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Antibacterial Evaluation of Ethanolic Leaf Extract of *Gongronema Latifolium* Benth on MDR Bacteria from Clinical Specimens

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Abstract

The rapid emergence of antibiotic resistance among hospital pathogens is a serious threat to the management of infectious diseases. The resistance problem demands that a renewed effort be made to seek effective antibacterial agents from bioactive phytochemicals of Plants. This study was carried out to evaluate the antibacterial activity of the ethanolic leaf extract of Gongronema latifolium on multidrug resistant clinical bacterial isolates. The antibacterial effects of ethanolic leaf extracts of *G. Latifolium* were evaluated by the agar well diffusion and micro broth dilution methods on multidrug resistant strains of *E. coli, S. aureus, S. saprophyticus* and *K. pneumoniae* isolated from tertiary hospital. Phytochemical screenings were also conducted on the *G. Latifolium* leaf extracts. Our study has shown high rate of resistance of *E. coli, S. aureus, S. saprophyticus* and *K. pneumoniae* isolates to some frontline antibiotics.

The Phytochemical analysis revealed that *G. Latifolium* leaf contains alkaloids, tannins, saponnins, cardiac glycosides, flavonoids, steroids and protein and these phytoconstituents can be said to be responsible for the antibacterial activity of the plant. The extract showed good activity against the multiple drug resistant bacteria in order of *K. pneumoniae > E. coli > S. aureus >* and *S. saprohyticus*. The ethanolic leaf extract of *G. latifolium* can be effectively used in the eradication/treatment of clinical infections caused by the multiple drug resistance isolates of *K. pneumoniae, E. coli, S. aureus* and *S. saprophyticus.*

Keywords: *Gongronema latifolium; MDR; Bacteria; clinical specimens; phytochemical; South-eastern Nigeria;*

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Introduction

Herbal plants by their high content of secondary metabolites are beneficial to man's health for the prevention and management of diseases/infections. The use of plant and/or plant products for the treatment of infections and as alternative medications is an age old custom in most of Nigerian homes [1-2]. Infections caused by resistant microorganisms are not responding to the standard treatment, resulting in prolonged illness, higher -health care expenditures and - risk of death [3-4]. Emerging resistance mechanisms are threatening our ability to treat common infectious diseases with consequences of death and higher medical cost.

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The development and spread of antibiotic resistant strains of pathogenic bacteria as well as the occurrence of undesirable side effects of some antibiotics have heightened the interest on exploiting plant materials as alternatives to some conventional antibiotics [5]. The resistance problem therefore renewed efforts/ strategies in searching for antibacterial agents effective against pathogenic bacteria resistant to current antibiotics [5-6]. One of the strategies involves localization of plant bioactive phytochemicals [4]. Thus new prototype antimicrobial agents are needed and plants are among the most important common sources of potentially valuable new drugs.

Plants have an almost limitless ability to synthesize aromatic substances, which serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. They are good natural sources of medications; provide raw materials for modern pharmaceuticals and are safe, less toxic and economical [4, 6-7]. *Gongronema latifolium* is a tropical rainforest plant of the order Gentiales and the family Apocynaceae. It belongs to the Subfamily Asclepiadaceae, and genus Gongronema [8]. In South-eastern Nigeria, the Igbos call it "Utazi" and it is utilized for its medicinal and culinary properties as spice and vegetable for sauces and soups [8-9].

It has been reported that *Gongronema latifolium* has an antimicrobial activity against some species of microorganisms [9-10]. In traditional medicine, *Gongronema latifolium* are used for the treatment of different bacterial infections such as the use in the treatment of stomach pains/infections. Therefore, this study was carried out to evaluate the antibacterial activity of the ethanolic leaf extract of *Gongronema latifolium* on multidrug resistant (MDR) clinical bacterial isolates.

Materials and Methods

Mueller-Hinton Agar was procured from Biomark Laboratory (India), Mannitol Salt Agar, MacConkey Agar, Nutrient Agar and Nutrient Broth (All procured from Titan Biotech. Ltd; India), Peptone Water (HiMedia Labs. Ltd; Mumbai, India) while Antibiotic Sensitivity disc was from Oxoid ltd. (England).

Collection and identification of plant materials

The leaves of the plant, *Gongronema latifolium* were bought from Eke-ututu market of Orsu-Ihiteukwa in Orsu Local Government Area of Imo state, Nigeria. The plant was identified and authenticated by a botanist Mr. Alfred Ozioko of University of Nigeria Nsukka and the Herbarium specimen deposited in the Pharmacognosy Department of Nnmadi Azikiwe University, Awka. The plant samples were washed using water and air-dried and then ground into uniform powder using Thomas Willey milling machine.

Preparation of the plant extract

Two hundred grams (200g) of the leaves of the plant was cold macerated with one liter of absolute ethanol in two separate maceration container containing 100g per 500 mL each for 48 hours. At the end of the extraction, the suspension was filtered with Whatman filter paper No. 1 (Whatman, Maidstone, England) and the filtrate was dried in a hot air oven regulated at 50°C (AMPUL dryer) and the dried filtrates was stored in the fridge at 4°C for further use.

Study Area

The urine, stool and wound samples were collected from both inpatients and outpatients of the Chukwuemeka Odimegwu Ojukwu University Teaching Hospital, (COOUTH) Awka, whereas the orthopaedic wound samples were collected from Orthopaedic hospital Enugu. This sample collection sites were chosen because they covers the urban area of the city of Anambra and Enugu (South-eastern Nigerian states) respectively where high population of individuals /referrals with varieties of infections are found. The study protocol was approved by the ethical committee of Chukwuemeke Odumegwu Ojukwu University Teaching Hospital (COOUTH) Awka (Refs: COOUTH/AA/VOI.I.002 & VOI.I.008).

The duration of the study was 5 months (January to May 2016). The study was carried out in the Microbiology Laboratory of the Department of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Agulu Campus.

Study Population

Various clinical samples (urine, stool and orthopaedic wound swabs) of patients comprising of both male and female patients both inpatients outpatients were collected after their informed consent was obtained.

Identification and Characterization of Isolates

Identification of bacterial isolates was done on the basis of their cultural, microscopic and biochemical characteristics [11].

Antibiotic Susceptibility Testing and detection of multidrug resistance isolates

Antibiotic susceptibility of pure cultures of confirmed isolates was performed on Mueller Hinton Agar by the standard Kirby Bauer disc diffusion method as described elsewhere [12]. The following antibiotics were tested : Levofloxacin (LEV) 5 µg, Ceftriaxone (CEF) 30 µg, Gentamycin (GEN) 30 µg, Azithromycin (AZM) 15µg, Penicillin (P) 10UNIT, Nitrofurantoin (NIT) 300 µg, Amoxicillin/Clavulanate (AMC) 30 µg.

The Inhibition Zone Diameter in millimeter (IZD in mm) produced were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2011) and British Society for Antimicrobial Chemotherapy (BSAC). After interpretation of the result, the organisms that were resistance to at least four (4) of the antibiotics used were considered multidrug resistant (MDR) and were selected for further evaluation of the antibacterial activities of the plant extracts.

Phytochemical Screening

Photochemical tests were carried out on the ethanolic extract and on the powdered specimens using standard procedures to identify the constituents [5].

Determination of antibacterial activity of the plant extract

Agar well diffusion method as described by Esimone., *et al*. [13] was used to determine the antibacterial activity of the extract. Briefly, 800 mg/mL concentration of the ethanolic extracts was prepared by dissolving 1.6g of the extract in 2 mL of 10% DMSO and then a 2-fold serial dilution was done to obtain 400 mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL concentrations for further use. Mueller Hinton agar was prepared according to the manufacturer's instructions. Overnight broth cultures of the organisms were standardized to 0.5 MacFarland standard. With the aid of a sterile 1mL syringe, 1mL of the standardized suspension of the isolates was mixed with 19 mL of the agar in sterilized petri-dishes. The inoculated agar plates were allowed to solidify and the plates were properly labeled. A sterile cork borer was used to bore eight holes in the agar with a diameter of 7 mm.

Using a micropipette, 0.1 mL of the reconstituted plant extracts were delivered into seven of the labeled wells; and 0.1 mL of a 10 µg/mL Gentamicin solution (Gentalek, India) was used as a positive control in the 8th hole. The plates were left for 30 minutes at room temperature to allow the extracts and the drug to diffuse into the agar. This was done in duplicates for each concentration of the extract against the three microorganisms used. The plates were incubated at 37°C for 24 hrs. After incubation, the plates were observed for inhibition zones around the wells. The diameters were measured and the mean inhibition zone diameter (IZD) was recorded to the nearest whole millimeter.

Determination of the minimum inhibitory concentration (MIC)

The MIC was determined using Agar dilution method as described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) [14]. An 800 mg of the extract was weighed using a sensitive balance and reconstituted in 4 mL of 10% DMSO to obtain a concentration of 200 mg/mL. A two-fold serial dilution was made to obtain concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL. A 19 mL of the molten Muller-Hinton agar was mixed with 1 mL of the plant extract dilutions in sterile Petri dishes and labelled accurately.

The mixture was allowed to solidify and a sterile wire loop was used to streak a loopful of the standardized inoculum on the surface of the dried agar. The plates were then incubated at 37°C for 24 hrs, after which the plates were observed for the presence of turbidity. The lowest dilution showing no turbidity was recorded as the MIC. A control experiment was run in parallel to study the impact of the solvent (i.e. DMSO) on the growth of the tested organism. The tests were done in duplicate against MDR *E. coli, K. pneumoniae S. aureus* and *S. saprophyticus* and the mean MIC was recorded to the nearest whole concentration.

Determination of Minimum Bactericidal Concentration (MBC)

This was done as an extension of the MIC assay as the agar plates showing no growth of visible colonies in the MIC evaluation were used to determine the MBC. The plates were further incubated at 37°C for 72 hours. The lowest concentrations showing no turbidity were recorded as the MBC for the different organisms.

Results and Discussion

The increasing incidence of antibiotic-resistant pathogens has drawn the attention of the pharmaceutical/ scientific communities towards research on the potential antimicrobial activity of plant-derived substances [5,15]. This study is designed to evaluate the antibacterial effects of *G. latifolium* (UTAZI) on multidrug resistant clinical bacterial isolates. A high degree of resistance of the bacterial isolates to multiple classes of antibiotics used was noted. Almost all the antibiotics proved less effective against all tested bacteria isolated from clinical samples.

Table 1 shows the results of the antibiotic resistance profile of the bacterial isolates. It revealed that most of the isolated bacteria were found to be resistant to the conventionally used antibiotics. All the bacterial isolates were highly resistant (60-100%) to Azithromycin, Penicillin, nitrofurantion, levofloxacin and penicillin. They all had good/moderate sensitivity to Amoxicillin – clavualnic acid as against penicillin and Ceftriaxone. This may be due to presence of clavulanic acid in the combination that expectedly should protect the Amoxicillin component against the activities of β-lactamases and increased indiscriminate and/ or unregulated use of the β-lactam antibiotics in the studied area leading to emergence of antibiotic resistant bacteria with consequent emergence of β-lactamases [16].

Isolates	AMC			GEN			LEV			NIT			PCN			AZM			CEF		
	S	MS	R	S	MS	R	S	MS	R	S	MS	R	S	MS	R	S	MS	R	S	MS	R
S. aureus	71.4	0	28.6	4.8	4.8	90.4	14.3	-	85.7	5	-	95	0	0	100	7.3	4.9	87.8	0	0	100
E. coli	30	20	50	0	10	90	20	20	60	20	-	80	0	0	100	0	0	100	0	0	100
K. pneumoniae	10	40	50	5	0	95	30	10	60	20	-	80	0	0	100	0	0	100	0	0	100
S. saprophyticus	60	0	40	5	0	95	0	5	95	5	-	95	0	0	100	0	0	100	0	0	100

Table 1: *Antibogram of the Bacterial Isolates.*

KEY: AMC = Amoxicillin-clavulanic acid, GEN = Gentamicin LEV = Levofloxacin PCN = Penicillin AZM = Azithromycin, CEF = Ceftriaxpne S = % Susceptible R= % Resistant MS = %Moderately sensitive

Antibiotics resistance occurs when antibiotics are over prescribed or misused [17-18]. It is this kind of MDR scenario or risk factors in the development of resistance amongst clinically important pathogens that necessitates development and search for novel sources (e.g. plants) of antimicrobial agents. The phytochemical analysis of the plant extract (Table 2) showed that ethanolic leaf extract of *G.latifolium* contain some phytoconstituents such as saponins, tannins, flavonoids, alkaloids, cardiac glycosides, proteins, steroids with no trace of terpenoids and these constituents can be associated with some of the beneficial antibacterial effects of the plant as shown in Tables 3-6.

S/No	Phytochemical test	Plant Extract
1	Alkaloid	+
2	Saponins	+++
3	Tannins	+++
4	Flavonoid	++
5	Steroid	+++
6	Terpenoid	_
7	Cardiac Glycoside	+
8	Protein	+

Table 2: *The photochemical screening of the plant extract.*

+ = mildly present, ++ = moderately present, +++ = abundantly present, - = absent.

The antibacterial activities of the ethanolic leaf extract of Gongronema latifolium Benth on MDR bacterial isolates was evaluated in terms of IZD, MIC and MBC. The ethanolic leaf extract of G. latifolium as observed from this work, have concentration dependent inhibitory effect on the MDR test organisms *E. coli, S. aureus, S. saprophyticus* and *K. pneumoniae* (Tables 3-6). The inhibition zone diameter ranged from 2.0-8.5 mm for MDR *E. coli, S. aureus* and *S. saprophyticus* and 2.0-9.5 mm for MDR *K. pneumonia.*

Inhibition zone diameter (mm)								
Isolates	**400 mg/mL**	**200 mg/mL**	**100 mg/mL**	**50 mg/mL**	**25 mg/mL**	**12.5 mg/mL**	**6.25 mg/mL**	**Positive control**
K1	8.5	6.5	5	4.5	5	5	5.5	0
K2	6.5	7	6	6	6	3.5	4.5	10
K3	6.5	7.5	4	5	3	2.5	2	11.5
K4	6.5	7	6	4	4	4	3	0
K5	7	5.5	5	3	4.5	4.5	3.5	6
K6	7.5	6.5	6	5	5.5	4.5	7	0

Table 3: *The Inhibition Zone Diameter of the Plant Extract against K. pneumoniae.*

Inhibition zone diameter (mm)								
Isolates	**400 mg/mL**	**200 mg/mL**	**100 mg/mL**	**50 mg/mL**	**25 mg/mL**	**12.5 mg/mL**	**6.25 mg/mL**	**Positive control**
E1	7	5	5	3.5	3.5	3	2	10
E2	8	6	5	4.5	4.5	4.5	6	0
E3	0	0	0	0	0	0	0	0
E4	6.5	6.5	6	5	2	3	4	0
E5	8.5	7.5	5.5	4.5	4	3	2.5	0
E6	8.5	8	6	4.5	2.5	2.5	0	13.5
E7	0	0	0	0	0	0	0	0
E8	7.5	7	7.5	4	0	0	0	8
E9	8	7.5	7.5	5.5	2.5	3	3	8

Table 4: *The Inhibition Zone Diameter of the Plant Extract against E. coli.*

Inhibition zone diameter (mm)								
Isolates	**400 mg/mL**	**200 mg/mL**	**100 mg/mL**	**50 mg/mL**	**25 mg/mL**	**12.5 mg/mL**	**6.25 mg/mL**	**Positive Control**
S1	7	7.5	6	5	3.5	0	0	0
S2	7.5	5.6	5.5	4.5	4	3.5	3	4.5
S3	8.5	7	7	4	3.5	3.5	2	9
S4	4.5	4	4.5	0	0	0	0	0
S5	7.5	6.5	5	2.5	0	0	0	0
S6	7	6.5	3.5	3.5	4	4.5	2.5	0
S7	8	7	6.5	5.5	3.5	3.5	2	10.5
S8	7.5	8.5	4	5	2.5	3	2	6.5
S9	7.5	6.5	6	5	4.5	4	3.5	8.5
S10	8.5	7.5	4.5	3.5	3.5	4	3.5	5

Table 5: *The Inhibition Zone Diameter Of The Plant Extract Against S.aureus.*

Inhibition zone diameter (mm)								
Isolates	**400 mg/mL**	**200 mg/mL**	**100 mg/mL**	**50 mg/mL**	**25 mg/mL**	**12.5 mg/mL**	**6.25 mg/mL**	**Positive Control**
Ss1	9.5	7	5.5	3	3.5	3	3	3
Ss2	7.5	7	4.5	3	3.5	2.5	3	12.5
Ss3	8	7.5	3.5	4	4	3	2	5.5
Ss4	7	6	7	5	4.5	3	3	5
Ss5	7	6	6	5	5	4	3.5	9
Ss6	8	7	6.5	6	5.5	4.5	3.5	5.5
Ss7	6	5	4	3.5	4	4	3	9
Ss8	7	6.5	5.5	5.5	4.5	3.5	3	8
Ss9	7.5	6	6	5.5	5	4.5	3	6.5

Table 6: *The Inhibition Zone Diameter of the Plant Extract against S. saprophyticus*

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the plant extract against the bacterial isolates is shown in Table 7. The MIC of the extract against *K. pneumoniae* isolates ranged from 0.3125-1.25 mg/ml and MBC range from 0.625-2.5 mg/ml, for *Escherichia coli* isolates the MIC of the extract ranged from 0.3125-2.5 mg/ml and MBC range from 1.25-2.5 mg/ml. The Minimum Bactericidal Concentration (MBC) of the plant extract against the *S. aureus* ranges from 0.625-2.5 mg, The Minimum Inhibitory Concentration (MIC) of the plant extract against the *S. saprophyticus* ranges from 0.625-2.5 mg and the MBC ranges from 1.25-10 mg.

Phytochemical analyses of different parts of *G. latifolium* have shown that the plant is rich in quite a good number of phytochemicals. The Phytochemical studies of the *G. latifolium* have shown that the antibacterial properties of these plants depend on certain active ingredients: saponins, tannins, steroids , flavonoids etc [19]. *G. latifolium* contain saponins and these have been known to be responsible for its antioxidant and antimicrobial properties [20-21]. The phytochemicals reported in this study have been documented for various antibacterial properties [22-23].

Concentration (mg/ml)								
	S. aureus		*K. pneumoniae*		*E. coli*		*S. saprophyticus*	
Isolates	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
1	0.625	0.625	0.3125	1.25	1.25	2.5	1.25	2.5
2	0.625	1.25	0.3125	0.625	0.3125	1.25	2.5	10
3	1.25	2.5	0.625	2.5	1.25	2.5	0.625	2.5
4	1.25	1.25	1.25	1.25	2.5	2.5	2.5	2.5
5	1.25	2.5	0.625	1.25	1.25	2.5	1.25	1.25

Table 7: *The Minimum Inhibitory Concentration (Mic) And Minimum Bactericidal.*

Flavonoids are known to have antiseptic and antibacterial properties, inhibitory to *S. aureus* and have been used in treatment of inflamed tissues [24]. Saponins also have been reported to be produced by plants to stop bacterial and fungal attacks, making them natural source of antimicrobials [22,25]. The rich amount of secondary metabolites in the leaf extract can explain why the ethanolic extracts of leaf exhibited good inhibitory effects on the MDR organisms. Aqueous and methanol leaf extracts of G. latifolium were reported to have exhibited antibacterial effects on a number of bacteria including *E. coli* and *S. aureus* [26]. Omodamiro and Ekeleme, [27] also reported that the ethanolic leaf extract showed a significant dose dependent inhibition of *S. aureus, S. pneumoniae, E. coli, Proteus mirabilis* and *Pseudomonas aeruginosa.*

Conclusively this study reports that the ethanolic leaf extract of Gongronema latifolium Benth, has activity against multiple drug resistant bacterial isolates. The order of activity against the multiple drug resistant strain was *K. pneumoniae > E. coli > S. aureus > and S. saprohyticus*. Therefore the ethanolic leaf extract of *G. latifolium* can be effectively used in the eradication/treatment of clinical infections caused by the multiple drug resistance isolates of *K. pneumoniae, E. coli, S. aureus* and *S. saprophyticus*. Antibacterial effect of *G. latifolium* which is evident from this study explains the long history of the use of these plants in traditional medicine for the treatment of different bacterial infections such as the use in the treatment of stomach pains/infections.

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