Novel Optimization Strategy of *Psidium guajava* Antibacterial Activity for Drug Discovery and Development.

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ABSTRACT

Developing a new antibiotic is difficult, with an estimated failure rate of 95%, a minor change in chemical structure (stereochemistry, geometry, functional group, removal of groups, derivative information, reduction, oxidation, hydrogenation, salt formation, chelate formation, *etc)* may modify a drug medicinal activity.

Rather focusing on isolation and concentration of the bioactive molecules in medicinal plants extracts which in most cases not effective against drug resistant pathogens, other synthetic reactions can be done with the crude medicinal plant extract before its isolation, concentration and purification. This study aim to develop new strategy(s) for optimizing antimicrobial properties of guava leave extract by simple reactions, either by self-reaction or combination reactions with a reagent/a drug/a different plant extract.

Seven (7) different combinatory samples were prepared, FTIR analysis revealed conjugation and formation of new functional group(s) which was further confirmed by weight analysis of the prepared samples, two preparations successfully inhibit the growth of drug resistant clinical isolates of *S.aureus* and *E.Coli* making the two preparations a broad spectrum antimicrobial, thus, showing that reacting plant extract alone or with another compound using an acid or alkali can effectively optimize its antimicrobial activities.

Considering the availability and vast types of natural products present in medicinal plants, Exploring this method in antifungal, anti-inflammatory, antiviral and anticancer drug discovery will create a new pathway for overcoming drug resistance threat worldwide.

Keywords: Antimicrobials, Drug resistance, Natural products, Drug discovery, *S.aureus, E.Coli,* Functional groups.

1.0 INTRODUCTION

Synthetic organic and medicinal chemistry is arguably reorienting the field of drug discovery in the same way as one century ago the field of organic chemistry. The area of drugs designing, discovery and development is the most focused and challenging task for the researchers.[1] Newer organic molecules synthesis with more favourable therapeutic properties which shows optimum medicinal activity should be safer, economic, less time consuming in order to prepare combinational molecule and also better creation against lethal diseases like malaria, tuberclosis, cancer *etc.* [2,3]

Natural products derived from nature stands as an infinite resource for drug development, novel chemotypes, pharmacophores, and scaffolds for amplification into efficient drugs for various disease indications and other valuable bioactive agents, it has been the backbone of traditional system of healing since time immemorial. [4-6]

Health care in the future might face enormous challenges considering the rising concern of drug resistance to existing chemotherapeutic regimens for fungal and bacterial infections, AIDS and cancer.[7-10]

The current level of investment in the development of natural- product- derived and synthetic small molecules stands in stark contrast to the increasing demand for new antimicrobials to treat infections caused by the global spread of multidrug- resistant bacterial pathogens. [11]

Novel agents displaying innovative chemistry and modes of action are desperately needed to tackle the public health menace posed by antimicrobial resistance which has resulted to yearly estimated dead of at least 700,000 people worldwide, and may rise to as much as 10 million by 2050 if the problem of antimicrobial resistance AMR is not addressed. [12-14]

Guava (*Psidium guajava)* fruit and leaves contains phytocompounds such as tannins, polyphenols, triterpenes, essential oil, isoquercitrin, guajanoic acid, saponin, carotenoids, [15,16] water extract and ethanolic extract of *Psidium quajava* leaves extracts were effective against *Salmonella typhi* (Typhoidal), *Salmonella Paratyphi A.* (Typhoidal) and *Salmonella typhimurium* (Non- Typhoidal) at 25,000 µg/mL.[17] In vitro as well as in vivo pharmacological research of Guava leaves has been widely used in co-treatment of different ailments such as Infectious and Parasitic Diseases, Neoplasms, Diseases of the Blood and Immune System, Diseases of the Skin and Subcutaneous Tissue, several randomized clinical trials has also been conducted during the last two decades to test the effect of guava leaf extract, [18,19] Some studies suggest that aqueous plant extracts may have comparable or even greater efficacy than some antibiotics in infection treatments, and that bacteria may not easily develop resistance to aqueous plant extracts. [20-23]

Concentrated tetraoxosulphate (vi) acid, and Sodium hydroxide, has always been used to initiate or catalyse certain reactions like esterification, saponification etc. [24] Their combined activity can be usedto optimize the antimicrobial activity in plant extract(s) alone or in combination with a different drugs like aspirin, acetaminophen, amoxicillin etc. at lower concentrations, because a minor change in chemical structure *viz.* change in stereochemistry, geometry, functional group, removal of groups, derivative information, oxidation, reduction, hydrogenation, chelate formation, salt formation *etc* may modify its medicinal activity. The effect of substitution is also of great significance in deciding medicinal activity of a drug. Similarly, drug activity depends upon physical and molecule structure, solubility, isosterism, partition coefficients *etc*. [2, 3]

Only two literatures detailed some synergistic combinations of antibiotic with medicinal plants extracts using concentrated sulphuric acid and/or sodium hydroxide in an effort to optimize their antimicrobial activities against drug resistant bacteria.[25,26] therefore, there is a pressing need for improved methodology, and tremendous development efforts in combinatorial synthesis where series of miscellaneous molecular hybrids can be obtained in drug discovery by a well-organized synthetic greener approaches. [3]

This study aim to induce reactions in plant extracts without the separation of its bioactive components, and also in a mixture of plant extract and aspirin to strategically optimize its antimicrobial activities against clinical isolates of multi-drug resistant *E.coli and S.aureus.* our objective is to use lower concentration of the combining species for the reaction and to test for its potency on resistant *E.coli and S.aureus* for a possible sign of bacteria growth inhibition, because if higher concentration of the reacting species is to be used, virtually all the product obtained might definitely show signs of bacteria inhibition thus maximizing toxicity potential. [3]

2.1 MATERIALS AND METHODS

The medicinal plant used for evaluating their antimicrobial (antibacterial) activity is *Psidium guajava*, the plant parts used for study are fresh leaves at fruit development stage.

ANACIN (ASA 300 mg) SKG-Pharmaceutical Limited Nigeria were purchased from pharmaceutical vendors within the Kaduna metropolis Kaduna North Kaduna, Nigeria. *Psidium guajava* (guava) leaves was harvested in a Florist garden along Television market, Kaduna South, and *E.coli and S.aureaus* was collected at Chemical Pathology, Haematology and Microbiology Diagnostic Laboratory of Oxford Hospital Makera, Kakuri, Kaduna State Nigeria.

2.2 Preparation of Extracts and Other Preparations: Leaf sample was properly washed, cut into smaller pieces, 5 g was weighed, mixed with 100 mL distilled water and was set to boil for 10 minutes, it was filtered using a filter paper and was kept in a conical flask for further analysis.

Aspirin solution of 2 mg/mL was prepared, 5% sodium hydroxide solution was prepared, concentrated sulphuric acid was also obtained.

2.3 Combinatorial process

The methods adopted by [26] was used with few modifications. 4 mL of **Guava extract** was added to 4 mL of **Aspirin solution** in a 50 mL beaker labelled **GAG**, it was set to boil in a water bath for few minutes, 0.4 mL of sodium hydroxide was added to it and allowed to

continue boiling for 5 minutes, a fresh 2 mL portion of the prepared **Guava extract** was added to the boiling mixture followed by addition of 0.3 mL of sulphuric acid, and was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

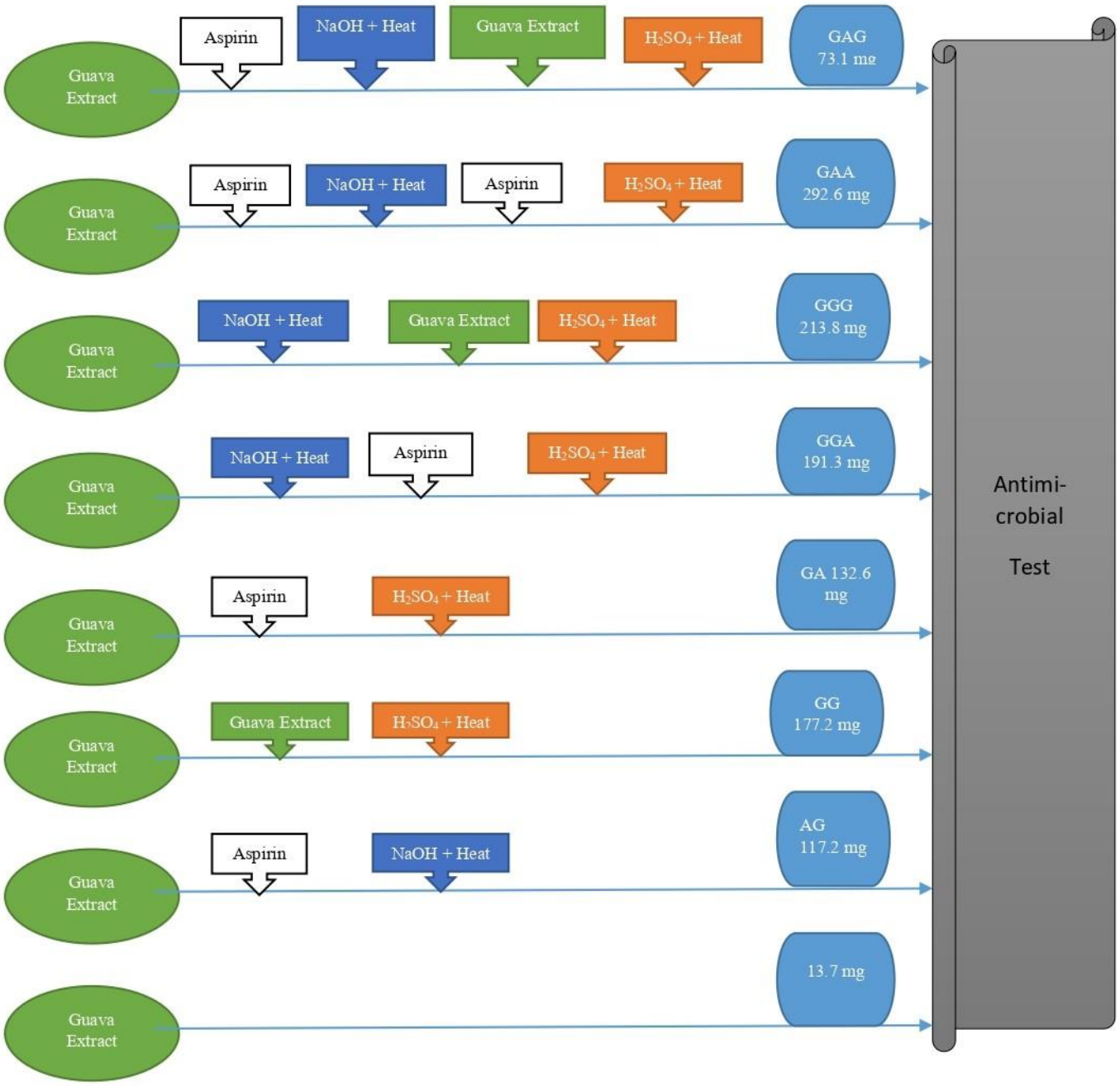

Fig. 1 Synthesis process designed for GA, GAA, GAG, GG, GGA, GGG, AG and Guava extract.

4 mL of **Guava extract** was added to 4 mL of **Aspirin solution** in a 50 mL beaker labelled **GAA**, it was set to boil in a water bath for few minutes, 0.4 mL of sodium hydroxide was added to it and allowed to continue boiling for 5 minutes, a fresh 2 mL portion of the prepared **Aspirin solution** was added to the boiling mixture followed by addition of 0.3 mL of sulphuric acid and was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

8 mL of **Guava extract** taken into a 50 mL beaker labelled **GGG**, it was set to boil in a water bath for few minutes, 0.4 mL of sodium hydroxide was added to it and allowed to continue boiling for 5 minutes, a fresh 2 mL portion of the prepared **Guava extract** was added to the boiling mixture followed by addition of 0.3 mL of sulphuric acid and was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

8 mL of **Guava extract** taken into a 50 mL beaker labelled **GGA**, it was set to boil in a water bath for few minutes, 0.4 mL of sodium hydroxide was added to it and allowed to continue boiling for 5 minutes, a fresh 2 mL portion of the prepared **Aspirin solution** was added

to the boiling mixture followed by addition of 0.3 mL of sulphuric acid and was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

5 mL of **Guava extract** was added to 5 mL of **Aspirin solution** in a 50 mL beaker labelled **GA**, it was set to boil in a water bath for few minutes, 0.4 mL of sulphuric acid was added to it, and it was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

10 mL of **Guava extract** was taken into a 50 mL beaker labelled **GG**, it was set to boil in a water bath for few minutes, 0.4 mL of sulphuric acid was added to it, and it was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

5 mL of **Guava extract** was added to 5 mL of **Aspirin solution** in a 50 mL beaker, it was set to boil on a water bath few minutes, 0.4 mL of sodium hydroxide was added to it, and it was allowed to boil for 10 minutes, after which it was transferred into a centrifugal tube for centrifugation.

10 mL of **Guava extract was** also transferred into a centrifugal tube for centrifugation. All the samples were dried in an oven at 48 ^{0}C for 24 hrs.

The test-tube containing the prepared samples as done in the methods above can be seen in Fig. 1, showing difference in volume and colouration based on the reaction conditions.

Fig. 2 prepared samples of GA, GAA, GAG, GG, GGA, GGG, Guava Aspirin base and Guava extract.

2.4 Agilent Technology Fourier transform infrared spectroscopy

Agilent Technology Fourier transform infrared spectroscopy, ATR-FTIR was used at a Sample scan of 30, Background scan of 16, Range 4000 cm-1 to 650 cm-1, resolution of 8 and system status Good for functional groups analysis of Guava extract, Aspirin, Guava Aspirin Guava and Guava Guava.

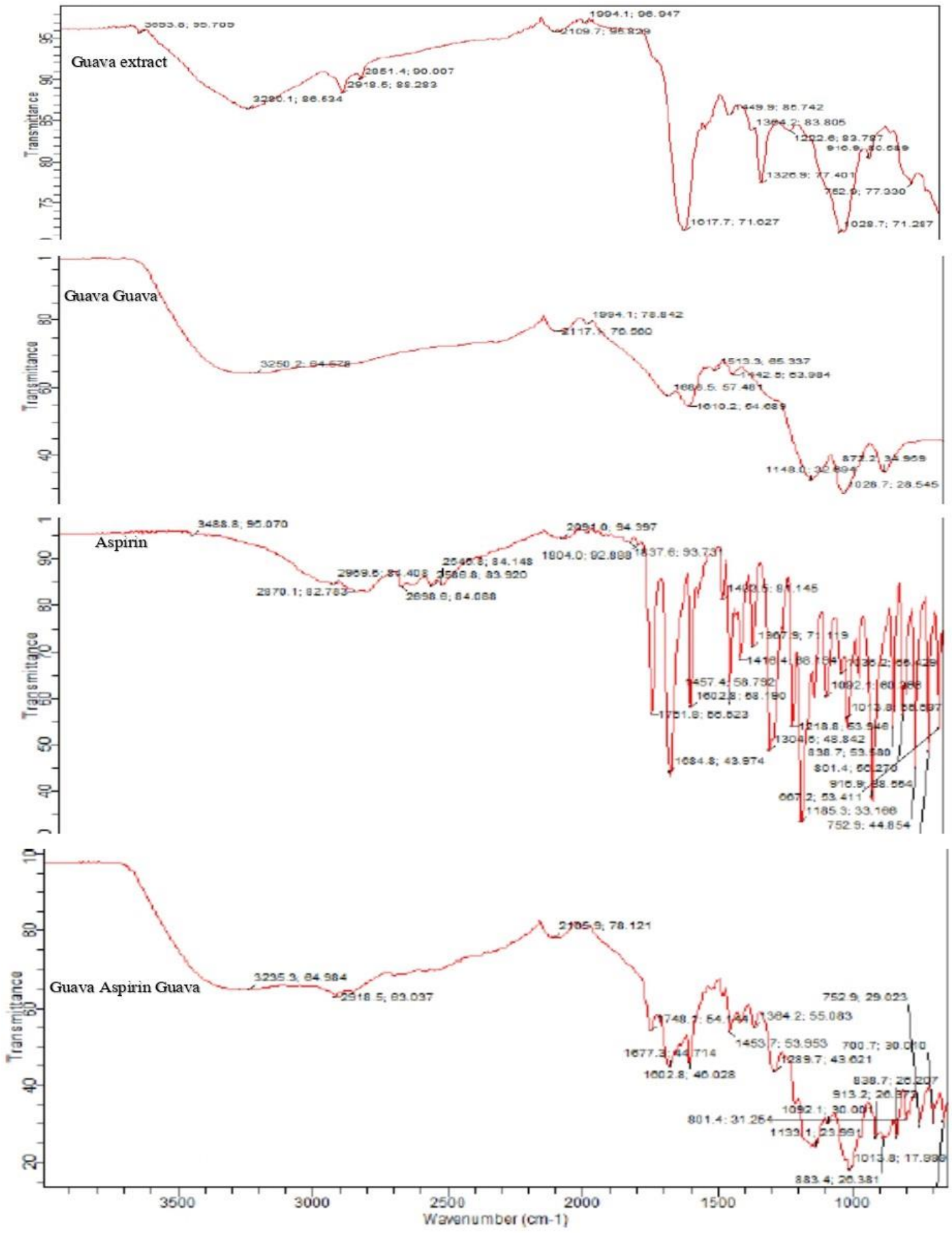

Fig. 3 ATR-FTIR analysis of Guava extract, Aspirin, Guava Aspirin Guava and Guava Guava

2.5 Antimicrobial screening

Confirmed multi-drug resistant clinical isolates of *Staphylococcus aureus* from High vaginal swab (HVS) and *Escherichia Coli* from (Stool) antimicrobial susceptibility test was carried out using the prepared samples listed below.

- Plane for Guava extract, GU
- Sc1 for Guava Guava, GG
- S10 for Guava Guava Guava, GGG
- Sg3 for Guava Guava Aspirin, GGA
- Tcl for Guava Aspirin Aspirin, GAA
- C20 for Guava Aspirin Guava, GAG
- Ag2 for Guava Aspirin, GA
- Sg2 for Aspirin Guava AG

Kirby- Bauer disk diffusion test using Mueller-Hinton Agar (MHA) was used, [27] disinfectant and open burner was used for the area sterilization, and a sterile loop was used to pick a well-isolated, fresh bacterial colony from a pure culture plate and transferred to the broth medium while maintaining sterility throughout the process.

The sterile loop was then used to inoculate the entire surface of a Mueller-Hinton agar plate by streaking over the agar in three directions (north-south, east-west, and diagonally) to ensure even distribution. The inoculated plate was allow to dry for a few minutes to allow the bacteria to adhere to the agar surface.

The prepared antimicrobial disks was placed on the surface of the agar using sterile forceps pressing them gently to ensure proper contact, the inoculated plates were then inverted and incubated for 24 hours at 37 °C. .After incubation, the plates was examined and the diameter of the zone of inhibition around each disk was measured and recorded as susceptible or resistance.

2.6 Bacterial Strains *I* Test organisms: The antibacterial activity of water extracts of guava and the seven (7) seven prepared samples were evaluated against 1 gram +ve bacteria, viz., *Staphylococcus aureus* 1 gram -ve bacteria, viz., *Escherichia coli.*

3.0 RESULT AND DISCUSSION

3.1 ATR-FTIR analysis of Guava extract, Aspirin, Guava Aspirin Guava and Guava Guava

In figure 2, the characteristic peaks at 3693 cm^{-1} to 2545 cm^{-1}, were attributed to N-H stretching, O-H stretching, and C-H stretching. Peaks observed at 2091 cm^{-1} to 1416 cm^{-1}were attributed to C=C, C=N, C=C=C, C=O, cyclic C=C, C≡C, C≡N, stretching vibration. Peaks observed at 1367 cm^{-1} to 913 cm^{-1} were also be attributed to C-O stretching, –CH2- bending vibrations, N-O stretch/nitro groups, while those observed at 838 cm^{-1} to 752 cm^{-1} were assigned to alkyl halides. [28] The observed disappearance of some of the peaks, increase and decrease in wave number of some peaks initially present in the unreacted guava extract and unreacted aspirin, this is as a result of conjugation of one molecular to another which is a factor affecting the location of peaks in infrared spectroscopy, [29] we also observed that the peaks at 2918 cm^{-1}, 1364 cm^{-1}, 752 cm^{-1} in guava extract and 1602 cm^{-1}, 1092 cm^{-1} 1013 cm^{-1} in Aspirin remain unchanged, meaning they were not engage in the reaction and their linkages were unaffected by the mass, bond strength or conjugation by the neighboring atom or molecules. Also the decrease of 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 an important information about the chemical changes that have occurred in the sample. [28-30]

Disappearance of some of the peaks, increase and decrease in wave number of some peaks initially present in the unreacted guava extract were observed, this was as a result of conjugation of one molecular to another, we could also see a new peak at 1688 cm^{-1} showing a new functional group is formed, while the peaks at 1994 cm^{-1} and 1028 cm^{-1} remain unchanged, indicating that they were not engage in the reaction and their linkages were unaffected by the mass, bond strength or conjugation by the neighboring atom or molecules. Also 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 an important information about the chemical changes that have occurred in the sample. [28-32]

Samples	Symbol on Disk	Product concentration obtained in mg/mL	Antimicrobial Activity	*S. aureus / E.coli*
Gu	Blank	1.37	Resistant	Both
GG	Sc1	17.72	Susceptible	Both
GGG	S10	21.38	Resistant	Both
GGA	Sg3	19.13	Resistant	Both
GAA	Tcl	29.26	Resistant	Both
GAG	C20	7.31	Susceptible	Both
GA	Ag2	13.26	Resistant	Both
AG	Sg2	11.72	Resistant	Both

Table 1. Antimicrobial test for Guava extract, Aspirin, Guava Aspirin Guava and Guava Guava

Guava Extract Gu, was resistant for both *S. aureus and E. coli* for 48 hours as shown in Table 1 and in Fig. 3 and 4, this is due to the resitant nature of the bacteria, lower concentration of the plant extract used and possibly the duration of the inoculation, the result obtain is similar to that of [33] where their observed zone of inhibition for ATCC *E. coli* was 0 mm and 16.1 mm for MRSA, although no concentration was specified but their sample was 0.7 g heavier than ours and the boiling time was thrice that of ours, this clearly shows a higher concentration as compared to the one used in this work.

Guava Aspirin Guava GAG and Guava Guava GG were susceptible in the inhibition of *S. aureus and E. coli* for 48 hours as shown in Fig. 6 and 7, GG showed a higher inhibition and a much clearer visibility at the edge of the prepared antimicrobial disk compared to GAG, although the visibility is best when the plates are positioned through a light source. The result obtained is similar to [25] where a drop of sulphuric acid in a mixture of plant extract and antibiotic increased the zone of inhibition of Clinical Isolates of *Streptococcus spp* (High Vaginal Swab), *Salmonella Typhi* (Stool), *Escherichia Coli* (Urine), *Shigella spp* (Stool), and *Staphylococcus Aureus* (High Vaginal Swab, and also similar to [26] where an addition of sodium hydroxide followed by sulphuric acid in the mixture of plant extract and aspirin increased the zone of inhibition of resistant *Salmonella spp*. The inhibition shown by GAG and GG is due to the structure extension which involves the addition of another functional group to the lead structure so as to probe for extra binding interactions with the target, ring size variation where by expanding or contracting a ring may put other rings in different positions relative to each other, and lead to better interactions with specific regions in the binding site, and finally the changing and formation of new functional groups as in the case of the new carbonyl C=O peaks observed in Guava Guava combination, and the disappearance of some of the peaks observed in Guava Aspirin Guava and Guava Guava in Fig 2. [34,30,31]

Figure 3. Prepared samples Antimicrobial susceptibility test on *E.coli*

this was further proven by the difference in mass of the centrifuged and dried sample of both 10 mL unreacted Guava extract **GU** and reacted guava extract **GG**, were unreacted Guava extract has a total mass of 13.7 mg and the reacted guava extract has 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 therefore greater weights than compounds with smaller and simpler molecules.

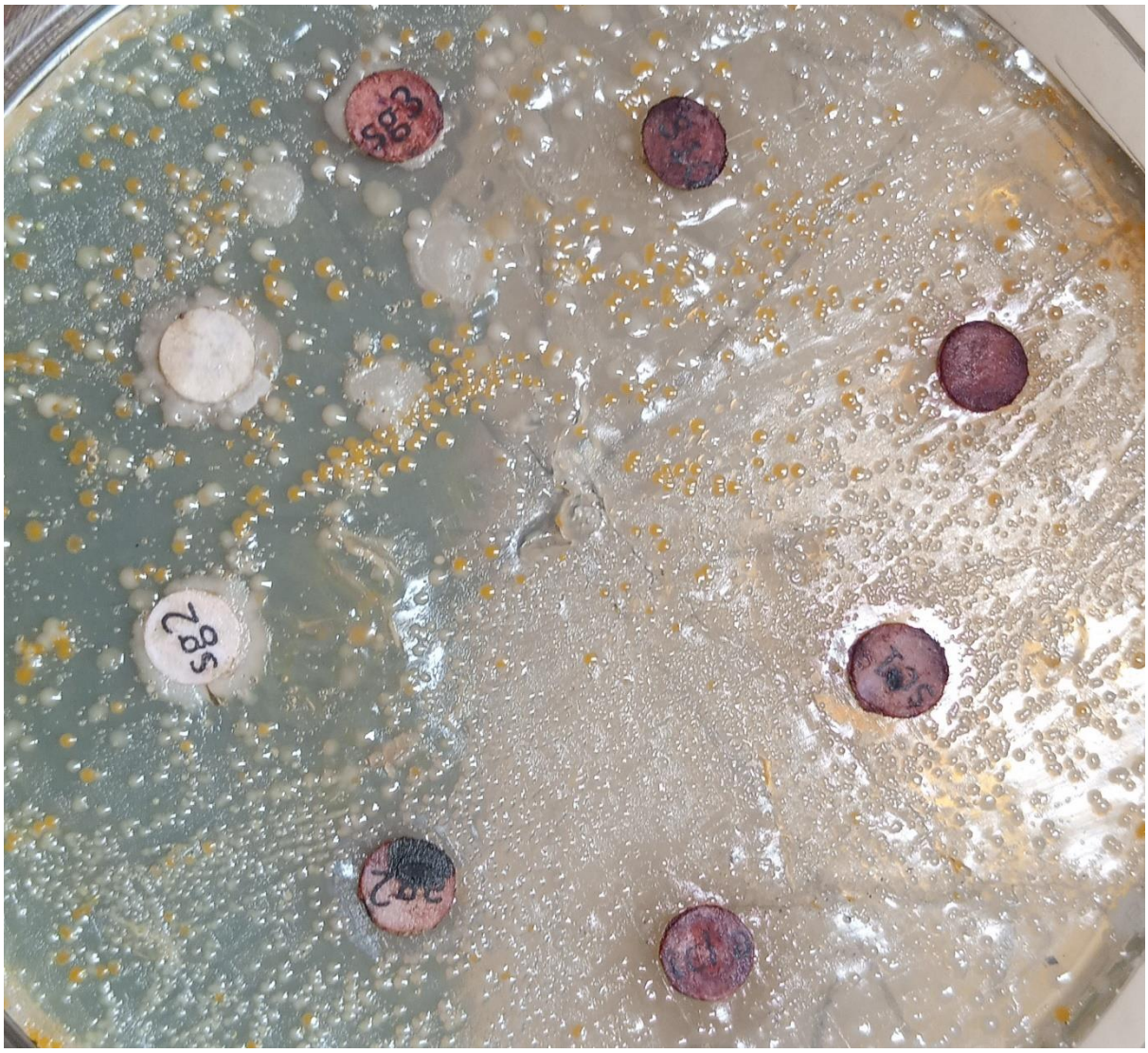

Figure 4. Prepared samples Antimicrobial susceptibility test on *S. aureus*

However, there can be exceptions to this rule, and other factors such as intermolecular forces, density, and molecular shape can also play a role in determining the weight of a compound. [32] The unreacted Guava extract and the remaining five (5) combinations did not show any inhibitory activity on both *S. aureus* and *E.coli*, which is similar to the results obtained by [25] where a drop of acid in the mixture of plant extract and azithromycin did not inhibit the growth of Salmonella spp due to a reduced concentration from 1 mg/mL to 100 µg/mL of antibiotics used, and also in line with the results obtained by [26] where a drop of sodium hydroxide followed by addition of sulphuric acid in a mixture of plant extract with Aspirin, Tetracycline and Co-trimoxazole was unable to inhibit the growth of Salmonella Spp. The no inhibition zone shown by The unreacted Guava extract and the remaining five (5) combinations is possibly due the lower concentration of plant extract used, to the addition of sulphuric acid and/or sodium hydroxide which initiated certain chemical reactions between the mixtures as show by the weight analysis but may not have favoured the formation of new active sites. [34,26]

4.0 CONCLUSION

In the various synthetic combinations carried out, Guava Aspirin Guava (**GAG, C20**) and Guava Guava (**GG, Scl**) showed inhibition of *S. aureus* and *E.coli* for 48 hrs at preparatory concentration, **Scl** shows a higher inhibition compared to **C20**, this could be from the new functional created and observed at 1688 cm^{-1} and also due to the larger molecular weight of **Scl** resulting to a total mass of 177.2 mg as to that of **C20** which is 73.1 mg. the results clearly shows that aqueous plant extract can be modify to overcome the threat of antimicrobial resistance alone or in combination with other compounds of known pharmacological activities, this methods can be explored and applied in antifungal, anti-inflammatory, antiviral and anticancer studies considering the availability and the vast types of natural products present in medicinal plants.

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Conflicts of Interest

The authors declare no competing financial interest.

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