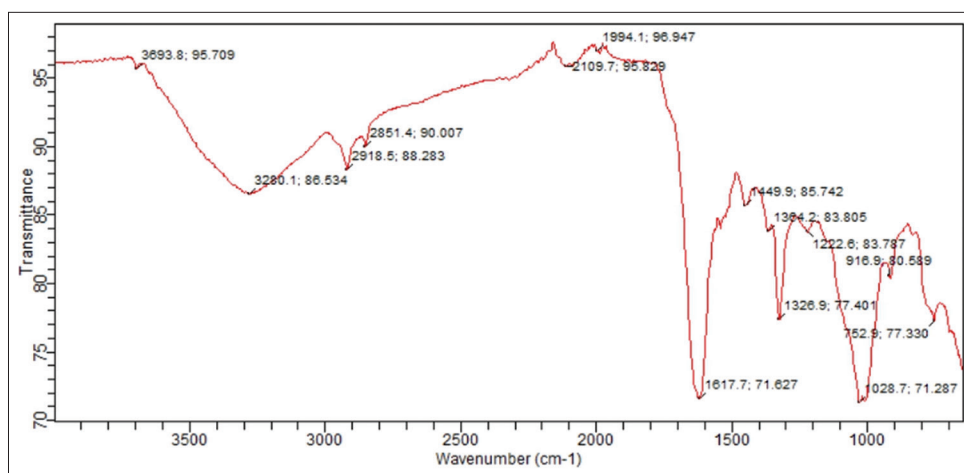


Figure 2. Agilent attenuated total reflectance-Fourier transform infrared spectroscopy analysis of guava extract (GU).

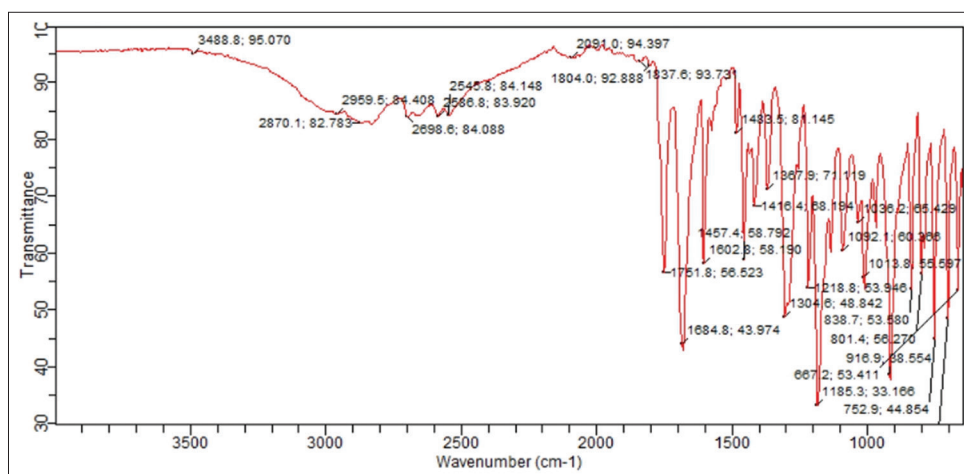


Figure 3. Agilent attenuated total reflectance-Fourier transform infrared spectroscopy analysis of aspirin.

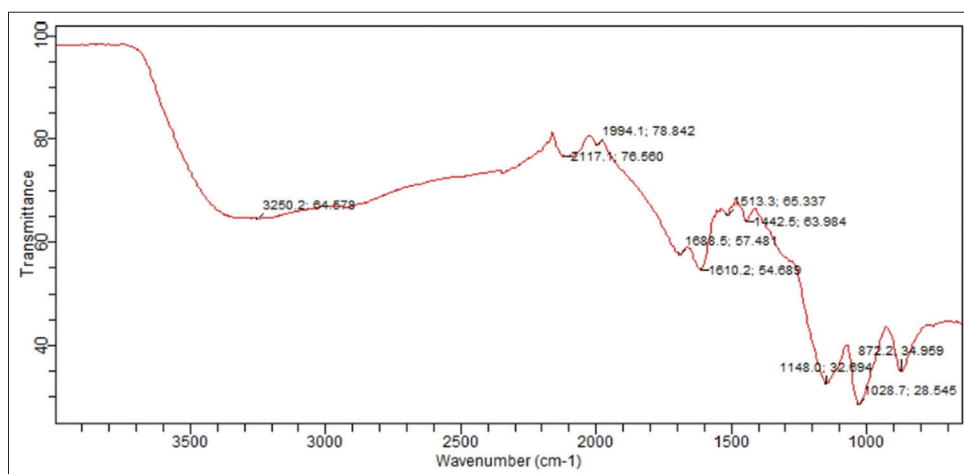


Figure 4. Agilent attenuated total reflectance-Fourier transform infrared spectroscopy analysis of Guava.

attributed to the resistant nature of the bacteria, the low concentration of the plant extract used, and possibly the duration of the inoculation. The results obtained are similar to those reported in a previous

study [26], where the observed zone of inhibition for *E. coli* was 0 mm and 16.1 mm for methicillin-resistant *S. aureus*. While the concentration of the extract in their study was not specified, their sample

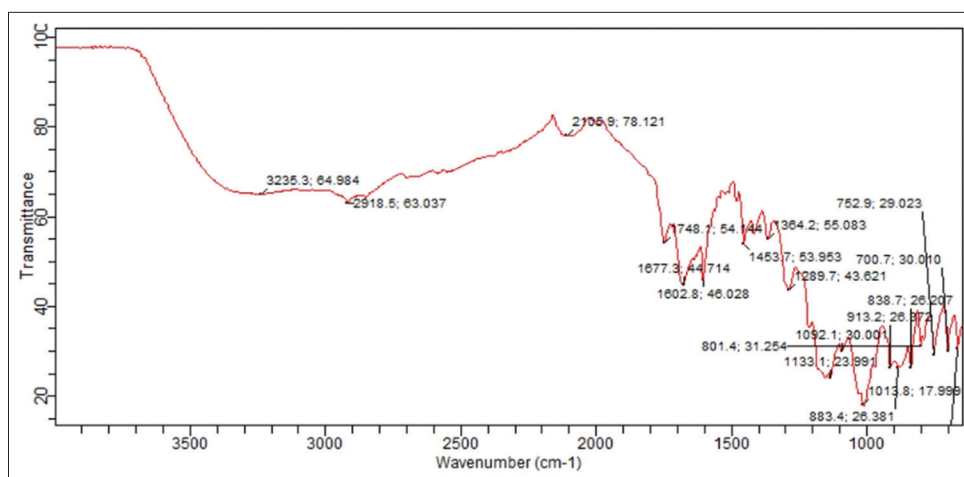


Figure 5. Agilent attenuated total reflectance-Fourier transform infrared spectroscopy analysis of Guava Aspirin Guava.

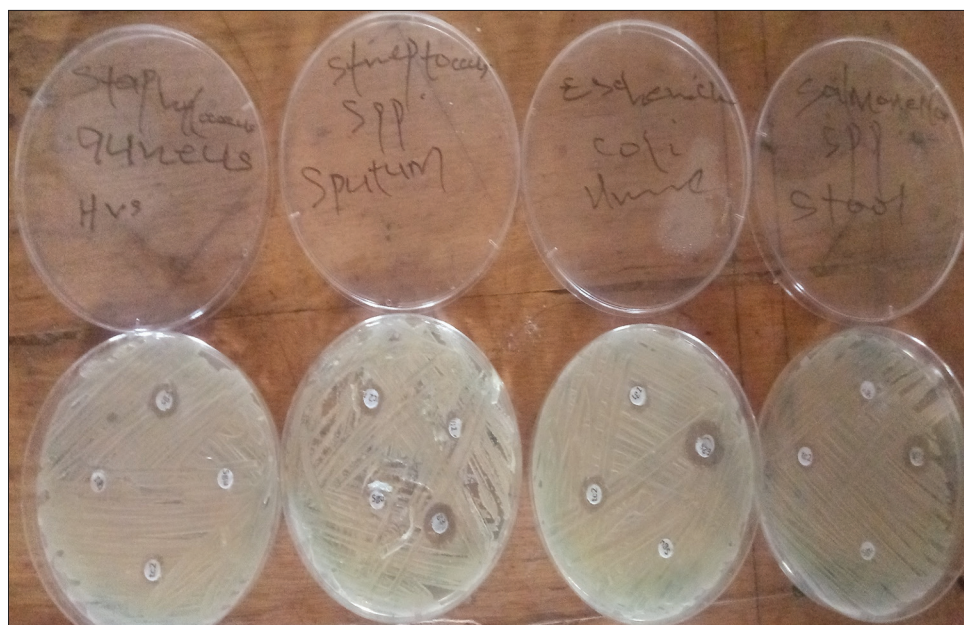


Figure 6. Antimicrobial susceptibility test on the prepared samples using *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and *Streptococcus* spp.

Table 1. Antimicrobial test for GE, GAG, and GG

Bacterial isolates	Diameter of inhibition zone (mm)			
	GE (Sgu)	GAG (Tg1)	GG (Tc2)	GG (Sg3)
<i>Salmonella</i> spp.	0.0	0.0	9.0	14.0
<i>E. coli</i>	0.0	5.0	12.0	16.0
<i>S. aureus</i>	0.0	0.0	7.0	12.0
<i>Streptococcus</i> spp.	0.0	5.0	10.0	13.0

Abbreviations: GE: Guava extract; GAG: Guava Aspirin Guava; GG: Guava Guava; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*

weighed 0.7 g heavier than that used in the current study, and the boiling time was three times longer than that of the present study. This discrepancy indicates a higher concentration compared to the one used in the present study.

At 0.1 mg/mL, GAG inhibited only *E. coli* and *Streptococcus* spp., with a zone of inhibition of 5.0 mm for both. On the other hand, GG at 0.1 mg/mL inhibited *E. coli*, *S. aureus*, *Salmonella* spp., and *Streptococcus* spp. with the zones of inhibition of 12.0 mm, 7.0 mm, 9.0 mm, and 10.0 mm, respectively. Bigger inhibition zones were observed when the concentration of GG was double,

reaching a maximum of 1.5 times the sizes obtained at 0.1 mg/mL. These results obtained are consistent with those reported in a previous study [18], where the addition of sulfuric acid in a mixture of plant extract and antibiotic increased the zone of inhibition against clinical isolates of *Streptococcus* spp. (HVS), *Salmonella typhi* (stool), *E. coli* (urine), *Shigella* spp. (stool), and *S. aureus* (HVS). Similarly, another study [19] also reported an increase in the zone of inhibition for resistant *Salmonella* spp. following the addition of sodium hydroxide followed by sulfuric acid in the mixture of plant extract and aspirin.

The inhibition shown by GAG and GG is attributed to three main factors: (i) The structure extension, which involves the addition of another functional group to the lead structure, allowing for extra interactions between the drug and its bacterial target. These interactions may include increased target binding [27], altered membrane penetration [28], and enhanced metabolic stability [29]. (ii) The variation in ring size, wherein expanding or contracting a ring can position other rings differently relative to each other, leading to improved interactions with specific regions in the binding site. And (iii) the changing and formation of new functional groups, as evident in the new carbonyl (C=O) peaks observed in GG combination, and the disappearance of some peaks observed in GAG and GG (**Figure 2**) [23,24,30]. The validity of these changes was further confirmed by the difference in mass between centrifuged and dried samples of unreacted guava extract (GU) and reacted guava extract (GG). The unreacted guava extract had a total mass of 13.7 mg, while the reacted guava extract had a total mass of 177.2 mg. When multiple elements combine to form a compound, the molar mass of the compound depends on the number and types of atoms present. In general, compounds with larger and more complex molecules tend to have higher molar masses and, consequently, greater weights than compounds with smaller and simpler molecules. However, exceptions to this rule, and other factors, such as intermolecular forces, density, and molecular shape, can also influence the weight of a compound [25].

4. Conclusion

In this study, we conducted a single-step acid combination reaction of guava plant extract and a double-step alkali-acid combination of guava extract

and aspirin. The results obtained demonstrated that the antimicrobial activity of medicinal plants can be improved or optimized through simple reactions of the plant extract with a non-antimicrobial drug like aspirin, or by adding a small volume of concentrated sulfuric acid to the plant extract with heating at a temperature range of 100 – 110°C. Among the two combinatory methods used, GG exhibited the best results, inhibiting the growth of all tested bacterial strains at a concentration of 0.1 mg/mL, compared to GAG, which was only inhibited the growth of only two bacterial strains, *E. coli*, and *Streptococcus spp.*, at the same concentration. These methods can be further explored and applied in various studies, including antifungal, anti-inflammatory, antiviral, and anticancer research, leveraging the availability and diverse range of natural products found in medicinal plants.

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Conflict of interests

The authors declare no competing financial interest.

Author contributions

This is a single-authored study.

Ethics approval and consent to participate

The ethical permit was obtained from the Ministry of Health in Kaduna State, Nigeria, and was strictly adhered to from sampling the clinical isolates to the antimicrobial test.

Consent for publication

Not applicable.

Availability of data

Not applicable.

Further disclosure

The paper has been deposited in a preprint server Chemrxiv.com, DOI: 10.26434/chemrxiv-2023-1z7nk-v2.

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