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ORIGINAL RESEARCH ARTICLE

Antibiogramic Efficacy of *Cymbopogan citratus*, *Ocimum gratissimum*, and *Kalanchoe pinnata* Methanolic and Aqueous Extracts on Colistin-Resistant Non-Clinical Isolates of Gram-negative Bacteria

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ABSTRACT

Antibiotic resistance is a significant global public health concern, with recent reports indicating that Gram-negative bacteria are developing resistance to colistin, a last-resort antibiotic. This study aimed to assess the antibacterial effects of methanolic and aqueous leaf extracts from *Ocimum gratissimum*, *Kalanchoe pinnata,* and *Cymbopogan citratus* medicinal plants against colistin-resistant bacteria. The extracts were obtained through maceration, followed by qualitative phytochemical analysis (including alkaloids, flavonoids, glycosides, saponins, sterols, phenolic compounds, and tannins) and quantitative evaluation of alkaloids, flavonoids, and tannins. Furthermore, antibacterial properties were tested against twelve colistin-resistant Gram-negative bacterial strains using the disc diffusion method. Qualitative analysis revealed the presence of flavonoids, proteins, carbohydrates, and tannins in all leaf extracts, while amino acids, fixed oil, and fats were absent. The highest flavonoid content was found in the methanolic extract of *C. citratus* (153 ± 2.7µg/ml) and the lowest in the aqueous extract of *K. pinnata* (0.0 ± 0.0µg/ml). Tannin levels were highest in the methanolic extract of *O. gratissimum* (282.4 ± 13.5µg/ml) and lowest in the aqueous extract of *C. citratus* (27.1 ± 5.7µg/ml). Phenolic compound concentrations ranged from 136.9 ± 58.0µg/ml (methanolic extract of *O. gratissimum*) to 14.5 ± 2.9µg/ml (aqueous extract of *K. pinnata*). The antibacterial activity of the methanolic extracts of *O. gratissimum, K. pinnata,* and *C. citratus* exhibited varied results, with all isolates resistant to all concentrations of the leaf extracts. However, the aqueous extract of *Ocimum* gratissimum inhibited *Providencia stuarti* at 250mg, while *Enterococcus dispar* and *Escherichia coli* were sensitive to the aqueous leaf extracts of *Ocimum* gratissimum across all concentrations. These findings indicate increasing resistance of Gram-negative bacteria to colistin and various concentrations of the methanolic and aqueous extracts of the three medicinal plants.

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INTRODUCTION

Pathogenic bacteria have long been recognized as a significant contributor to illness and death in humans. Despite the development of numerous new antibacterial agents by pharmaceutical companies in recent years, resistance to these medications has escalated and is now a worldwide concern (Adwan and Mhanna, 2008). Antibiotics are natural substances produced by certain microorganisms or synthetic products that tend to inhibit the effect of other microorganisms. They are generally used in cases of infections caused by bacteria (Moussaoui and Alaoui, 2016). However, many bacteria have become antibiotic-resistant, especially those commonly used. The increased resistance is caused by several factors, such as high drug pressure due to anarchic prescription by hospitals and self-medication, amongst others (Yala *et al.*, 2016). This massive use of antibiotics contributes to developing acquired and natural antibiotic resistance (Yala *et al.*, 2020). Consequently, many therapeutic failures are reported worldwide (Yala *et al.*, 2016).

Multi-drug resistant (MDR) bacteria are becoming more widespread, severely restricting the efficacy of the medications already on the market and contributing to treatment failure (Hancock and Speert, 2000). The upregulation of MDR efflux pumps could be the root cause of bacterial resistance to antimicrobial drugs that are

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chemically distinct, posing a significant public health concern (Sharma et al., 2005; Li et al, 2010).

The elevated levels of innate and acquired antibiotic resistance often linked with Gram-negative bacteria stem from the interplay of efflux pumps and diminished drug absorption, facilitated by a dual membrane barrier (Lomovskaya, 2007). Many of these multidrug-resistant (MDR) efflux pumps belong to the resistance-nodulation-cell division of tripartite efflux pumps, prevalent in Gram-negative bacteria (Lomovskaya, 2007).

Due to the resistance of most microorganisms to common antibiotics, colistin, belonging to the polymyxin family, is recommended. Colistin is an old and useful antibiotic that has been reintroduced as a last-resort therapy option due to the growth of multidrug-resistant (MDR) and extensively drug-resistant Gram-negative bacteria and the lack of innovative medicines against infections caused by these bacteria (Baron et al., 2016). This antibiotic, belonging to the polymyxin class, possesses lipophilic and hydrophilic properties. Among the five chemical compounds comprising the polymyxin group, only two (polymyxins B and C) are utilized in clinical practice (Gallardo-Godoy et al., 2016), with colistin also referred to as polymyxin E (Cassir *et al*., 2014).

When managing infections induced by MDR Gram-negative bacteria, such as those that produce carbapenemase and are caused by the Enterobacterales, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, colistin has been used in humans as a last-resort antibiotic in recent years (Biswas et al., 2012). The World Health Organization (WHO) classified colistin as having "very high importance for Human Medicine" (Abaji and Omoruyi, 2022). Interestingly, there was also an emergence of colistin-resistant strains with a frequency of 1.2% between 2011 to 2015 in humans distributed between *E. coli* and *Klebsiella pneumoniae* (Liu *et al.,* 2016). One of the most prevalent resistance mechanisms in Gram-negative bacteria is active efflux through resistance-nodulation-cell division (RND) pumps (Omoruyi and Ojubiaja, 2022).

For decades, colistin has been used in food animals worldwide for growth promotion, preventive, and therapeutic purposes (Poirel *et al.,* 2017). Some countries previously permitted the use of colistin as a growth enhancer in agriculture and livestock before a recent ban was imposed due to concerns about antimicrobial resistance (AMR) (Wang *et al*., 2018; Liu and Liu, 2018). However, the ban specifically prohibits using colistin as a feed supplement, while their applications as a treatment for sick animals remain allowed (Walsh and Wu, 2016).

Thus, a plethora of alternative solutions are needed, and one potential option lies in medicinal plants commonly utilized in traditional therapy. The use of plant species for medicinal purposes has gained increasing importance, with a growing demand for raw materials sourced from medicinal plants experiencing an annual growth rate of 15–25% (Omoruyi and Emefo, 2012).

Increased antibiotic resistance fuels the search for new drugs to combat resistant bacteria. Due to the wide range of secondary metabolites found in plants and their potential to display antimicrobial properties, plant species commonly employed in herbal medicine appear to provide biologically active components extracted from the plant and could represent a promising alternative. Numerous medicinal plants have been reported to demonstrate effectiveness in fighting infectious diseases, including those that are resistant to multiple antibiotics. *Ocimum gratissimum*, *Kalanchoe pinnata,* and *Cymbopogan citratus* have all been previously reported as effective against multidrug-resistant bacteria. *Ocimum gratissimum* has been reported to have an antibiogramic effect on several Gram-negative bacteria, such as *Pseudomonas aeruginosa* (Silva et al., 2022). Similar studies by Chanthaboury et al. (2022) have also reported the inhibitory effects of *O. gratissimum* against *Streptococcus pyogenes*, *Streptococcus mutans,* and *Staphylococcus aureus* and noted *O. gratissimum* as a weaker antibacterial when compared with other plant extracts from the same genus, such as *O. santum*. Despite the claim by Chanthaboury et al. (2022), *O. gratissimum* has been reported to have a strong bactericidal effect against diarrhoea causing bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Shigella* sp., and *Salmonella* sp.) and could be a suitable alternative to synthetic antibiotics in the treatment of diarrhoea caused by these bacteria (Amengialue et al., 2013).

On the other hand, *Cymbopogon citratus* has reported evidence of antibacterial activity against pitted keratolysis caused by bacteria such as *Kytococcus sedentarius*, *Dermatophilus congolensis,* and *Bacillus thuringiensis* (Schweitzer et al., 2022) as well as against other clinically important bacteria such as methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecalis*, *Staphylococcus aureus*, *S. epidermidis* and methicillin-sensitive *Staphylococcus aureus* (Subramaniam et al., 2020). Similarly, *Kalanchoe pinnata* has been reported to inhibit the growth of *Helicobacter pylori* (Zakharchenko et al., 2017), *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Cryer et al., 2017; Loi et al., 2020), *Escherichia coli* and *Salmonella* Typhi (Abubakar and Haque, 2020; Loi et al., 2020) amongst others.

Despite the available information on the antibiogramic activities of these plant extracts, there is a paucity of data on the effectiveness of these plants against colistin-resistant bacteria. The current study aimed to investigate the antibacterial efficacy of commonly utilized medicinal plants against Gram-negative bacteria resistant to colistin.

MATERIALS AND METHODS

Obtaining Plant Samples

In December 2021, we obtained the ripe leaves of *Cymbopogon citratus, Kalanchoe pinnata*, and *Ocimum gratissium* from the Ekiosa market in Benin City, Edo state, Nigeria. Prof. Osondu Christopher Akoma from the Botany Unit, Department of Biological Sciences, Benson Idaho University, authenticated the samples. Subsequently, the plant materials underwent air drying in the sun and were

washed with running tap water until reaching a consistent weight. To facilitate further analysis, the dried leaf samples were finely powdered.

Preparation of Extracts

Methanol Extraction

Following air drying and coarse grinding, the plant materials were extracted with 100% methanol using maceration techniques. Specifically, 500ml of methanol was combined with 250g of each powdered sample and allowed to macerate for 72 hours. Subsequently, the mixture was filtered through a double-layered muslin cloth into a clean container, and the filtrate was subjected to a rotary evaporator to obtain the extract, which was then concentrated to a gel-like consistency using an oven set at 70°C. The resulting concentrate was then stored in a universal container at room temperature (35°C) to shield it from sunlight and moisture.

Aqueous Extraction

Each powdered substance in a total of 250g was separately mixed with 1000ml of distilled water for 72 h, following which it was filtered through a double-layered muslin cloth into a clean bucket, and the filtrate was placed in a Rotary Evaporator to get the extract. Once the sterile extract was collected, it was placed in a sterile McCartney bottle and kept warm at 40C until needed.

Phytochemical Screening

As Omoruyi and Emefo (2012) described, the phytochemicals identified in the qualitative evaluation were subsequently examined quantitatively. Following the methods outlined by Trease and Evans (1989) and Sofowora (1993), the crude extracts from each plant were assessed for the presence or absence of various phytochemicals, including alkaloids, flavonoids, glycosides, phenolic compounds, saponins, sterols, and tannins as detailed below.

Powdered plant samples (5 g) were extracted with a 4:1 mixture of methanol and water (150 ml) using a Soxhlet apparatus for 12 h. The extract was then cooled and filtered through Whatman No. 1 filter paper. The remaining residue was further extracted with 125 ml of ethyl acetate, and the percentage of crude fibers was calculated. The fats and waxes were determined by evaporating the ethyl acetate.

The methanol-water filtrate was concentrated to about 1/10th of its original volume and acidified with 2M H_2SO_4. This solution was then extracted with 75 ml of chloroform in three portions of 25 ml each using a separating funnel. The chloroform layer containing phenolics and terpenoids was separated and evaporated to dryness in a water bath at 45°C.

The aqueous layer from the separation was adjusted to pH 10 with 2M NaOH and further extracted with 60 ml of a 3:1 chloroform-methanol mixture, followed by 40 ml of chloroform in a separating funnel. The aqueous basic layer was then evaporated to dryness in a water bath, yielding a residue containing quaternary alkaloids and N-oxides. The organic layer (chloroform-methanol) was transferred to a beaker, and the solvent was evaporated to dryness, resulting in a basic extract containing alkaloids.

Determination of total phenolic content

The total phenolic contents in medicinal plants were determined spectrophotometrically using the Folin-Ciocalteu method (Singleton *et al.*, 1999). Gallic acid was used to set up the standard curve. The samples' phenolic compounds were expressed as gallic acid equivalents (GAE) in mg/g dry weight. All samples were analyzed in triplicates.

Determination of total flavonoid content

The $AlCl_3$ method (Ordonez *et al.*, 2006) was used to quantify the total flavonoid content of the methanolic plant extracts. Briefly, 20 µl of the sample extract was added to a solution of 2% $AlCl_3.6H_2O$. The mixture was vigorously shaken and diluted with double distilled water to make a total volume of 10 ml. The absorbance of the reaction mixture was measured after 10 min incubation at 440 nm. Flavonoid contents were expressed as quercetin equivalents in mg/g dry material. All the determinations were performed in triplicates. Correlation coefficients (R2) to determine the relationship between two variables (between different RSA tests and content of total phenolic and flavonoid compounds) were calculated using MS Excel software.

Determination of Tannin

The Folin-Ciocalteu method (CI and Indira, 2016) was applied to quantify the tannin content. Approximately 0.1 ml of the sample extract was added to a 10 ml volumetric flask containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent. Then, 1 ml of 35% sodium carbonate solution was added, and the mixture was diluted to 10 ml with distilled water. The solution was thoroughly mixed and left at room temperature for 30 min. Reference standard solutions of tannic acid (20, 40, 60, 80, 100 µg/ml) were prepared similarly. The absorbance of the test and standard solutions was measured at 700 nm using a UV/Visible spectrophotometer against a blank. The tannin content was determined in triplicate and expressed as mg of tannic acid equivalents per gram of dried sample.

Bacterial Isolates

Twelve (12) different Gram-negative bacterial isolates: *Pseudomonas aeruginosa*, *Enterobacter ludwigii*, *Providencia stuarti*, *Enterococcus* dispar, *Providencia rustigianii, Providencia rettgeri*, *Providencia burhodogranariea*, *Escherichia coli* 1, *Klebsiella quasipneumoniae*, *Enterococcus saccharolyticus*, *Salmonella enterica,* and *Escherichia coli* 2 were confirmed to be colistin-resistant in a previous study and identified to their species level by 16S rRNA at Inquaba Biotechnology (Ibadan, Oyo State,

Nigeria), as reported in an earlier study by Omoruyi *et al.*, (2023).

Standardization of Bacterial Isolates

Standardizing the bacterial inoculum involved adding a loop of each bacterium to a sterile nutrient broth and culturing it for 24 h at 37°C. The isolates' turbidity was adjusted to match the 0.05 McFarland Standards (Omoruyi and Ojubiaja, 2022).

Determination of Antibacterial activity of the Leave Extracts

With minor adjustments, we utilized the agar-well diffusion method (Gashe and Zelek, 2017) to assess the antibacterial activity of the crude extracts. Initially, a sterile swab stick was employed to inoculate 0.2 ml of the adjusted slurry onto Muller Hilton agar independently. Subsequently, a sterile cork borer with an 8 mm diameter was utilized to punch out the agar medium, and sterile forceps were employed to aseptically remove the cut agar discs. Then, 0.1 ml of each crude extract was added to the 8 mm holes in the agar medium containing the colonies using a sterile micropipette. The samples were left at room temperature for an hour to allow the substrates to diffuse before the organisms could proliferate. Finally, the plates were aerobically incubated for 24h at 37°C. A zone of inhibition surrounding the wells containing the extracts indicated the antibacterial activity of the extracts against the test organisms.

Statistical analysis

Analysis of variance (ANOVA), correlation, cluster, and regression analysis were performed using Python 3 software.

RESULTS

The current study's findings indicate that the leaf extracts of *C. citratus*, *O. gratissimum*, and *K. pinnata*, both methanolic and aqueous, contain flavonoids, proteins, carbohydrates, and tannins. However, amino acids, fixed oil, and fats were absent in all leaf samples (methanol and aqueous), as shown in Table 1.

Meanwhile, alkaloids were only present in the aqueous extracts of all three leaf samples, saponin was present in all samples (methanol and aqueous extracts) except for one, the aqueous extract of *O. gratissimum*. Reducing sugar was also present in only the methanolic extract of *O. gratissimum* and the aqueous extracts of *C. citratus*. Interestingly, glycosides were present in all leaf samples investigated (methanolic and aqueous extracts) except for the methanolic extract of *O. gratissimum* and the aqueous extract of *C. citratus*.

Table 2 shows the quantitative phytochemical composition of the methanolic and aqueous extracts of *C. citratus, O. gratissimum,* and *K. pinnata*. The concentration of flavonoid was 153.4 ± 2.7 x 10^3 µg/ml, 267.6 ± 2.7 x 10^3 µg/ml, and 52.6 ± 2.7 x 10^3 µg/ml in the methanolic extracts of *C. citratus, O. gratissimum,* and *K. pinnata* respectively, while the flavonoid concentration of the aqueous extracts was significantly lower than the methanolic extracts, with the aqueous extract of *C. citratus, O. gratissimum,* and *K. pinnata* reported to be 26.2 ± 2.7 x 10^3 µg/ml, 18.7 ± 2.7 x 10^3 and 0.00 ± 0.0 µg/ml respectively.

Similarly, the concentration of tannin was highest in the aqueous extract of O. *gratissimum* (282.4 ± 13.5 x 10^3 µg/ml) and lowest in the aqueous extracts of *C. citratus* (27.1 ± 5.7 x 10^3 µg/ml). Phenolic content was also highest in the methanolic extracts of *O. gratissimum* and lowest in the aqueous extracts of *K. pinnata* (14.5 ± 2.9 x 10^3 µg/ml).

Figure 1 shows the box plot of the total concentration of phytochemicals in the methanolic and aqueous leaf extracts of *Cymbopogan citratus*, *Ocimum gratissimum,* and *Kalanchoe pinnata*, with the methanolic leaf extract of *Ocimum gratissimum* having the highest concentration, while the aqueous leaf extract of *Kalanchoe pinnata* had the lowest concentration range.

As demonstrated in Tables 3, 4, and 5, the methanol extracts from the leaves of *Ocimum gratissimum*, *Kalanchoe pinnata*, and *Cymbopogan citratus* show average antimicrobial activities with no zones of inhibition at any concentration, indicating resistance of the organisms to the methanol extract at varying concentrations. The average antimicrobial activity of the aqueous extracts from *Ocimum gratissimum, Kalanchoe pinnata,* and *Cymbopogan citratus* leaves, presented in Tables 6, 7, and 8, reveals zones of inhibition at some concentrations, indicating sensitivity of some organisms to the aqueous extract at various concentrations. According to Table 6, the aqueous extract of *Ocimum gratissimum* inhibited *Providencia stuarti at* 250mg, while the growth of *Enterococcus dispar* and *Escherichia coli* 2 was inhibited at concentrations of 250mg, 125mg, 67.5mg, and 33.7mg. The aqueous extract of *Kalanchoe pinnata* inhibited *Klebsiella quasipneumoniae* at 250mg and 125mg, as well as *Providencia stuarti* and *Escherichia coli* 2 at concentrations of 250mg, 125mg, 67.5mg, and 33.7mg (Table 7). The aqueous extract of *Cymbopogan citratus* only inhibited *Escherichia coli* 2 at 250mg, 125mg, 67.5mg, and 33.7mg (Table 8).

The correlation analysis showing the relationship between the different concentrations of phytochemicals and antimicrobial efficacy is represented with a hit map, as shown in Figure 2, with aqueous extracts of *Kalanchoe pinnata* and *Cymbopogan citratus* showing more potential for use as an alternative to synthetic antimicrobials in the treatment of infections caused by colistin-resistant bacteria. The result of the analysis of variance (ANOVA) gave F-statistics of 10.101, which suggests that there is more variability between the different plant types than the type of leaf extracts in their efficacy against colistin-resistant Gram negative bacteria. At the same time, a p-value of 0.000560 is much less than the common significance level (alpha) of 0.05, indicating that the antibacterial efficacy of the different plants and leaf

extracts is not the same. The type of extract had a significant impact on the concentrations of the phytochemicals being measured, as well as the antibacterial activities of the extract.

Table 1: Qualitative Phytochemical Constituent of the methanolic and aqueous leaf extracts of *Cymbopogan citratus, Ocimum gratissimum,* and *Kalanchoe pinnata*

	METHANOL EXTRACT			AQUEOUS EXTRACT		
	C. citratus	***O. gratissimum***	***K. pinnata***	***C. citratus***	***O. gratissimum***	***K. pinnata***
Alkaloids	-	-	-	+	+	+
Saponins	+	+	+	+	-	+
Flavonoids	+	+	+	+	+	+
Amino Acids	-	-	-	-	-	-
Proteins	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+
Reducing Sugars	-	+	-	+	-	-
Fixed Oil and fats	-	-	-	-	-	-
Glycosides	+	-	+	-	+	+
Tannins	+	+	+	+	+	+

Key: +: Positive; -: Negative

Table 2: Quantitative analysis of the methanolic and aqueous leaf extracts of *Cymbopogan citratus, Ocimum gratissimum,* and *Kalanchoe Pinnata*

	Concentration [µg/ml (×10³)]					
	E1	**E2**	**E3**	**E4**	**E5**	**E6**
Flavonoid	153.4 ± 2.7	267.6 ± 2.7	52.6 ± 2.7	26.2 ± 2.7	18.7 ± 2.7	0.0 ± 0.0
Tannin	75.1 ± 7.4	282.4 ± 13.5	126.4 ± 27.8	27.1 ± 5.7	33.1 ± 4.9	51.1 ± 3.0
Phenolic	57.5 ± 43.0	136.9 ± 58.0	58.5 ± 32.6	14.9 ± 7.3	17.5 ± 4.5	14.5 ± 2.9

KEY: E1: *Cymbopogan citratus* methanolic extract; E2: *Ocimum gratissimum* methanolic extract; E3: *Kalanchoe pinnata* methanolic extract; E4: *Cymbopogan citratus* aqueous extract; E5: *Ocimum gratissimum* aqueous extract; E6: *Kalanchoe pinnata* aqueous extract.

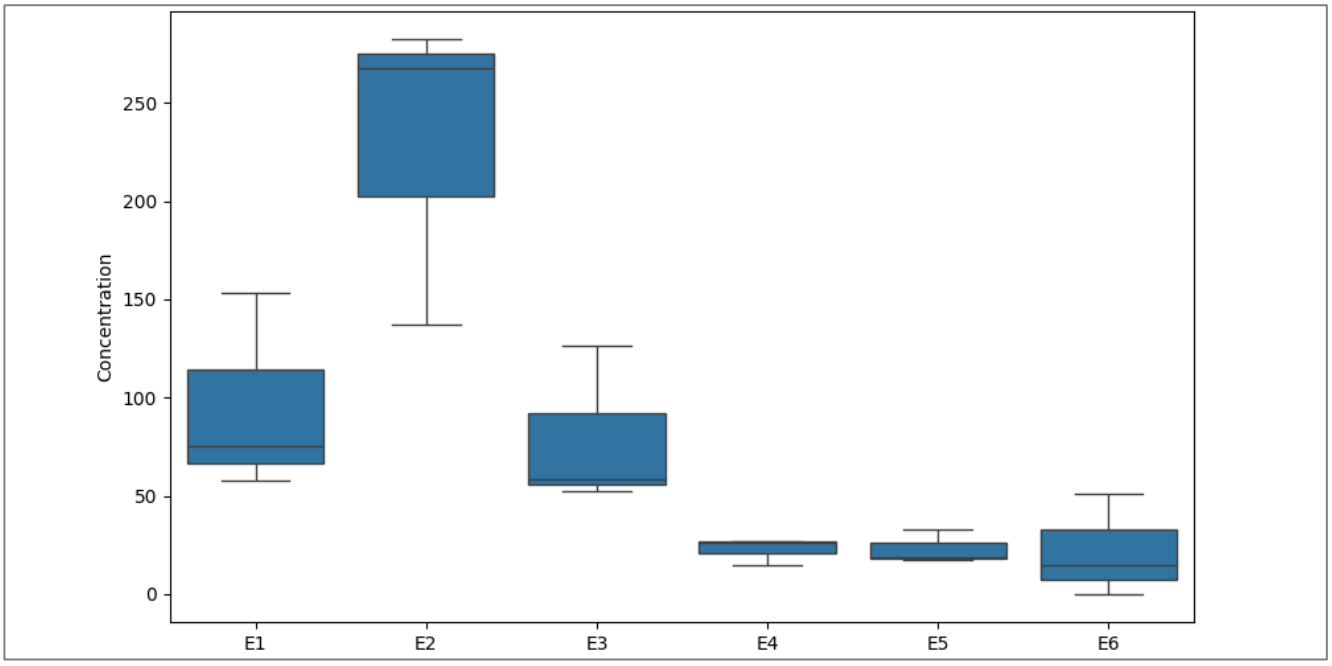

Figure 1: Box plot showing the concentration of phytochemicals in methanolic and aqueous leaf extracts of *Cymbopogan citratus*, *Ocimum gratissimum,* and *Kalanchoe pinnata*

KEY: E1: *Cymbopogan citratus* methanolic extract; E2: *Ocimum gratissimum* methanolic extract; E3: *Kalanchoe pinnata* methanolic extract; E4: *Cymbopogan citratus* aqueous extract; E5: *Ocimum gratissimum* aqueous extract; E6: *Kalanchoe pinnata* aqueous extract.

Table 3: Bactericidal effect of the methanolic extracts of *Ocimum gratissimum* against some non-clinical colistin-resistant bacteria

Isolates	250mg	125mg	67.5mg	33.7mg
Pseudomonas aeruginosa	-	-	-	-
Enterobacter ludwigii	-	-	-	-
Providencia stuartii	-	-	-	-
Enterococcus dispar	-	-	-	-
Providencia rustigianii	-	-	-	-
Providencia rettgeri	-	-	-	-
Providencia burhodogranariea	-	-	-	-
Escherichia coli 1	-	-	-	-
Klebsiella quasipneumoniae	-	-	-	-
Enterococcus saccharolyticus	-	-	-	-
Escherichia coli 2	-	-	-	-
Salmonella enterica	-	-	-	-

Key: -: Resistant

Table 4: Bactericidal effect of the methanolic extracts of *Cymbopogan citratus* against some non-clinical colistin-resistant bacteria

Isolates	250mg	125mg	67.5mg	33.7mg
Pseudomonas aeruginosa	-	-	-	-
Enterobacter ludwigii	-	-	-	-
Providencia stuartii	-	-	-	-
Enterococcus dispar	-	-	-	-
Providencia rustigianii	-	-	-	-
Providencia rettgeri	-	-	-	-
Providencia burhodogranariea	-	-	-	-
Escherichia coli 1	-	-	-	-
Klebsiella quasipneumoniae	-	-	-	-
Enterococcus saccharolyticus	-	-	-	-
Escherichia coli 2	-	-	-	-
Salmonella enterica	-	-	-	-

Key: -: Resistant

Table 5: Bactericidal effect of the methanolic extracts of *Kalanchoe pinnata* against some non-clinical colistin-resistant bacteria

Isolates	250mg	125mg	67.5mg	33.7mg
Pseudomonas aeruginosa	-	-	-	-
Enterobacter ludwigii	-	-	-	-
Providencia stuartii	-	-	-	-
Enterococcus dispar	-	-	-	-
Providencia rustigianii	-	-	-	-
Providencia rettgeri	-	-	-	-
Providencia burhodogranariea	-	-	-	-
Escherichia coli 1	-	-	-	-
Klebsiella quasipneumoniae	-	-	-	-
Enterococcus saccharolyticus	-	-	-	-
Escherichia coli 2	-	-	-	-
Salmonella enterica	-	-	-	-

Key: -: Resistant

Table 6: Bactericidal effect of the aqueous extracts of *Ocimum gratissium* against some non-clinical colistin-resistant bacteria

Isolates	250mg	125mg	67.5mg	33.7mg
Pseudomonas aeruginosa	-	-	-	-
Enterobacter ludwigii	-	-	-	-
Providencia stuarti	35(S)	-	-	-
Enterococcus dispar	32(S)	21(S)	20(S)	18(S)
Providencia rustigianii	-	-	-	-
Providencia rettgeri	-	-	-	-
Providencia burhodogranariea	-	-	-	-
Escherichia coli 1	-	-	-	-
Klebsiella quasipneumoniae	-	-	-	-
Enterococcus saccharolyticus	-	-	-	-
Escherichia coli 2	26(S)	18(S)	22(S)	17(S)
Salmonella enterica	-	-	-	-

Key: -: Resistant; S: Sensitive

Table 7: Bactericidal effect of the aqueous extracts of *Kalanchoe pinnata* against some non-clinical colistin-resistant bacteria

Isolates	250mg	125mg	67.5mg	33.7mg
Pseudomonas aeruginosa	-	-	-	-
Enterobacter ludwigii	-	-	-	-
Providencia stuarti	26(S)	23(S)	17(S)	15(S)
Enterococcus dispar	-	-	-	-
Providencia rustigianii	-	-	-	-
Providencia rettgeri	-	-	-	-
Providencia burhodogranariea	-	-	-	-
Escherichia coli 1	-	-	-	-
Klebsiella quasipneumoniae	14(S)	11(S)	-	-
Enterococcus saccharolyticus	-	-	-	-
Escherichia coli 2	27(S)	17(S)	17(S)	20(S)
Salmonella enterica	-	-	-	-

Key: -: Resistant; S: Sensitive

Table 8: Bactericidal effect of the aqueous extracts of *Cymbopogan citratus* against some non-clinical colistin-resistant bacteria

Isolates	250mg	125mg	67.5mg	33.7mg
Pseudomonas aeruginosa	-	-	-	-
Enterobacter ludwigii	-	-	-	-
Providencia stuarti	-	-	-	-
Enterococcus dispar	-	-	-	-
Providencia rustigianii	-	-	-	-
Providencia rettgeri	-	-	-	-
Providencia burhodogranariea	-	-	-	-
Escherichia coli 1	-	-	-	-
Klebsiella quasipneumoniae	-	-	-	-
Enterococcus saccharolyticus	-	-	-	-
Escherichia coli 2	18(S)	15(S)	12(S)	16(S)
Salmonella enterica	-	-	-	-

Key: -: Resistant; S: Sensitive

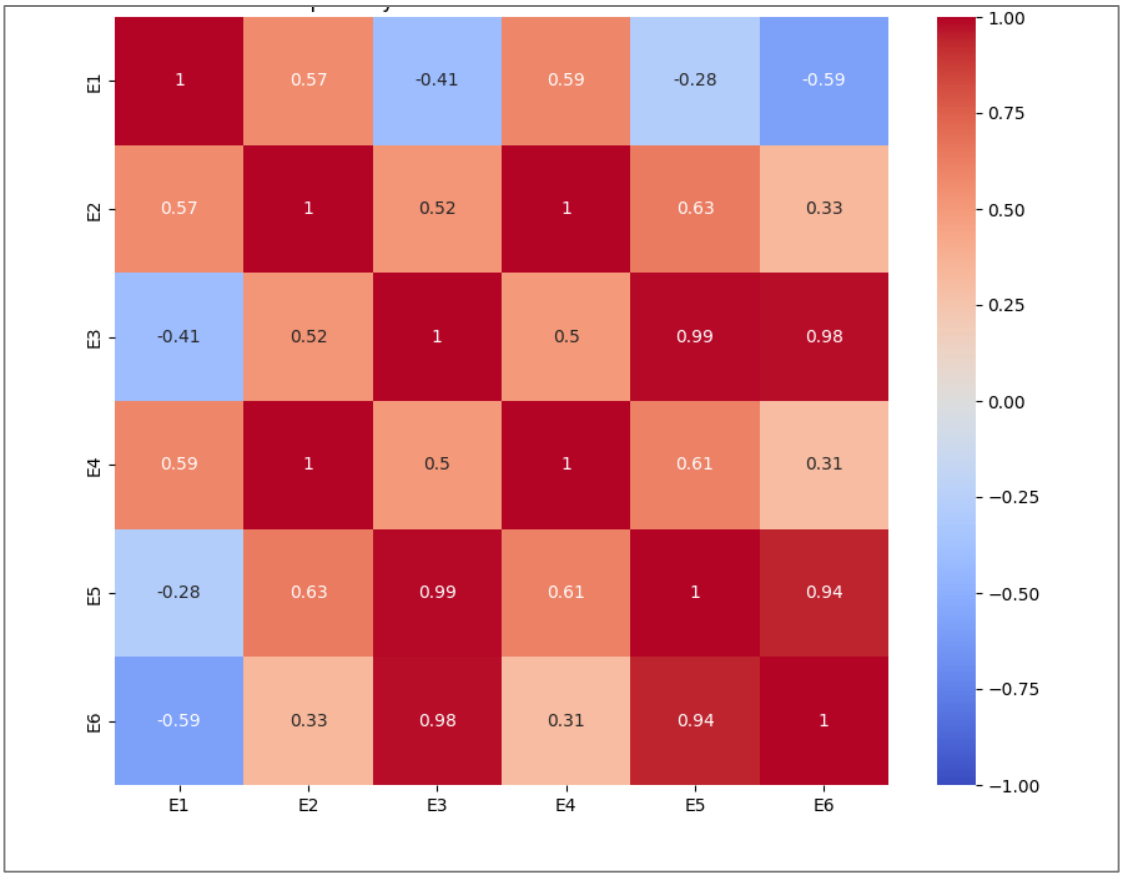

Figure 2: Correlation hit map of phytochemical concentrations across different environments

DISCUSSION

Since colistin is considered a last-resort antibiotic, it is concerning whether resistance to this medication arises outside medical facilities. The community's resistance mechanisms and colistin resistance carriage are poorly understood in developing nations. This investigation aimed to assess the antibiogramic efficacy of aqueous and methanolic extracts of *Cymbopogan citratus*, *Ocimum gratissimum*, and *Kalanchoe pinnata* against a range of Gram-negative bacteria obtained from non-clinical environments and resistant to colistin.

The qualitative phytochemical screening results indicated the presence of flavonoids, alkaloids, phenolic compounds, glycosides, saponins, proteins, reducing sugar, and carbohydrates in varying amounts across the different leaf extracts. This finding suggests that the plant extracts contain essential phytochemicals, as reported in previous studies. Plants remain an undiminished source of new pharmaceuticals and are mainly based on the presence/abundance of phytochemicals in them. Flavonoids are reported to be abundant in fruits, vegetables, and certain beverages and are known to have antimicrobial, anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties coupled with their capacity to modulate key enzyme functions (Yang et al. 2014; Panche et al., 2016). The presence of flavonoids in both aqueous and methanolic leaves extracts of all 3 plants investigated in the current study is in keeping with that of previous studies (Akimoladun *et al.* 2007; Macdonald *et al.*, 2010; Ajiboye *et al.,* 2014; Chetia *et al.,* 2014; Ohadoma *et al.,* 2015), and is an indication of their potential as used in the treatment of infections caused by a wide range of antimicrobials. However, in the current study, methanolic extracts of *C. citratus* did not have any antibacterial effect on all 12 Gram-negative bacterial isolates used, while aqueous extract only affected 1 (*Escherichia coli* 2) isolate and at all concentration levels investigated. In a previous study, the leave extract of *Cymbopogan citratus* was reported to be more effective against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) when compared with Gram-negative bacteria: *Escherichia coli* and *Vibrio cholerae* (Hasan et al., 2022), giving justification to the outcome reported in our study. *C. citratus* is known to contain an abundance of terpenes, especially the older plants (Lawal et al. 2017), and terpenes are well known to inhibit the growth of Gram-positive bacteria but are inactive against Gram-negative bacteria (Subramaniam et al. 2020).

The presence of phytochemicals and antimicrobial potential of *Ocimum gratissimum,* on the other hand, have been studied quite extensively, especially against Gram-positive bacteria as well as fungi, with the majority of studies reporting promising antimicrobial effects (Nakamura et al., 1999; Adebolu and Oladimeji, 2005; Chanthaboury et al., 2022; Silva et al., 2022). In a recent study, Hao and Quoc (2024) reported the plant extracts of *O. gratissimum* to have more antibacterial activities on Gram-negative bacterial (*Escherichia coli* and *Salmonella*

Typhimurium) when compared with *Bacillus cereus* and *Staphylococcus aureus*. In the current study, the methanolic extracts of *O. gratissimum* did not show any antibacterial effect on colistin-resistant Gram-negative bacterial isolates, while the aqueous extracts had an effect against *Providencia stuarti*, only at the highest concentration used (250 mg), and on *Enterococcus dispar* as well as *E. coli* 2 at all concentrations (250 mg, 125 mg, 67.5 mg, and 37.25mg). This is the first reported case of the antibacterial activity of *O. gratissimum* against *Enterococcus dispar*, and further evidence is warranted. It is also worth mentioning that most publications on the antimicrobial properties of different plant extracts focused on popular bacteria and fungi species such as *E. coli*, *Staphylococcus aureus*, *Vibrio cholerae,* etc, leaving out other very important pathogens of public health importance. For instance, this is the first study on the antibacterial activities of *O. gratissimum* against four different *Providencia* species (*P. stuartii*, *P. rustigianii*, *P. rettgeri*, *P. burhodogranariea*). Additionally, *Klebsiella quasipneumoniae* is a novel isolate first reported from the blood culture of a patient in 1997 (Brisse et al., 2014) and the environment in 2023 (Omoruyi et al., 2023), and there is no comparable literature on the antibacterial activities of different plant extracts against it. However, the compelling evidence is that the isolates investigated in the current study are known colistin-resistant Gram-negative bacteria predominantly of environmental origin (Omoruyi et al. 2023), and their resistance to the methanolic and aqueous plant extracts is a confirmation of their resistance to several antibiotics including colistin and therefore, a source of public health concern.

Similarly, Okwu and Nnamdi (2011) documented the presence of flavonoids and alkaloids in the methanolic leaf extract of *K. pinnata*. The aqueous leaf extract of this plant was the only tested leaf extract that had an effect against *Klebsiella quasipneumoniae* and could be a source of antibacterial against this very important pathogen of public health significance. A similar outcome was also reported for *Providencia stuarti* and *E. coli* 2. The methanolic leaf extracts of all samples exhibited minimal antibacterial activity against colistin-resistant bacteria at varying concentrations. While the exact reason for this observation remains unclear, certain test bacterial isolates displayed sensitivity to the aqueous extract at different concentrations.

Alkaloids were also found in only the aqueous leaf extracts of all three plants and were absent in their methanolic counterparts. This outcome is not surprising when compared with the results of previous studies, as alkaloids have been reported to be absent in the methanol, hexane, ethyl acetate, chloroform, and butanol leaf extracts of different plant leaves (Hossain et al., 2013). Also, aqueous extracts of all 3 plant leaves were found to have better and more promising antibacterial effects when compared with methanolic leaf extracts. This finding contradicts a review article on phytochemical screening and plant extract extraction, which suggested that using different solvents for plant extraction may result in more consistent antibacterial activity compared to using water as the extraction solvent (Tiwari *et al.*, 2011) as well as findings by Pattewar *et al.* (2013) who reported a higher inhibition rate by the methanolic extracts of *K. pinnata* when compared with the aqueous extracts.

Overall, the results obtained with these plants' leaf extracts were ineffective against most of the organisms tested against, particularly the methanolic extracts. The bacterial strains used in this investigation are known to be resistant to carbapenem (Mabika et al., 2020). The resistance rate was higher than that observed in many studies, estimated to be 6.91% and 6.2% (Liu *et al.,* 2016; Wang *et al.,* 2018), although the organisms studied were quite distinct. The high prevalence obtained with colistin in this study could be explained by modifying the charges on the organisms' outer membrane, influencing the electrostatic interactions between the positive and negative charges of the outer membrane. Indeed, these changes are due to the alteration of lipopolysaccharides (LPS) by covalent modifications of lipid A with the addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) on the phosphate groups of the lipid A and by acylation/deacylation (Lee *et al.,* 2014). These covalent modifications neutralize the negative charges of lipid A, thus conferring resistance to colistin. The continuous use of colistin in the veterinary field may be responsible for the bacteria resistant to colistin in this study. In general, resistance to colistin has been shown in the Enterobacteriaceae family (Del Bianco *et al.,* 2018). This high rate of resistance in this study is worrisome and calls for concern. Correlation analysis further revealed a positive correlation between some plant leaf extracts and the antibacterial activities recorded, while the analysis of variance showed variation in the concentration of phytochemicals and antibacterial activity.

CONCLUSION

Methanolic and aqueous leaf extracts of *Cymbopogan citratus*, *Ocimum gratissimum*, and *Kalanchoe pinnata* were found to contain phytochemicals such as flavonoids, tannin and saponin in varying degrees, while alkaloid was only present in the aqueous extract of all 3 leaf extracts. Additionally, the methanolic leaf extract of all 3 plants did not have any antibacterial effect on the 12 colistin-resistant bacterial isolates, while aqueous leaf extracts of *Cymbopogan citratus*, *Ocimum gratissimum*, and *Kalanchoe pinnata* had varying degrees of antibacterial activity against 1 (*Escherichia coli* 2), 3 (*Providencia stuarti*, *Enterococcus dispar,* and *E. coli* 2) and 3 (*Providencia stuarti*, *Klebsiella quasipneumoniae* and *Escherichia coli* 2) colistin-resistant bacterial isolates respectively. Overall, extracts of all 3 plants were more effective against *E. coli* 2, followed by *Providencia stuarti* and *Klebsiella quasipneumoniae*. The outcome of this study shows a promising effect on the use of aqueous leaf extracts of *Cymbopogan citratus*, *Ocimum gratissimum*, and *Kalanchoe pinnata* in the treatment of infection caused by selected colistin-resistant bacteria.

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