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Research Article

Synergistic Combinatorial Strategy for Combating Antimicrobial Resistance (AMR) in Clinical Bacteria by Combining Antibiotics with Plant Extracts

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Abstract: Bacteria resistance to antibiotics used for the treatment of infections and diseases is of global concern. Medicinal plants have been used as the primary sources of plants' active ingredients or lead compounds in drug development. The combination of various antimicrobial agents to obtain a synergistic effect is considered an ideal strategy for combating bacteria resistance. In this work, a constant repetitive synergy in all combinations was achieved by adding 0.3 mL of concentrated tetraoxosulphate (vi) acid, H_2SO_4 in a mixture of *Calotropis procera* extract separately with (a) 1 mg/mL Amoxicillin, (b) 1 mg/mL Ampicillin, (c) 100 µg/mL Azithromycin and (d) 100 µg/mL Ampicillin and were heated at 110 °C for 20 minutes. Higher zones of inhibitions were observed at 16.7 mm for *Salmonella spp*, 16.4 mm for *Shigella spp*, 16.8 mm for *Staphylococcus aureus*, 21.3 mm for *Escherichia coli* and 22.4 mm for *Streptococcus spp* in situations where the antibiotics alone zone of inhibition was 0 mm at the same concentration of a, b, c, and d. These increase the regular probability model of obtaining synergism in plant extracts combination with antibiotics as shown by multiple literatures from 33% to 66% at antibiotic concentration of 100 µg/mL and 100% at antibiotic concentration of 1 mg/mL. The validation process using *Piliostigma reticulatum* extract shows that a volume of 0.1 mL of concentrated tetraoxosulphate (vi) acid in 2 mL of the mixture was enough to induce synergism to combat bacteria resistance. This work shows a cost-effective method where the antimicrobial activity of ineffective antibiotics can be enhanced and optimized using plant extracts. It can also be explored and applied in different ways to identify novel compounds, and isolate and purify their active principles for selectivity, efficacy, safety and their development as clinical trial candidates in antiviral and anticancer research to overcome enormous health challenges.

***Keywords*:** antibiotics, bacteria, *Calotropis procera*, drug resistance, infections, combination, ethnopharmacology

1. Introduction

Antibiotic are drugs used to treat bacterial infections, having different modes of action against these pathogens. But over the last few decades, some of these pathogens have developed resistant to these antimicrobial agents, which has become a major problem for clinical health, environmental health, community health, food security and development worldwide.[1]

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International trade and travel borders have become vulnerable to new forms of antibiotic resistance, as they can spread remarkably faster due to interrelationship between people from different countries.[2] Bacteria or fungi do not need to be Resistance to every available antibiotic to be considered dangerous, serious problems can arise by resistance to just one antibiotic; resistance to antibiotics caused more than 23,000 deaths a year in the United States alone, reports by the Centre for Disease Control (CDC) United States classify group B Streptococcus, non-typhoidal *Salmonella*, Methicillin-resistant *Staphylococcus aureus*, and drug-resistant *Shigella* as bacteria of urgent threat.[3]

The discovery of novel antibiotics is declining whereas persistent bacteria causing severe infections and diseases are hampering drug efficacy in their treatments due to antimicrobial resistance.[4] Several factors such as Mutation, Gene Transfer, Inappropriate Use and Diagnostics, and prescription of antibiotics to persistent patient which are yet undiagnosed are significant ways in which antimicrobial resistance happens faster.[5] Identifying novel methods to develop lead compounds for selectivity, efficacy, and safety for a clinical trial candidate remains a scientific challenge.[6] In most developing countries, medicinal plants possess complex mixtures of bioactive and inactive substances that are still in use to treat various diseases, these bioactive substance comprise of one-fourth of the active ingredients in most prescribed drugs.[7]

Medicinal plants have been shown to contain various types of simple and complex phytocompound/chemicals depending on the plant species, geographical location and also the types and nature of the phytocompounds/chemicals extracted is highly dependent on the extracting solvent.[8-11] When antibiotics are combined with plant extract for synergistic antimicrobial property, if the bacteria is already resistant to that antibiotic where no zone of inhibition is observed, it will be highly difficult near to impossible to have a sensitive antimicrobial activity at same concentration except either the extract concentration or antibiotic concentration is higly increase (overdosing). Only few literature were lucky to be able to have one out of many of their combinations to work for a specific bacteria in such manner.[12-14]

Medicinal plants also were shown to alter the spectrum of activity of synthesized nanoparticles directing it antimicrobial activity towards a specific bacteria or fungi, silver nanoparticles synthesize using *B. ciliata* based AgNPs, aqueous root extract was Nil for *E. coli*, *S. haemolyticus*, *B. cereus*. *R. dantatus* based AgNPs, aqueous root extract was positive to *E. coli* and Nil to *S. haemolyticus*, *B. cereus*. *R. hastatus* based AgNPs, aqueous root extract was positive for *B. cereus* and Nil for *S. haemolyticus* at 90 µg/well using the same reaction conditions and method of synthesis.[15] Medicinal plant extract were used to synthesize silver nanoparticle and were also combined with antibiotics to fight resistant bacteria.[16]

The commonly known milkweed *Calotropis procera*, its fresh juice of leaves and flowers were administered orally in the treatment of Malaria and intermittent fever, and decoction for Gonorrhoea treatment.[17] The 70% methanol-water extract of the stems fruit, leaves, and flowers of *C. procera* and its n-hexane, ether, chloroform and water fraction were shown to contain Glycosidic Flavonoids, Sterol, Tanins, Terpenoids, Saponins, combined Anthraquinones, and Cardiac glycosides.[18]

For centuries, concentrated tetraoxosulphate (vi) acid, H_2SO_4 and Sodium hydroxide, NaOH have been used to catalyse various reactions leading to the formation of new compounds; for example, in the formation of esters, ethers, alcohols, etc., they can be used to induce reactions between *C. procera* extracts with antibiotics at different concentrations, pH and temperature.[19] The process might adopt the principle of random selectivity of reacting specie in order of their affinity or chemical reactivity in bond breaking and bond formation in the mixture.[6]

There is limited or no available literature over the past ten years which proposed a novel or different combinatorial strategy to achieve an overall stable synergistic effect in boosting the antimicrobial properties of ineffective antibiotics using plant extracts to overcome drug-resistant bacteria at lower concentrations.

This current work seeks to demonstrate a new and consistent one-step combinatorial strategy where the antimicrobial properties of a mixture of ineffective antibiotics with medicinal plant extract can be optimized using tetraoxosulphate (VI) acid or Sodium hydroxide to overcome resistant clinical isolates of *Streptococcus spp*, *Salmonella spp*, *Staphylococcus aureus*, *Shigella spp*, and *Escherichia coli*.

2. Methods

2.1 *Sampling*

Amoxicillin 500 mg and Ampicillin 500 mg 10 capsules from MEDREICH LIMITED, Virgonagar, Bangalore-560049, INDIA, 500 mg Azithromycin VATROMAX oral capsules Vivax from Pharmaceutical Co., LTD. CHINA were purchased from pharmaceutical vendors within the Kaduna metropolis Kaduna North Kaduna, Nigeria.

Calotropis Procera and *Piliostigma reticulatum* specimens were collected in March 2022 in Sabon Tasha Kaduna and *Calotropis Procera* was authenticated by a plant taxonomist with voucher number V/N-ABU900086.

Clinical Isolates of *Streptococcus spp* (High Vaginal Swab), *Salmonella Typhi* (Stool), *Escherichia Coli* (Urine), *Shigella spp* (Stool), and *Staphylococcus Aureus* (High Vaginal Swab) were collected at Chemical Pathology, Hematology and Microbiology diagnostic laboratory of Oxford Hospital Makera, Kakuri, Kaduna State Nigeria.

2.2 *Experimental*

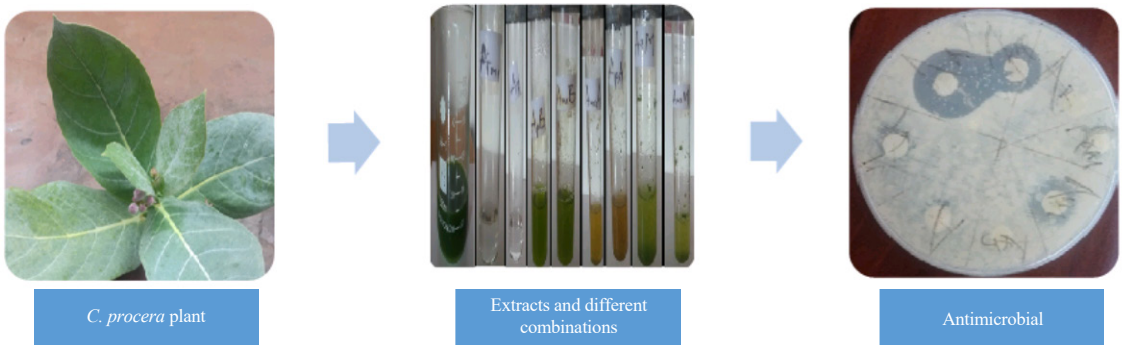

Figure 1. Showing experimental process diagram

Fresh leaves and flowers of *Calotropis Procera* combine weighing 10 g (as shown in Figure 1) were washed with distilled water, chopped with a knife, squeezed with 50 mL of distilled water, and filtered using a hand sieve to extract its juice. 1 mg/mL Ampicillin solution was prepared and labelled Ap, 1 mg/mL Amoxicillin solution was also prepared and labelled Amx. In contrast, 10% Sodium hydroxide (NaOH) was prepared to be used for alkaline combination reactions.

1 mL of the prepared Amoxicillin solution was added to a test tube containing 1 mL of *C. procera* extract, 0.3 mL of conc. Tetraoxosulphate (vi) acid (H_2SO_4) was added to it (as shown in Figure 1), the test tube was labelled AmxA, it was then heated in a water bath at 110 °C for 20 minutes, this procedure was repeated for 1 mL Ampicillin solution and was also labelled ApA.

1 mL of Amoxicillin solution was added to 1 mL of *C. procera* extract in a test tube, 0.3 mL of sodium hydroxide, NaOH was added, it was labelled AmxB, and it was heated in a water bath at 110 °C for 20 minutes; this procedure was repeated for Ampicillin solution, and it was labelled ApB.

1 mL of Amoxicillin solution was added to 1 mL of *C. procera* extract in a test tube; it was labelled AmxM and then heated in a water bath at 110 °C for 20 minutes; this procedure was repeated for Ampicillin solution and was labelled ApM, a schematic representation of this process can be seen in Figure 2.

2.3 *Screening for antibiotic resistance and antimicrobial test of prepared samples*

2.3.1 *Materials required*

- Mueller-Hinton agar
- Antibiotic discs

• Cotton swabs
• Petri dishes
• 0.5 McFarland Turbidity standard
• Inoculum
• Forceps
• Metric ruler or caliper

Streptococcus spp (High Vaginal Swab) *Salmonella spp* (Stool), *Escherichia Coli* (Urine), *Shigella spp* (Stool), and *Staphylococcus Aureus* (High Vaginal Swab) were isolated, characterized, and identified. High profile positive/negative 10 tipped multiple susceptibility antibiotic discs containing Amoxicillin, Perfloxacin, Erythromycin, Septrin, Streptomycin, Ciprofloxacin, Rocephin, Zinnacef, Ampiclox, Gentamycin, Sparfloxacin, Chloramphenicol were used for screening of resistant bacteria, while the prepared sample mixtures were used for antimicrobial test against the screened resistant bacteria. The patterns for antimicrobial susceptibility of confirmed clinical isolates of *E. coli*, *Salmonella spp*, *Shigella spp*, and *S. aureus* strains for selected antibiotics were done by Kirby-Bauer disk diffusion test using Mueller-Hinton Agar (MHA),[20] The area was Sterilize with disinfectant and open burner, a sterile cotton swab was dipped into the inoculum and excess medium was removed by pressing the swab onto the wall of the tube, the surface area of the plate was swab completely by rotating the plate. The plates were allowed to dry for 5 minutes so that the medium will absorb the inoculum properly, alcohol sterilize forceps were used in picking up the antibiotic discs and were lightly touched with the forceps to ensure that it is in good contact to avoid misplacement. The plates were then incubated upside down for 24 hours at 37 °C. After 24 hours of incubation, a metric ruler was used to measure the Zone Of Inhibition (ZOI), which include the diameter of the disc in the measurement and the results were then reported as Susceptible (S), Intermediate (I), or Resistant (R) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).[20] The antibiotics used were the common and most readily available in Nigerian markets.

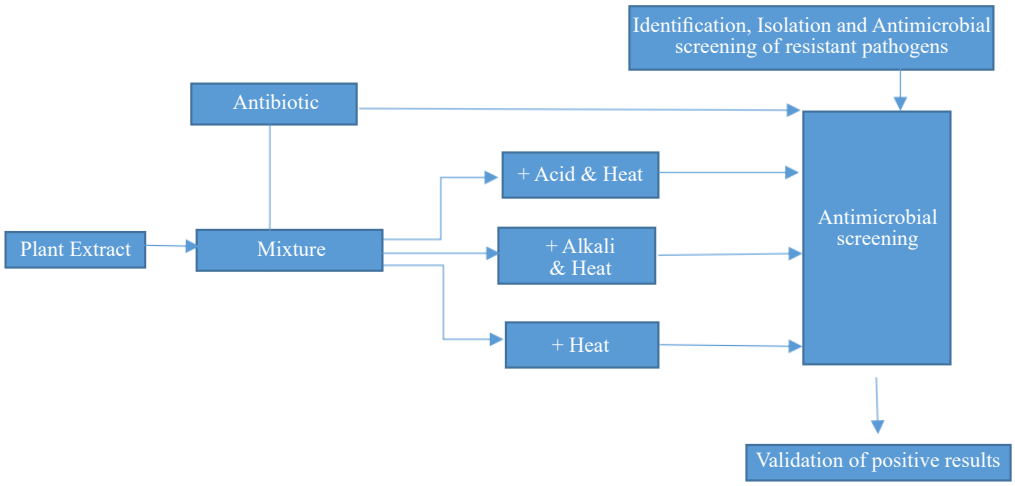

Figure 2. Schematic representation of the strategy used in the combinatorial process

2.4 *Validation of positive results*

Piliostigma reticulatum[21] was harvested, washed with distilled water, dried in the shade at 27 °C and pulverized. Exactly 10 g was transferred into a 250 mL beaker, heated to boil for 15 minutes in 100 mL distilled water and filtered using filter paper. 100 µg/mL of azithromycin solution and 100 µg/mL of Ampicillin solution were prepared and were labelled A, and B respectively.

1 mL of Ampicillin solution was added to 1 mL of *P. reticulatum* extract, and 0.1 mL of conc. H_2SO_4 was added to it and heated in a water bath at 110 °C for 20 minutes and labelled A1, this procedure was repeated for Azithromycin solution and was labelled B1.

1 mL of Ampicillin solution was added to 1 mL of *P. reticulatum* extract, 0.2 mL of conc. H_2SO_4, was added to it and heated in a water bath at 110 °C for 20 minutes and labelled A2, this procedure was repeated for Azithromycin solution and was labelled B2.

1 mL of Ampicillin solution was added to 1 mL of *P. reticulatum* extract, 0.3 mL of conc. H_2SO_4 was added to it and heated in a water bath at 110 °C for 20 minutes and labelled A3, this procedure was repeated for Azithromycin solution and was labelled B3.

3. Results and discussion

The results of susceptibility pattern of antibiotics against five microorganisms and zones of inhibition (mm) of resistant bacterial strains responses are shown in Tables 1 & 2.

Table 1. Susceptibility pattern of antibiotics against five selected microorganisms

S/N	Antibiotic	Micro grams	*Streptococcus spp*	*S. aureus*	*Salmonella spp*	*Shigella spp*	*E. coli*
1	Amoxicillin	30	+	-	-	-	-
2	Pefloxacin	10	++	+++	-	++	+++
3	Erythromycin	10	++	NT	NT	NT	NT
4	Septrin	30	+++	+++	-	+	+
5	Streptomycin	30	+++	+++	-	+	++
6	Ciprofloxacin	10	+++	+++	-	+++	+++
7	Rocephin	25	-	+++	NT	NT	NT
8	Zinnacef	20	-	NT	NT	NT	NT
9	Ampiclox	20	-	NT	NT	NT	NT
10	Gentamycin	10	++	NT	+	++	+++
11	Spafloxacin	10	NT	++	-	++	++
12	Chloramphenicol	30	NT	++	-	-	+
13	Augmentin	30	NT	-	-	-	+
14	Tarivid	10	NT	-	-	+	+

Diameter of inhibition zone: NT-Not Tested; no inhibition (-); 5-15 mm (+); 16-25 mm (++); 26-35 mm (+++)

Streptococcus spp were resistant to Rocephine, Zinnacef, and Ampiclox at 20 µg, and intermediate for Amoxicillin. *S. aureus* showed resistance to 10 µg Tarivid, 30 µg of Amoxicillin, and Augmentin. *Salmonella spp* were resistant to all tested antibiotics except for Gentamycin at 10 µg. *Shigella spp* showed resistance to 30 µg of Amoxicillin, Chloramphenicol, and Augmentin. *Escherichia Coli* was only resistant to Amoxicillin at 30 µg.

3.1 *Antibacterial activity of Amx, AmxA, AmxM, AmxB, Ap, ApA, ApM, and APB*

The antimicrobial susceptibility test in Table 2, combination of ampicillin solution in combination with *Calotropis Procera* extract using NaOH (ApB) showed a decrease in Zone Of Inhibition (ZOI) compared to the ZOI of the

antibiotic alone for *Salmonella spp*, *Shigella spp*, and *E. coli* (as shown in Figure 3), meaning NaOH might have altered the functionality of the active site present in the antibiotic or might have eventually reduce the concentration of the antibiotic present in the mixture.[13,19]

Table 2. Zone of inhibition (mm) of resistant bacterial strains in response to prepared samples

Bacteria	Samples							
	Amoxicillin (Amx)	Ampicillin (Ap)	AmxAcid	ApAcid	AmxBase	ApBase	AmxMixture	ApMixture
Salmonella spp	0	5.6 ± 0.3	16.7 ± 1.2	17.4 ± 1	5.8 ± 0.6	0	5.5 ± 0.4	0
Shigella spp	0	5.8 ± 0.5	15.4 ± 0.9	16.6 ± 0.7	0	5.3 ± 0.5	0	5.1 ± 0.3
S. aureus	0	0	16.2 ± 0.8	25.9 ± 1.2	0	0	5.0 ± 0.2	16.8 ± 0.8
E. coli	0	10.8 ± 1.2	21.3 ± 1.2	18.3 ± 0.8	0	0	0	13.4 ± 0.9

Amoxicillin solution in combination with *Calotropis Procera* extract using NaOH (AmxB) was increased a little from 0 mm to 5.8 mm for *Salmonella spp*[12,13] and remain unchanged for *Shigella spp*, *S. aureus* and *E. coli* (as shown in Figure 3). When drops of NaOH were added to the mixture, a change from light green to deep green was observed, this clearly shows that NaOH was not a favourable reagent that can be used to enhance synergism in the mixture.[19]

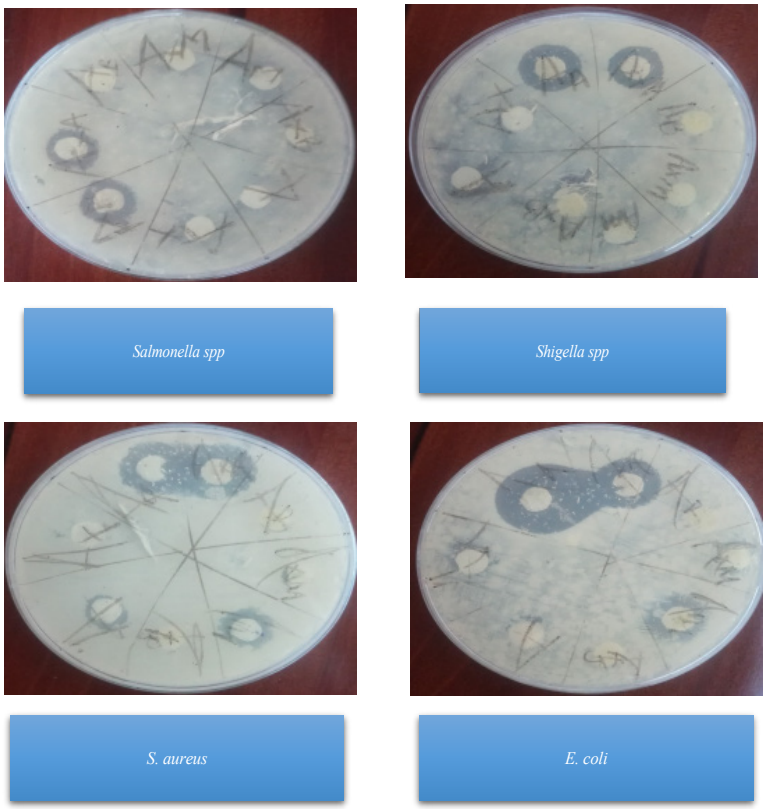

Figure 3. Showing Anti-bacterial activities of prepared samples against resistant bacteria strains

Ampicillin solution in combination with *Calotropis Procera* extract plus heat (ApM) showed a decrease in ZOI compared to the antibiotic alone in *Salmonella spp* and *Shigella spp*, this result was in similarity to that observed when NaOH was used, on the other hand the combination was synergistic for *S. aureus* with a ZOI from 0 mm to 16.8 mm, and as an additive with slight increase from 10.8 mm to 13.4 mm for *E. coli*.[22-24]

Amoxicillin solution in combination with *Calotropis Procera* extract plus heat (AmxM) showed a slight increase in ZOI from 0 mm to 5.5 mm and 5.0 mm for *Salmonella spp* and *Shigella spp* respectively, while no change in ZOI was observed for *S. aureus* and *E. coli*. These results further confirmed that plant extract combined with antibiotic for synergism is a probability based model.[12,13,23,24]

Ampicillin solution in combination with *Calotropis Procera* extract using H_2SO_4 (ApA) shows a colour change from light green to light brown,[19] there were successive increase in the ZOI of +11.5 mm, +10.8 mm, +25.9 mm and +7.5 mm for *Salmonella spp*, *Shigella spp*, *S. aureus* and *E. coli* respectively in Figure 3, while amoxicillin solution in combination with *Calotropis Procera* extract using H_2SO_4 (AmxA) also shows an increase in ZOI from 0 mm for all tested bacteria strains to a ZOI of +16.7 mm, +15.4 mm, +16.2 mm and +21.3 mm for *Salmonella spp*, *Shigella spp*, *S. aureus* and *E. coli* respectively.[12,13]

These characteristic properties exhibited by both conc. H_2SO_4 aided combination might be due to some secondary reactions in the mixture such as functional group(s) conversion/inter-conversion, ring expansion/opening/closing or extensive conjugation between the compositions of the precursors in the medium which favours the antimicrobial activity of the mixture.[19]

3.2 *Validation of positive results*

The positive results were validated from the first combinatorial method as shown in Figure 4, and Table 2 was carried out using the extract of *Piliostigma reticulatum*,[21] with an additional antibiotic (azithromycin), and an additional bacteria strain (*streptococcus spp*). The validation results in Table 3, the combination of azithromycin solution with *Piliostigma reticulatum* extract using conc. H_2SO_4 shows a stepwise increase in ZOI for *Streptococcus spp* from 0 mm to a maximum value of 22.4 mm while the same combination with ampicillin also shows the same stepwise increase in ZOI from 0 mm to a maximum value of 21.2 mm, this confirms and validate the ZOI obtained in Table 2, and also shows that an increase in the volume of acid added also increase the ZOI in *Streptococcus spp* by a minimum value of 4 mm and maximum value of 9 mm.

Table 3. Zone of inhibition (mm) of prepared sample against resistant bacteria strains

	Samples							
Bacteria	Azithromycin (A)	A 0.1	A 0.2	A 0.3	Ampicillin (B)	B 0.1	B 0.2	B 0.3
Streptococcus spp	0	11.3 ± 1.5	15.5 ± 1.5	22.4 ± 1.2	0	13.5 ± 0.8	19.7 ± 1	21.2 ± 0.9
S. aureus	13.40 ± 0.9	21.4 ± 1.2	19.2 ± 0.7	21.1 ± 1.1	0	12.2 ± 0.9	17.2 ± 1.2	16.6 ± 0.8
Salmonella spp	0	0	0	0	0	0	0	0

The combination of azithromycin solution with *Piliostigma reticulatum* extract using H_2SO_4 shows a stable increase in ZOI for *S. aureus* from 13.4 mm to 21.4 mm and an increase from 0 mm to 12.2 mm for ampicillin and *Piliostigma reticulatum* extract combination, this also revalidates the results shown in Table 2 and Figure 4 even at a reduced concentration of 100 ug/mL of antibiotics.

Both combinatory methods do not show any antimicrobial activity on *Salmonella spp*, this may be due to the reduced concentration of both antibiotics as comparing to the concentration use in Table 2 as each antibiotic has a threshold Minimum Inhibitory Concentration (MIC)[20] for different bacteria strains, and also may be due to the

phytocompounds present in *Piliostigma reticulatum*[21] because it is not used to treat intermittent fever like *Calotropis Procera*.[11]

Successive increase in ZOI was observed in *Streptococcus spp* with an increasing volume of acid from 0.1 to 0.3 mL, while an increase in ZOI values was observed in *S. aureus* upon the addition of 0.1 to 0.2 mL of acid, this was not observed upon addition of 0.3 mL of acid thus, showing that 0.1 mL of the acid is enough in 2 mL of antibiotic and plant extract to initiate reactions which may overcome resistance in bacteria.

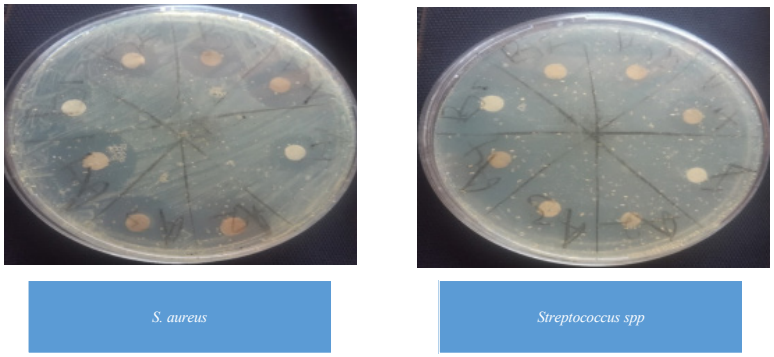

Figure 4. Showing anti-bacterial activities of prepared samples against *Streptococcus spp* and *S. aureus*

The resistance of *Salmonella spp* to all prepared samples as shown in Table 3 and Figure 4 may be due to the reduced concentration of the antibiotic by a factor of 10 because ampicillin solution of 1 mg/mL showed a ZOI of 5.6 mm in Table 2, it may also be due to the nature of phytochemicals present in *Piliostigma reticulatum*.[21]

Table 4. Comparison of acid-enhanced combination with other methods for resistant bacteria inhibition

	1	2	3	4
No. of test/bacteria strains	No. of Antibiotic used	Change in MIC from 0 to X^n	Percentage of change in MIC %	Reference
15/3	5	5	33.33	[12]
38/4	5	0	0.00	[22]
30+/9	4	0	0.00	[23]
60/2	5	0	0.00	[24]
200/10	4	21	10.5	[13]
14/7	3	12	85.7	Current work

The comparison of the results of this current work to the results other literatures expressed in percentage is shown in Table 4, and also from Figure 5, it can be seen that there is an increase of ZOI for all combinations against *Streptococcus spp* and *S. aureus* which was higher for 0.1 mL of acid with reference to the initial antibiotic concentration compared to 0.2 mL and 0.3 mL of acid used, upon subsequent addition of the volume of acid, average increase, uniformity and slight decreased in ZOI were observed for all combination against the tested bacteria strains. These validation results obtained for *Streptococcus spp* and *S. aureus* further confirm that a volume of 0.1 mL in mL of the mixture is sufficient enough to induce synergism that will overcome resistance in bacteria.

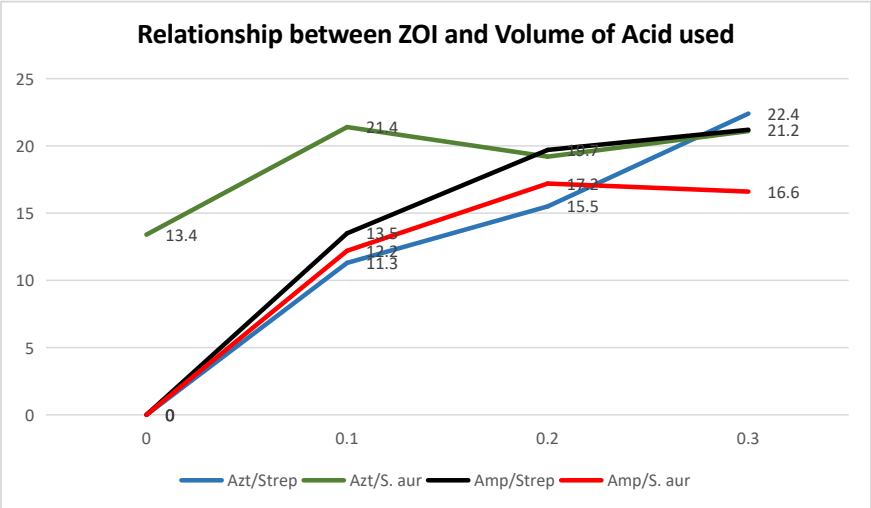

Figure 5. Further, the line intersects the data

4. Conclusions

In this work, antibiotic resistance in bacteria was overcome by adding few volumes of conc. H_2SO_4 in a mixture of *Calotropis procera* extract with three different antibiotics (amoxicillin, ampicillin, and azithromycin), higher zones of inhibitions were observed at 16.7 mm *Salmonella spp*, 16.4 mm *Shigella spp*, 16.8 mm *S. aureus*, 21.3 mm *E. coli* and 22.4 mm *Streptococcus* in situations where antibiotics ZOI was 0 mm, these removed the probability model of obtaining synergism in plant extract combination with antibiotics as shown by multiple literatures. The validation process shows that a volume of 0.1 mL in 2 mL of the mixture is enough to overcome antibiotic resistance in bacteria, this present research shows a cost-effective method which the antimicrobial activity of antibiotics can be enhanced to overcome resistance in bacteria using plant extracts, this method can be used to optimized ineffective antibiotics which their used is almost phased out due to antimicrobial resistance threat, it can also be explored and applied in different ways to identify novel compounds, isolates, and purify their active principles for selectivity, efficacy, safety and their development to the clinical trial candidate in antiviral and anticancer research to overcome enormous health challenges.

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Legal ethics

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.

Conflicts of interest

No conflict of interest associated to this work.

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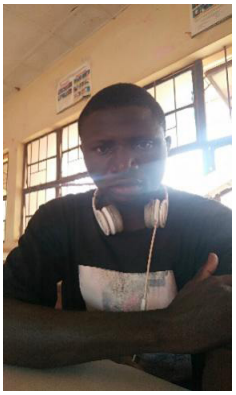
Biography



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