

Alchornea Cordifolia (Christmas Bush) Leaves Extract Shows Activity Against Extended-Spectrum Beta-Lactamase-Producing Klebsiella Pneumoniae That Cause Multi-Drug Resistant Urinary Tract Infections

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Abstract

Klebsiella pneumoniae are one of the most common cause of multi-drug resistant urinary tract infections such as cystitis and pyelonephritis. Extended-spectrum beta-lactamases are plasmid-mediated enzymes commonly found in the *Klebsiella pneumoniae* and other Gram negative bacteria. They are capable of hydrolysing beta-lactams except carbapenems and cephamycins. Extended-spectrum beta-lactamases also confer resistance to several non-beta-lactam antibiotics, limiting treatment options for urinary tract infections caused by extended-spectrum beta-lactamases -producing *K. pneumoniae*. This study determined the *in vitro* efficacy of *Alchornea cordifolia* on extended-spectrum beta-lactamases -producing *K. pneumoniae*. Serial dilutions of the ethanolic extract of the leaves were prepared and tested against the extended-spectrum-beta-lactamases-producing *K. pneumoniae*. The phytochemical screening was performed to determine the antibacterial compounds. The Christmas bush leaves extracts concentrations ranging from 50 mg/ml – 200 mg/ml showed significant active diameter zone of inhibition. The ethanolic extract of *A. cordifolia* leaves had minimum inhibition concentration and minimum bactericidal concentration of 3.13 mg/ml indicating significant antibiotic activity against the ESBL isolates. The phytochemical screening of the Christmas bush leaves showed the presence of antimicrobial phytochemicals such as flavonoids. Ethanolic extracts of Christmas bush leaves is proving to be efficacious against multi-drug resistant urinary tract infections. This offers hope for the development of effective antibiotics due to the presence of flavonoids in the *A. cordifolia* leaf extracts. Therefore, there is the need to determine the toxicological effect and clinical trials of the active antimicrobial compounds isolated in the leaf extracts of *A. cordifolia* shrub.

Keywords: Alchornea. cordifolia, Flavonoids, Multi-drug resistant, Extended-spectrum beta-lactamase, Klebsiella pneumoniae

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1.0 Introduction

Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated beta lactamases that are capable of hydrolysing beta-lactams except carbapenems and cephamycins. They are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. They have been found in the *Enterobacteriaceae* and other Gram negative bacilli. ESBL producing isolates are predominantly *Klebsiella pneumoniae* and *Escherichia coli* (Paterson and Bonomo, 2005).

Klebsiella pneumoniae are inhabitants of the intestinal flora and are among the most common causes of urinary tract infections such as cystitis (bladder infection) and pyelonephritis (kidney infection). It is estimated that about 150 million people develop urinary tract infection in every given year (Flores-Mireles et al., 2015) and are more common in women than men (Salvatore et al., 2011). *Enterobacteriaceae* are the source of community and hospital acquired infections. Because ESBL enzymes are plasmid mediated, the genes encoding these enzymes are easily transferable among different bacteria. Most of these plasmids not only contain DNA encoding ESBL but also carry genes conferring resistance to several non-beta-lactam antibiotics (Nordmann et al., 2011). Consequently, most ESBL isolates are not only resistant to penicillins, cephalosporins and monobactams but also to other classes of antibiotics including aminoglycosides, fluoroquinolones, tetracyclines, nitrofurantoin and sulfamethoxazole-trimethoprim.

Since 2000, spread of community acquired enterobacterial isolates (*Klebsiella pneumoniae*) that produce extended-spectrum β -lactamases (ESBLs) capable of hydrolyzing almost all cephalosporins except carbapenems has been reported worldwide (Pitout and Laupland, 2008). Previous studies by Hackman et al., (2013) in Accra

established that extended-spectrum beta-lactamase (ESBL) producers were increasingly becoming a public health nuisance due to community acquired and nosocomial infections.

Treatment of these multiple drug-resistant organisms has proven to be a therapeutic challenge. ESBL-producing isolates pose serious public health, financial and logistics challenges because of the limited choice of antibiotics for the treatment of infections by ESBL producing isolates. The screening of natural products has been the source of innumerable therapeutic agents. Higher plants, as a source for new potential drugs is still largely unexplored and only a small percentage of them has been subjected to phytochemical investigation and the fractions submitted to pharmacological screening is very low (Saraswathy *et al.*, 2011).

The leaves, stems and roots of *Alchornea cordifolia* (Schumach. and Thonn.) Müll. Arg are used as traditional medicine in many African countries for the management of gastrointestinal, respiratory and urinary tract infections as well as for the treatment of wounds (Siwe Noundou *et al.*, 2016).

According to George *et al.* (2010), the potency of the *A. cordifolia* leaves extract could be attributed to various classes of compounds such as steroids, tri-terpenoids, phenols and polyphenols, alkaloids, flavonoids, anthraquinones, saponins and tannins, already known to be present in *A. cordifolia* leaves.

Siwe Noundou *et al.*, (2016) isolated seven compounds from *A. cordifolia* with antibacterial activities namely stigmaterol, stigmasta-4,22-dien-3-one, friedelin, friedelane-3-one-28-al, 3-O-acetyl-aleuritic acid, 3-O-acetyl-erythrodiol and methyl-3,4,5-trihydroxybenzoate (methyl gallate).

In this study, the antibacterial activity of ethanolic extract of *Alchornea cordifolia* leaf was tested against ESBL-producing *K. pneumoniae*. Phytochemical screening of the plants extract was performed to determine the various compounds. The study was based on the hypothesis that *Alchornea cordifolia* will possess phytochemical compounds that will have antimicrobial properties against ESBL-bacterial infections through *in vitro* analysis.

2.0 Materials and Methods

2.1 Study Site

This research work was carried out at the Centre for Plant Medicine Research, Mampong-Akuapim in the Eastern Region of Ghana.

2.2 Sampling of Test Plant Material and Extracts Preparation

Thousand gram (1000g) of the plant leaves were collected and authenticated at the herbarium at the Centre for Plant Medicine Research (CPMR), Mampong-Akuapim. The sampling was based on the assumption that the plant is healthy and uninfected. The plant parts were cut into pieces, air dried at $26 \pm 2^\circ\text{C}$ for ten days and milled into coarse powder with the aid of an electric blender.

2.3 Ethanolic Extraction, Evaporation and Freeze Drying of Extracts

Three hundred grams (300g) of the powdered plant part was soaked in 2L of 96% absolute ethanol for 72 hours with occasional stirring and was filtered through Whatman's No.1 filter paper. The filtrate was concentrated with the aid of a rotary evaporator and was freeze-dried using the freeze drying machine at a temperature and reduced pressure of 35°C and 100-80hPa respectively. The dried extract was stored under refrigeration condition (4°C) until needed.

2.4 Reconstitution of Serial Concentrations of the Crude Extract

Eight hundred milligrams (800mg) of the dried crude extracts were dissolved in 4ml of 5% v/v dimethyl sulphoxide (DMSO) to prepare 200mg/ml concentration of the leaves extract. Subsequent concentrations of 100, 50, 25, and 12.5 mg/ml were achieved using the conventional two folds dilution technique.

2.5 Preparation of Mueller Hinton Agar (MHA) as a Culture Media

The culture media were prepared according to the manufacturers instruction. Thirty eight grams (38g) of the powder was suspended in 1000 ml of demineralized water and heated to boil with agitation to completely dissolve the powder. The medium was then sterilized by autoclaving at 121°C for 15 minutes.

2.6 Detection of ESBL-producing *K. pneumoniae* using Combined Disc Synergy Method

MAST ID™ ESBL Detection Discs (Mast Group, UK) were used to screen and confirm the ESBL phenotypes. The MAST ID™ ESBL Detection Discs comprise of cefpodoxime 30µg disks, cefpodoxime 30µg + clavulanic acid 10µg disks; ceftazidime 30µg disks, ceftazidime 30µg + clavulanic acid 10µg disks and cefotaxime 30µg disks, cefotaxime 30µg + clavulanic acid 10µg disks (Paterson and Bonomo, 2005).

Using a pure culture of the test organism, a suspension in distilled water equivalent in density to a McFarland 0.5 opacity standard was prepared. Using a sterile swab, the suspension was spread uniformly across the surface of Mueller-Hinton agar plate. Using a sterile forceps, one of each MAST ID™ ESBL Detection Discs was placed onto the inoculated medium ensuring that they were evenly spaced. The plates were incubated aerobically at 35-

37°C for 18 – 20 hours. The diameter of any zones of inhibition that were observed were measured and recorded. The zone of inhibition for the cefpodoxime, ceftazidime and cefotaxime was compared to that of the cefpodoxime, ceftazidime and cefotaxime plus clavulanic acid combination disks. An increase in zone diameter of =5mm in the presence of clavulanic acid from any or all of the sets of MAST ID™ ESBL Detection Disks indicates the presence of ESBL in the test organism. isolates.

2.7 Subculturing of Extended-spectrum Beta-lactamase Producing *K. pneumoniae*

The test organism was subcultured onto Mueller Hinton agar plate and was incubated for 24 hours to obtain a pure culture and to ascertain the viability of the organism. At least four colonies from the pure culture were transfer into sterile peptone water using an inoculation needle and adjusted to turbidity equivalent to 0.5 McFarland Standard.

2.8 Antimicrobial Susceptibility Testing Using Agar Well-Diffusion Method

Antimicrobial activity of the shrub extracts was assessed using the agar- well diffusion method. A 3ml inoculum was prepared in peptone water and adjusted to 0.5 McFarland and uniformly spread on the MHA plate using the glass spreader. Wells (6mm diameter) were created in the plate using cork borer and 20µl of the shrub extract concentrations (200 mg/ml – 12.5 mg/ml) were pipetted into the wells at their respective concentration. Amikacin (30µg/ml) and colistin (10µg/ml) disks were used as positive controls and 5% v/v DMSO and sterile distilled water (SDW) were used as the negative controls. They were allowed to diffuse at room temperature for 30 minutes and the plates were then incubated at 37°C for 24 hours. All experiments were carried out in triplicate.

After incubation, the diameter zone of inhibition (DZI) were measured and expressed in millimeters. DZI < 7mm was considered inactive; 7-10 mm partially active; 11-16 active and > 16 very active as described by Junior and Zanil (2000).

2.9 Determination of Minimum Inhibition Concentration (MIC)

Hundred microlitres (100µl) of ethanol extract of the active shrub were added to 100 µl of 5% DMSO in the first well of the 96-well microplate and mixed well with a micropipette; 100 µl of this dilution was transferred to the next well in the column. This two-fold serial dilution was continued to obtain dilutions ranging from 10¹ to 10¹⁰ with the aid of a micropipette. The final volume in each well was 100 µl. Solutions of a reference antibiotic amikacin (30µg/ml) and colistin (10 µg/ml) were also serially diluted in different columns of the microplate as a positive control. The wells were inoculated with 5µl of microbial suspension of turbidity equivalent to 0.5 McFarland and incubated at 37°C for 24 hours. After incubation, 40µl of 0.2mg/ml INT (p-Iodonitrotetrazolium violet) dissolved in sterile distilled water was added to each of the wells. The microplates were examined after additional 30 to 120 minutes of incubation. Bacterial growth was indicated by a red colour of INT reduced to the formazan. The lowest concentration at which a decreased in the red colour is apparent compared to the next dilution was taken as the MIC value.

2.10 Determination of Minimum Bactericidal Concentration (MBC)

The MBC values were deduced from those wells with lowest concentrations at which no growth took place after culture for 24 hours of incubation. A small sample from each of those wells was transferred to fresh nutrient agar plates and was incubated overnight at 37°C and plates were examined for the presence or absence of living organisms. Plates with no microbial growth were regarded as the minimum bactericidal concentrations.

2.11 Phytochemical Screening

Phytochemical screening methods described by Fong *et al.*, (1977) and Ciulei (1982) were used to screen the various extracts for the presence of saponins, reducing compounds, polyuronides, phenolic compounds, alkaloids, triterpenes, phytosterols, flavonoids and anthracenosides.

3.0 RESULTS

3.1 Susceptibility of Extended-spectrum Beta-lactamase Producing *K. pneumoniae* against Ethanol Extract of *Alchornea cordifolia*.

The ethanolic extract of Christmas leaves showed varied zones of inhibition as in Table 1:

Table 1: Susceptibility of Extended-spectrum Beta-lactamase-Producing *K. pneumoniae* to ethanolic extract of *Alchornea cordifolia* leaves

Standards		Diameter of zones of inhibition (mm)					
Amikacin (30 µg/ml)	Colistin (10µg/m)	SDW/ DMSO	200 mg/ml	100 mg/ml	50 mg/m	25 mg/m	12.5 mg/ml
22.0 ± 0.5	17.0 ± 0.5	0.0	16.3 ± 1.0	13.7 ± 0.5	11.9 ± 1.3	8.5 ± 2.5	0.0 ± 0.0

Values are mean inhibition zone (mm) ± S.D of three replicates

Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) shown in Table 2 were determined using the 96 well microplate.

Table 2: MIC and MBC of ethanol leaves extracts of *Alchornea cordifolia* against ESBL-producing *K. pneumoniae*.

Plant extract	MIC (mg/ml)	MBC (mg/ml)
<i>Alchornea cordifolia</i>	3.13	3.13

3.2 Phytochemical Constituents

The phytochemical constituents of the leaf extract were saponins, phenolic compounds, reducing sugars, polyuronoids and flavonoids as shown in Table 3. Previous work by Siwe Noundou *et al.*, (2016) had identified the antibacterial compounds in *A. cordifolia* leaves and elucidated their chemical structure.

Table 3: Phytochemical constituents of *Alchornea cordifolia* leaves

Phytochemical	<i>A. cordifolia</i>
Saponins	+
Phenolic compounds	-
Reducing sugars	+
Polyuronoids	-
Alkaloids	-
Triterpenes	-
Phytosterols	-
Flavonoids	+
Anthracenosides	-

Detected = (+); Not Detected = (-)

4.0 Discussion

The outcome of this study suggested that ESBL-producing *K. pneumoniae* are sensitive to varied concentration (50 mg/ml – 200 mg/ml) of the ethanolic extracts of *Alchornea cordifolia* leaves. More so, the ethanolic leaves extracts of Christmas bush leaves had MIC and MBC of 3.13 mg/ml. This is an indication that the ethanolic extracts of *Alchornea cordifolia* leaves have significant antibacterial activity against multi-drug resistant *K. pneumoniae*. This may be because *Alchornea cordifolia* leaves possess rich phytochemicals such as saponins, reducing sugars and flavonoids.

This may be because *Alchornea cordifolia* leaves possess phytochemicals such as saponins, reducing sugars and flavonoids (George *et al.*, 2010). Flavonoids are known to possess the antibacterial effect. According to Siwe Noundou *et al.*, (2016) antibacterial compounds such as stigmaterol, stigmasta-4,22-dien-3-one, friedelin, friedelane-3-one-28-al, 3-O-acetyl-aleuritic acid, 3-O-acetyl-erythrodiol and methyl-3,4,5-trihydroxybenzoate (methyl gallate) were isolated from Christmas bush. It could be that the extract used in this study may contain these compounds since they fall under flavonoids and the phytochemical screening of the test plant revealed that it contains flavonoids as shown in Table 1. There might be other phytochemicals that were not determined in this study, which may be responsible for the antibacterial activity of the *A. cordifolia* leaves.

The activity of the standards (amikacin and colistin) used in this study as positive control corresponds with the results of Hackman *et al.*, (2017) although amikacin (30 µg/ml) used in this study shows a higher diameter zone of inhibition. According to Junior and Zani (2000) diameter zone of inhibition of 11-16 is considered active and >16 is considered very active. From the results, concentrations of the test plant ranging from 50 mg/ml – 200 mg/ml showed active diameter zone of inhibition. From Table 2, *Alchornea cordifolia* had an MIC of 3.13 mg/ml with MBC of 3.13 mg/ml with percentage inhibition index of 2.94. This indicate that *Alchornea cordifolia* is able to inhibit the growth of the ESBL-producing *K. pneumoniae* at the stated concentrations.

5.0 Conclusion

The hypothesis for this study proved positive since the plant under study showed activity against the ESBL-producing *K. pneumoniae*. Ethanolic extracts of *A. cordifolia* leaves is proving to be efficacious against multi-drug resistant ESBL-producing *K. pneumoniae*, which a major cause urinary tract infections. Phytochemical screening of the leaves showed that it possesses flavonoids, saponins and reducing sugars, which is responsible for the antibacterial efficacy of the leaves extracts. This offers hope for the development of effective antibiotics for the treatment of multi-drug resistant systemic infections. Therefore, there is the need to determine the toxicological effect and perform clinical trials of the active antimicrobial compounds isolated in the leaves extracts of *A. cordifolia*.

Abbreviations

CPMR: Centre for Plant Medicine Research

DMSO: Dimethyl Sulphoxide

DZI: Diameter Zone of Inhibition

K. pneumoniae: *Klebsiella pneumoniae*

ESBL: Extended-spectrum beta-lactamase

INT: p-Iodonitrotetrazolium violet

MIC: Minimum Inhibitory Concentration

MBC: Minimum Bactericidal Concentration

MHA: Mueller Hinton Agar

SDW: Sterile Distilled Water

Declarations

Ethics Approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HKH designed the study. DB, BN, MA carried out the experiment and collected the data. HKH, LA and REA performed the statistical analysis. HKH, LA, BKA and REA interpreted the clinical data. All authors' read and approved the final version of the manuscript.

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