One-Step Combinatorial Strategy for Optimization of Antibiotics With Plant Extract Against Drug Resistant Clinical Bacteria

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Research Article

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Abstract

Antimicrobial resistance (AMR) is of global concern, resistance to every antibiotic is not an essential requirement for bacteria or fungi to be considered dangerous, and a severe problem can arise from resistance to just one antibiotic. Medicinal plants are the primary sources of active ingredients used in formulating drugs. This current work demonstrates a one-step combinatorial strategy where antibiotics can be optimized using random selectivity of phytochemicals present in aqueous plant extract, which is effective against resistant clinical isolates of *Streptococcus spp*, *Salmonella spp*, *Staphylococcus aureus*, *Shigella spp*, and *Escherichia coli*. Concentrated sulphuric acid and 10% sodium hydroxide were used in the combination of *Calotropis Procera* extract with Amoxicillin and Ampicillin at 1 mg/mL. To validate the positive results obtained in stage one, *Piliostigma reticulatum* extract was combined with 100 µg/mL of azithromycin and separately with 100 µg/ml of ampicillin, varying the volume of the acid. Higher inhibitions zones were observed at 16.7 mm for *salmonella spp*, 16.4 mm for *shigella spp*, 16.8 mm for *S. aureus*, 21.3 mm for *E.coli*, and 22.4 mm *streptococcus spp* in situations where antibiotics inhibitions zones were 0 mm.

The results of this present work report a cost-effective method by which antibiotics can be enhanced to overcome resistance in bacteria using various phytochemicals present in plant extracts. This method can 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, which may lead to being applied in antiviral and anticancer research to overcome enormous health challenges.

Introduction

Clinical, environmental, community health, food security, and development are currently threatening by antimicrobial resistance (AMR). International borders are vulnerable to new forms of antibiotic resistance, as they can remarkably spread faster due to international trade and travel\(^1\). Resistance to every available antibiotic is non-essential requirement for bacteria or fungi 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 Center 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.\(^2\)

The Discovery of new antibiotics is slow; almost all clinical bacteria isolates have slowly developed resistance mechanisms. Persistent bacteria causing severe infections and diseases hamper drug efficacy in their treatments due to antimicrobial resistance.\(^3\)

Mutation, Gene Transfer, Inappropriate Use and Diagnostics, and prescription of antibiotics to persistent yet undiagnosed patients are significant ways resistance in bacteria happens faster.\(^4\)
Identifying novel methods to develop lead compounds for selectivity, efficacy successfully, and safety for a clinical trial candidate remains a scientific challenge. Medicinal plants possess complex mixtures of bioactive and inactive substances still in use to treat various diseases in most developing countries. Also, these bioactive substances comprise one-fourth of the active ingredients of drugs prescribed in developed countries. Limited literature data on the combination of plant extract with antibiotics shows synergy to combat resistant bacteria better than the antibiotic used in the combination. Plant extract were used to synthesize silver nanoparticle and were also combined with antibiotics to fight resistant bacteria, there are limited or no available literature over the past ten years which proposed a novel or different combinatorial strategy in molecular modification of ineffective antibiotics with plant extract to improve their antimicrobial activity greater than the antibiotic used for combination at lower concentrations to overcome drug-resistant bacteria.

The commonly known milkweed *Calotropis procera*, has been used in various forms for the treatment of common infections and diseases. Fresh juice of *Calotropis procera* leaves and flowers were administered orally in the treatment of Malaria and intermittent fever, decoction for Gonorrhoea treatment.

Various reactions leading to the formation of new compounds reversibly or irreversibly are often catalyzed by acids or alkalis; for example, in the formation of esters, ethers, alcohols, etc., plant extracts and antibiotics are polyfunctional organic molecules with different degrees of reactivity at various concentrations, pH and temperature. The optimization of antibiotics in this research adopts the principle of random independent selectivity of reacting specie in order of their affinity in bond breaking and bond formation since plant extracts is a mixture of complex organic compounds containing various phytochemicals, these phytochemicals are allowed to independently conjugate with the antibiotic in any way possible to give an extended antibiotic which may pose new antimicrobial properties against human and animal pathogens.

Side-effect associated with antimicrobials and the development of bacteria resistance have shifted the focus of research toward Ethnopharmacology; this current work seeks to demonstrate a one-step combinatorial strategy where antibiotics can be optimized using random selectivity of the phytochemicals present in aqueous plant extract, making it highly effective against resistant clinical isolates of *Streptococcus spp*, *Salmonella spp*, *Staphylococcus aureus*, *Shigella spp*, and *Escherichia coli*.

## 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 was purchased pharmaceutical vendors within the Kaduna metropolis Kaduna North Kaduna, Nigeria.
Calotropis Procera and Piliostigma reticulatum specimens were collected 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.

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

1 mL of Amx was added to a test tube containing 1 mL of plant extract, 0.3 mL of tetraoxosulphate (vi) acid (H₂SO₄) was added, the test tube was labeled AmxA and was heated in a water bath at 110 °C for 20 minutes, this procedure was repeated for Ap and was labeled ApA.

1 mL of Amx was added to 1 mL of plant extract in a test tube, 0.3 ml of NaOH was added, labeled AmxB, and heated in a water bath at 110 °C for 20 minutes; this procedure was repeated for Ap and was labeled ApB.

1 mL of Amx was added to 1 mL of plant extract in a test tube; it was labeled AmxM and was heated in a water bath at 110 °C for 20 minutes; this procedure was repeated for Ap and was labeled ApM.

2.2 Screening for antibiotic resistance and antimicrobial test of prepared samples

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. 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) adopting the method used by, High profile positive /negative 10 tipped multiple susceptibility antibiotic discs containing Amoxicillin, Perfoxacin, Erythromycin, Seprin, Streptomycin, Ciprofoxacin, Rocephin, Zinnacef, Ampiclox, Gentamycin, Sparfoxacin, Chloramphenicol, Augmentin and Tarivid were placed on the top layer of agar plates, incubated at 37°C for 24 h. The isolates resistant to antimicrobials were considered drug-resistant strains according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The antibiotics used were common and readily available in Nigerian markets.

The identified resistant bacteria strains were cultured and the zone of inhibition (ZOI) in millimeters (mm) for Amx, Ap, AmxA, APA, AmxB, APB, AmxM, and ApM were recorded.
2.3 Validation of positive results

*Piliostigma reticulatum* 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 and 100 µg/mL of Ampicillin were prepared and labeled A, and B respectively.

1 mL of A was added to 1 mL of plant extract, and 0.1 mL of H<sub>2</sub>S0<sub>4</sub> and heated in a water bath at 110 °C for 20 minutes and labeled A, this procedure was repeated for B and was labeled B1.

1 mL of A was added to 1 mL of plant extract, 0.2 mL of H<sub>2</sub>S0<sub>4</sub>, and heated in a water bath at 110 °C for 20 minutes and labeled A, this procedure was repeated for B and was labeled B1.

1 mL of A was added to 1 mL of plant extract, 0.2 mL of H<sub>2</sub>S0<sub>4</sub>, and heated in a water bath at 110 °C for 20 minutes and labeled A, this procedure was repeated for B and was labeled B1.

**Results And Discussion**

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

**Table 1.** Susceptibility pattern of antibiotics against different microorganisms.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Antibiotic</th>
<th>µg</th>
<th><em>Streptococcus spp</em></th>
<th><em>S. aureus</em></th>
<th><em>Salmonella spp</em></th>
<th><em>Shigella spp</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amoxicillin</td>
<td>30</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Pefloxacin</td>
<td>10</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>Erythromycin</td>
<td>10</td>
<td>++</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>Septrin</td>
<td>30</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Streptomycin</td>
<td>30</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>Ciprofloxacin</td>
<td>10</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>Rocephin</td>
<td>25</td>
<td>-</td>
<td>+++</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>Zinnacef</td>
<td>20</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>Ampiclox</td>
<td>20</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>Gentamycin</td>
<td>10</td>
<td>++</td>
<td>NT</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>11</td>
<td>Spafloxacin</td>
<td>10</td>
<td>NT</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>Chloramphenicol</td>
<td>30</td>
<td>NT</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Augmentin</td>
<td>30</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Tarivid</td>
<td>10</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
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.


The antimicrobial susceptibility test in Table 2 showed that *Salmonella* spp were resistant to Amx, ApB, and ApM with no zone of inhibition in mm (ZOI), slightly intermediate to Ap (ZOI 5.6±0.3), AmxB (ZOI 5.8 ± 0.6), and AmxM (ZOI 5.5±0.4), was susceptible to AmxA (ZOI 16.7±1.2), ApA (ZOI 17.4±1). *Shigella* spp were resistant to Amx, AmxB, and AmxM with no ZOI, slightly intermediate to Ap (ZOI 5.8±0.5), ApM (ZOI 5.1±0.3), susceptible to AmxA (ZOI 15.4±0.9), ApA (ZOI 16.6±0.7).

*S. aureus* were resistant to Amx, Ap, AmxB, and ApB with no ZOI, slightly intermediate to AmxM (ZOI 5.0±0.2), were susceptible to AmxA (ZOI 16.2±0.8), ApA (ZOI 25.9±1.2), and ApM (ZOI 16.8±0.8). *E. coli* were resistant to Amx, AmxB, ApB, and AmxM with no ZOI and susceptible to Ap (ZOI 10.8±1.2), AmxA (ZOI 21.3±1.2), ApA (ZOI 18.3±0.8), and ApM (ZOI 13.4±0.9).


<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Salmonella</em> s</td>
<td>0</td>
<td>5.6±0.3</td>
<td>16.7±1.2</td>
<td>17.4±1</td>
<td>5.8±0.6</td>
<td>0</td>
<td>5.5±0.4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td><em>Shigella</em> s</td>
<td>0</td>
<td>5.8±0.5</td>
<td>15.4±0.9</td>
<td>16.6±0.7</td>
<td>0</td>
<td>5.3-0.5</td>
<td>0</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td>3</td>
<td><em>S. aureus</em></td>
<td>0</td>
<td>0</td>
<td>16.2±0.8</td>
<td>25.9±1.2</td>
<td>0</td>
<td>0</td>
<td>5.0±0.2</td>
<td>16.8±0.8</td>
</tr>
<tr>
<td>4</td>
<td><em>E. coli</em></td>
<td>0</td>
<td>10.8±1.2</td>
<td>21.3±1.2</td>
<td>18.3±0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13.4±0.9</td>
</tr>
</tbody>
</table>

Higher ZOI was observed in AmxA and ApA greater than their corresponding group mixtures, in all tested bacteria strains, higher ZOI of 16.8±0.8, and 13.4±0.9 were shown in ApM for *S. aureus* and *E. coli*, still this ZOI are less compared to the corresponding ZOI of ApA which were 25.9±1.2 and 18.3±0.8 for *S. aureus*, and *E. coli*.

The increase in ZOI for acid-catalyzed synthesis is due to the linking of functional groups, substituents, or change in ring size of possibly the non-active sites of the antibiotics and the different phytochemicals.
present in *Calotropis procera*; fig 5 confirms a reaction in the mixture with a color change from light green to brown.

No observable changes in ZOI of alkali combinatorial synthesis, this may be due to the linking of functional groups, substituents, or change in ring size of possibly the non-active sites of the antibiotics, and the phytochemicals present in *Calotropis procera* with colour change from light to dark cloudy green solution. The heat catalyzed mixture of both antibiotics were able to overcome resistance with ZOI increase from 0 to 5.5±0.4 mm for *salmonella spp*, 0 to 5.0±0.2 *E. coli* in amoxicillin and a +16.8 mm increase in *S. aureus* for ampicillin only, thus confirming the antimicrobial properties exhibited by the plant *Calotropis Procera* extract.

3.2 Validation of positive results

The positive results were validated from the first combinatorial synthetic method as shown in Figure 5, and Table 2 was carried out using extract of *Piliostigma reticulatum* with an additional antibiotic (azithromycin), and an additional bacteria strain (*streptococcus spp*).

*Streptococcus spp* were resistant to A and B with no ZOI, susceptible to A1 (ZOI 11.3±1.5), A2 (ZOI 15.5±1.5), A3 (ZOI 22.4±1.2), B1 (ZOI 13.3±0.8), B2 (ZOI 19.7±1), and B3 (ZOI 21.2±0.9). *S. aureus* were resistant to B, with no ZOI, and were susceptible to A (ZOI 13.4±0.9), A1 (ZOI 21.4±1.2), A2 (ZOI 19.2±0.7), A3 (ZOI 21.1±1.1), B1 (ZOI 12.2±0.9), B2 (ZOI 17.2±1.2), and B3 (ZOI 16.6±0.8). *Salmonella spp* were resistant to A, A1, A2, A3, B, B1, B2, and B3 with no ZOI.

The successive increase 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.

The resistance of *Salmonella spp* to all prepared samples is due to the reduced concentration of the antibiotic by a factor of 10 because ampicillin and 1 mg/mL showed a ZOI of 5.6±0.3 in Table 2, and may also be due to the nature of phytochemicals present in *Piliostigma reticulatum*.

**Table 3** Zone of inhibition (mm) of 3 resistant bacterial strains in response to A, A1, A2, A3, B, B1, B2, and B3
This further confirms the necessity of prerequisite knowledge of the plant’s ethnopharmacological usage and the resistant pathogen before any combinatorial method.

A decrease in color intensity and smell was observed with successive addition of acid from 0.1 to 0.3 mL as shown in Figure 6, at 0.3 mL no smell was detected in the case of ampicillin, this implies that at 0.1 and 0.2 mL of acid added the antibiotic still retains some of the functional groups or substituents responsible for its characteristic smell, but on the addition of another 0.1 mL of acid the smell was not detected, indicating that particular functional groups or substituents were involved in conjugation or might be substituted.14

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

<table>
<thead>
<tr>
<th>Bacteria strain</th>
<th>Antibiotic 1</th>
<th>Antibiotic 2</th>
<th>Mixture MIC</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. chlororaphis</td>
<td>Cefotaxime</td>
<td>Nil</td>
<td>12.5 µg/ml</td>
<td>9</td>
</tr>
<tr>
<td>P. monteilii</td>
<td>Rifampicin</td>
<td>Nil</td>
<td>0.195 µg/ml</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>Ticarcillin 5 µg/ml</td>
<td>Nil</td>
<td>16.00 mm</td>
<td>8</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>Chloramphenicol 30 µg</td>
<td>Nil</td>
<td>18.33 mm</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>Tetracycline 30 µg</td>
<td>Nil</td>
<td>11.00 mm</td>
<td>7</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
<td>12.00 mm</td>
<td></td>
</tr>
</tbody>
</table>

As shown in Table 4, the former combinatorial method is a trial based on a probability approach where the combination may or may not inhibit bacterial growth with the effect being either additive, antagonistic or synergistic even with the right medicinal plants. Still, in acid catalyze methods at lower concentrations with the right medicinal plants, there is a higher possibility that all combinations will be synergistic.

Conclusions
In this work, antibiotic resistance was overcome using acid catalyzes the combinatorial synthesis 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. The results of this present research proposed cost-effective methods by which antibiotics can be enhanced to overcome resistance in bacteria using various phytochemicals present in plant extracts, this method can 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, it may also be applied in antiviral and anticancer research to overcome enormous scientific challenges.

**Declarations**

**Acknowledgments**

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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 conflicts of interest are associated with this work.

**Funding**

This research work is self-funded.

**References**


Figures

**Figure 1**

Schematic representation of the combinatorial synthesis process.
Figure 2

Schematic representation of results validation process in fig. 1

Figure 3

showing antimicrobial activity of purchased antibiotic disk against tested bacteria strains.

1. Streptococcus spp, 2. Salmonella spp 3. S. aureus
Figure 4


Figure 5

Showing plant extract in beaker and labeled test-tubes containing different products of the synthesis.
Figure 6

showing plant extract, acid catalyse combination before heating and after heating.

Figure 7

showing Anti-bacterial activities of A1, A2, A3, A4, B1, B2, B3, and B4 against tested bacteria strains.