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ANTIBACTERIAL ACTIVITY OF *TERMINALIA GLAUCESCENS, MANGIFERA INDICA* AND *MITRACARPUS VILLOSUS* ON CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

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ABSTRACT

The root of *Terminalia glaucescens*, stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* were screened for antibacterial activities against 23 carbapenem-resistant Enterobacteriaceae (CRE) isolates. The phyto-constituents of the plants were extracted by cold maceration. Disc-diffusion and broth microdilution methods were used to determine the antibacterial activity and the minimum inhibitory concentration, respectively. The sensitivity of the isolates to the methanol extracts of the plant parts was between four to eight isolates (10 – 26.5 mm) including *Enterobacter aerogenes, Proteus mirabilis* and *Escherichia coli,* with the highest activity shown by *Mitracarpus villosus.* An overall higher activity was however observed with the ethanol extracts of the plant parts with potency on twelve to fifteen isolates (9 – 18.5 mm) including *Enterobacter aerogenes, Proteus mirabilis, Escherichia coli* and *Klebsiella pneumoniae*. Generally for all methanol extracts, a constant MIC value 100 mg/ml was observed for the susceptible isolates except two *Enterobacter aerogenes* isolates with MIC of 1 mg/ml while the MIC value of the ethanol extracts ranged from ≤ 0.1 – 100 mg/ml. Ethanol extracts of stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* exhibited considerably higher activities compared to other extracts with low MIC values. The phytochemical screening showed that the extracts contained at least five bioactive metabolites with alkaloids, tannin and flavonoids present in all. The plants used in this study show promising antibacterial activity that can be explored in the treatment of multi-drug resistant Enterobacteriaceae infections.

ACTIVITÉ ANTIBACTÉRIENNE DE TERMINALIA GLAUCESCENS, MANGIFERA INDICA ET MITRACARPUS VILLOSUS SUR CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

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ABSTRACT

La racine de Terminalia glaucescens, écorce de la tige de Mangifera indica et les feuilles de Mitracarpus villosus ont fait l'objet d'une activité antibactérienne contre 23 à l'épreuve des carbapénèmes Enterobacteriaceae (CRE) isolats. Les phyto-constituants de la plante ont été extraits par macération à froid. Disc-diffusion et de microdilution en méthodes ont été utilisées pour déterminer l'activité antibactérienne et la concentration minimale inhibitrice, respectivement. La sensibilité des isolats à l'usine de méthanol extraits des parties était de quatre à huit isolats (10 - 26,5 mm) y compris l'Enterobacter aerogenes, Escherichia coli et Proteus mirabilis, avec la plus forte activité affichée par Mitracarpus villosus. Une activité plus globale a toutefois observé avec l'éthanol extrait de la parties de plantes à l'activité sur 12 à 15 isolats (9 - 18,5 mm) y compris l'Enterobacter aerogenes, Proteus mirabilis, Escherichia coli et Klebsiella pneumoniae. En général pour tous les extraits au méthanol, une constante valeur MIC 100 mg/ml a été observée pour les isolats sensibles à l'exception de deux isolats Enterobacter aerogenes avec micro de 1 mg/ml alors que la valeur de la CMI d'extraits d'éthanol allaient de ≤ 0,1 - 100 mg/ml. Extraits de l'éthanol de l'écorce des tiges de Mangifera indica et les feuilles de Mitracarpus villosus présentaient des activités beaucoup plus élevé par rapport à d'autres extraits avec de faibles valeurs de CMI. La phytochemical dépistage préliminaire a montré que les contenus des extraits au moins cinq métabolites bioactifs alcaloïdes avec, de tanins et de flavonoïdes présents dans tous. Les plantes utilisées dans cette étude montrent une activité antibactérienne prometteuses qui peuvent être explorés dans le traitement des infections à entérobactéries résistantes aux médicaments

Keywords: Carbapenem – resistant Enterobacteriaceae (CRE), *Terminalia glaucescens, Mangifera indica, Mitracarpus villosus*, Antibacterial

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INTRODUCTION

The increase in the rate of antimicrobial resistance exhibited by bacteria, especially the Gram negative populace, and of major emphasis the Enterobacteriaceae family, is a threat to public health (1). Medicinal plants for centuries have been recognized for their use as remedies for infectious diseases because of the presence of biological components with therapeutic value (2). Carbapenem resistance in Enterobacteriaceae had been a negligible phenomenon before year 2000 (3). Carbapenem – resistant Enterobacteriaceae (CRE) are selected members of Enterobacteriaceae with hydrolytic activities on β-lactam drugs including carbapenems revered to possess the broadest antibacterial spectra over Gram negative bacteria (4, 5).

CRE cause serious infections in debilitated and immune-compromised patients, in association with prolonged hospitalization and increased fatality ranging from 24% to 70% (1). The CRE isolates used in this study - *Klebsiella pneumoniae, Escherichia coli, Enterobacter aerogenes* and *Proteus mirabilis* were isolated from urine and blood samples of in-patients attending three different selected tertiary hospitals in Ekiti, Osun and Oyo states, Nigeria.

Terminalia glaucescens is one of the about 100 species of the large flowering tree genus *Terminalia* belonging to the family Combretaceae. The plant is distributed in tropical, sub-tropical and savannah regions of the world. The root and stem of the plant have reportedly shown efficient bactericidal action against *Streptococcus mutans*, *Candida albicans* and *Staphylococcus saprophyticus* (6, 7, 8). *Mangifera indica* L, commonly called *mango* (English) is a large evergreen tropical tree in the family *Anacardiacea*. Mada et al. (8) have reported the use of the leaves, bark and root to treat oral candidiasis, malaria, skin infection, dysentery, diarrhea, thrush and shingles. *Mitracarpus villosus* is a member of the family Rubiaceae. In various parts of tropical Africa, it is traditionally used for treatment of sore throat, ringworm and eczema, fresh cuts, wounds and ulcer (9, 10, 11). Previous studies also revealed that the plant contains biologically active substances such as fatty acids, flavonoids and other phenolic compounds with potential antifungal, antimicrobial and anti-inflammatory activities (12, 13, 14, 15, 16, 17).

The anti-hemolytic, antibacterial and attenuation of quorum sensing and biofilm formation of few plants and essential oils against carbapenem-resistant isolates have been reported (16, 18); thus this study intends to provide information on plants with prospective efficacy against CRE. Based on this background, the susceptibility of 23 CRE isolates to ethanol and methanol extracts of *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus* was evaluated and compared.

Materials and Methods

Source of the isolates

The details on the source of the isolates - *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae* and *Proteus mirabilis* are provided in Table 1.

Screening of herbal extracts

The medicinal plants used in this study are *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus*.

Collection of plant materials: The root, stem-bark and leaves of *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus* respectively were sourced from farms by herbal practitioners in Ondo town, Ondo state, Nigeria. Identification and authenticated at an herbarium in Ondo state, Nigeria.

***Preparation of plant extracts*:** The plant parts were air-dried and pulverized into fine powder using a milling machine, then extracted by cold maceration. One hundred grams of the powdered plant parts was soaked in 500 ml of ethanol 96% and methanol, each. These mixtures were kept on the rotator shaker for 72 hours for agitated extraction. The mixtures were then filtered using Whatman filter paper no 1. The filtrate was concentrated using a water bath at 60 ˚C until solvent was completely removed. The crude was stored in an air-tight container and kept in a refrigerator at 4 ˚C until use.

Disc preparation: Discs of 6-mm in diameter were cut from Whatman no. 5 filter paper. The discs were wrapped in aluminium foil and sterilized in hot-air oven for 15 minutes. Then 50 µl of the reconstituted extracts was impregnated into the discs accordingly, based on prior absorbance test.

Antibacterial screening of plant extracts: The standardized organisms (adjusted to 0.5 McFarland) were seeded onto solidified Mueller Hinton Agar (Rapid Labs, UK) plates by transferring 100 µl of the bacterial suspension to the agar surface. Then a glass spreader was used to evenly cover the agar surface with the inoculum. The plates were left on the work bench for 30 minutes, and then the discs were placed firmly on surface of the agar using sterile forceps. The plates were left for 1 hour for diffusion of the extracts and then incubated at 37 ˚C for 18 – 24 hours (18). After incubation, the zones of inhibition generated by the antibiotics were measured on three axis using a ruler; the mean and standard error mean (SEM) of the values were calculated and recorded in millimeter (mm).

Determination of Minimum Inhibitory concentration (MIC): The broth microdilution method as described by CLSI (19) was adopted for the MIC with slight modifications. Varying

concentrations (10 mg/ml, 1 mg/ml and 0.1 mg/l) of the extracts were prepared with Mueller Hinton broth (MHB) (Rapid Labs, UK) at 1:10 dilution from the stock concentration of 100 mg/ml and kept in tubes. The wells of the 96-well microtitre plate were filled with 100 µl of the plants extracts. Then, 100 µl of the standardized bacterial suspensions were inoculated into each well. Dimethyl sulfide was used as a control and MHB as a negative control. Imipenem was used as positive control for the isolates. The plate was incubated at 37 ˚C for 18 - 24 hours. The plate was read by optical density at 650 nm to observe microbial growth. The minimum concentration that showed no visible growth was taken as the MIC of the extract for each organism. This assay was carried out in duplicates.

Determination of Minimum Biocidal Concentration (MBC): The content of the wells that showed no microbial growth were subcultured on MHA and incubated at 37 ˚C for 18 – 24 hours. The least concentration that showed no visible growth on plate was taken as the MBC.

Phytochemical analysis of plant extracts: The extracts of *Terminalia glaucescens, Mangifera indica* and *Mitracarpus villosus* were analyzed for the presence of alkaloid, saponins, anthraquinone, steroids, tannin, flavonoid, and cardiac glycosides according to standard methods (19, 20).

RESULT

Antibacterial screening of plant materials

The root of *Terminalia glaucescens,* stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* were screened for antibacterial activities against carbapenem-resistant Enterobacteriaceae isolates. Generally, the ethanolic extract of the plant parts exhibited more antibacterial activity than the methanol extract. The methanol extract of the root of *Terminalia glaucescens* showed potency on four (4) out of 23 isolates including *E. aerogenes* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 12 – 24 mm. The methanol extract of stem-bark of *Mangifera indica* was effective on five (5) of 23 isolates including *E. aerogenes* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 10 – 26.5 mm. The methanol extract of leaves of *Mitracarpus villosus* showed inhibitory effect on eight (8) isolates including *E. coli, E. aerogenes* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 9 – 26 mm (Figure 1). Overall, *E. coli* was highly resistant to the methanol extracts of the plant parts with just one of eight isolates showing susceptibility to *Mitracarpus villosus.* Also, *K. pneumoniae* showed no sensitivity to any of the extracts while a total of fifteen (15) isolates showed complete resistance to all methanol extracts of the plant parts.

The ethanolic extract of *Terminalia glaucescens* was effective on twelve (12) isolates including *E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 9.5 – 20 mm. The ethanolic extract of *Mangifera indica* showed potency on fifteen (15) isolates including *E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 9.5 – 18.5 mm. Lastly, the ethanolic extract of *Mitracarpus villosus* was effective on thirteen (13) isolates *E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis* from urine and blood samples, with zone of inhibition ranging from 10 – 19.5 mm. Six isolates showed complete resistance to all the three plant extracts (Figure 2).

Minimum Inhibitory Concentration/ Minimum Biocidal Concentration

For the methanol extract, lower concentrations of the extracts were less effective. A constant MIC value of 100 mg/ml was obtained for *Terminalia glaucescens* against four isolates including *E. aerogenes* and *P. mirabilis*. For *M. indica,* the MIC value of 10 (*E. coli, E. aerogenes, K. pneumoniae* and *P. mirabilis*) out of the 11 isolates that were inhibited was 100 mg/ml while one of the *E. aerogenes* isolates had MIC of 1 mg/ml. For *M. villosus,* seven isolates were inhibited at 100 mg/ml while two isolates had MIC of 1 mg/ml (Table 2).

Ethanol extract of *Terminalia glaucescens* had MIC value of 100 mg/ml on five isolates and then a lower value of ≤ 0.1 mg/ml on *Klebsiella pneumoniae*. The MIC value of ethanol extract of *M. indica* for fourteen isolates ranged from ≤ 0.1to 100 mg/ml while the MIC of ethanol extracts of *M. villosus* for thirteen isolates also ranged from ≤ 0.1to 100 mg/ml (Table 2). Succinctly, none of the extracts showed bactericidal properties on any of the isolates.

Qualitative Phytochemical analysis of the plant extracts

Table 3 shows the phytochemicals present in the extracts of the three plants. The ethanol extracts of both *Terminalia glaucescens* and *M. indica* contained all seven phytochemicals assayed for- alkaloid, saponin, tannin, anthraquinone, flavonoid, cardiac glycosides and steroids. The ethanol extract of *M. villosus* contained five of the seven phytochemicals except saponins and steroids.

The methanol extract of *Terminalia glaucescens* contained lesser phytochemicals than the ethanol extract. All phytochemicals were present except anthraquinones and cardiac glycosides. The methanol extract of *M. indica* contained all phytochemicals except steroids while *M. villosus* contained all except anthraquinones.

TABLE 1: SOURCE OF ISOLATES

S/N	Isolates	Sample	Age	Gender
U37	*Escherichia coli*	**Urine**	**70 +**	**M**
U50	*Escherichia coli*	**Urine**	**70 +**	**M**
B9	*Ent. Aerogenes*	**Blood**	**41 – 50**	**F**
B41	*Ent. Aerogenes*	**Blood**	**61 – 70**	**M**
U12	*Proteus mirabilis*	**Urine**	**51 – 60**	**M**
U31	*Proteus mirabilis*	**Urine**	**70 +**	**F**
U1	*Escherichia coli*	**Urine**	**21 – 30**	**M**
B16	*Escherichia coli*	**Blood**	**21 – 30**	**F**
B18	*Ent. Aerogenes*	**Blood**	**31 – 40**	**F**
B19	*Ent. Aerogenes*	**Blood**	**51 – 60**	**M**
B28	*Ent. Aerogenes*	**Blood**	**21 – 30**	**F**
U30	*Proteus mirabilis*	**Urine**	**41 – 50**	**F**
U42	*Proteus mirabilis*	**Urine**	**41 – 50**	**F**
B9	*Escherichia coli*	**Blood**	**51 – 60**	**M**
U18	*Escherichia coli*	**Urine**	**31 – 40**	**M**
U34	*Escherichia coli*	**Urine**	**41 – 50**	**F**
U50	*Escherichia coli*	**Urine**	**51 – 60**	**M**
U5	*Ent. Aerogenes*	**Urine**	**21 – 30**	**M**
U12	*Ent. Aerogenes*	**Urine**	**21 – 30**	**M**
U47	*Ent. Aerogenes*	**Urine**	**70 +**	**M**
B2	*Ent. Aerogenes*	**Blood**	**41 – 50**	**M**
B25	*Klebsiella pneumoniae*	**Blood**	**31 – 40**	**M**
U36	*Proteus mirabilis*	**Urine**	**51 – 60**	**F**

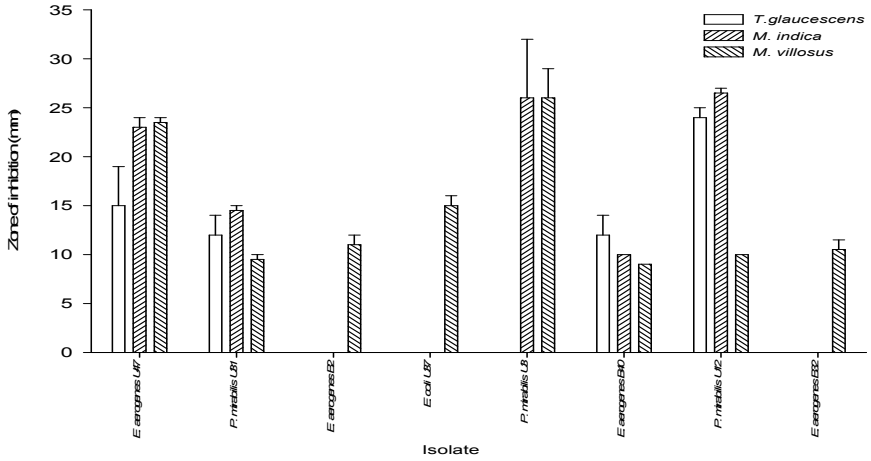

FIGURE 1: ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACTS OF THE PLANTS

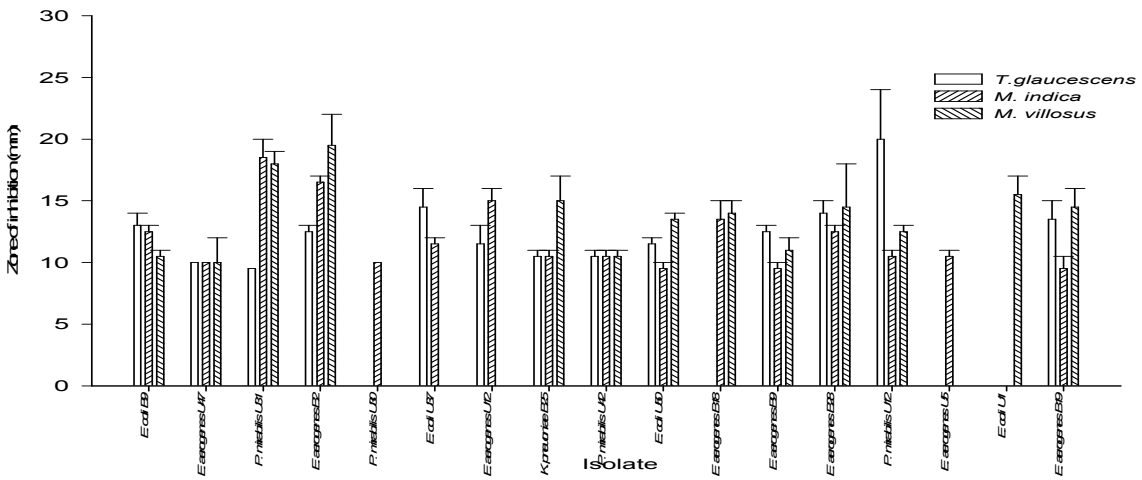

FIGURE 2: ANTIBACTERIAL ACTIVITY OF ETHANOL EXTRACTS OF THE PLANTS

TABLE 2: MINIMUM INHIBITORY CONCENTRATION OF THE METHANOL AND ETHANOL EXTRACTS PLANTS

Tag	Isolates	Methanol (× 10^3 µg/ml)			Ethanol (×10^3µg/ml)		
		T. glaucescens	*M. indica*	*M. villosus*	*T. glaucescens*	*M. indica*	*M. villosus*
U37	*Escherichia coli*	--	100	--	--	100	100
U50*	*Escherichia coli*	--	--	--	--	--	--
B9	*E. aerogenes*	--	100	100	--	0.1	0.1
B41	*E. aerogenes*	--	--	--	--	--	--
U12	*Proteus mirabilis*	100	100	100	100	100	100
U31	*Proteus mirabilis*	100	100	100	100	1	1
U1	*Escherichia coli*	--	--	--	--	--	100
B16	*Escherichia coli*	--	--	--	--	--	--
B18	*E. aerogenes*	--	--	--	--	0.1	100
B19	*E. aerogenes*	--	--	--	--	--	--
B28	*E. aerogenes*	100	100	100	100	100	100
U30	*Proteus mirabilis*	--	--	--	--	100	--
U42	*Proteus mirabilis*	--	100	100	100	0.1	100
B9	*Escherichia coli*	--	100	100	--	100	--
U18	*Escherichia coli*	--	--	--	--	--	--
U34	*Escherichia coli*	--	--	--	--	--	--
U50**	*Escherichia coli*	--	100	--	--	100	100
U5	*E. aerogenes*	--	--	--	--	0.1	1
U12	*E. aerogenes*	--	--	--	--	--	--
U47	*E. aerogenes*	100	100	1	100	100	0.1
B2	*E. aerogenes*	--	1	1	--	0.1	0.1
B25	*K. pneumoniae*	--	100	100	0.1	100	0.1
U36	*Proteus mirabilis*	--	--	--	--	--	--

- *Indicates 'no MIC' value*

TABLE 3: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN THE PLANT EXTRACTS

Extracts	Phytochemicals						
	Alkaloids	**Saponins**	**Tannin**	**Anthraquinone**	**Flavonoid**	**Cardiac glycosides**	**Steroids**
Terminalia glaucescens [a]	+	+	+	+	+	+	+
Mangifera indica [a]	+	+	+	+	+	+	+
Mitracarpus villosus [a]	+	--	+	+	+	+	--
Terminalia glaucescens [b]	+	+	+	--	+	--	+
Mangifera indica [b]	+	+	+	+	+	+	--
Mitracarpus villosus [b]	+	+	+	--	+	+	+

'a' represents ethanol extract 'b' represent methanol extract '+' represents present '-' represent absent

DISCUSSION

This study reports significant antibacterial activities of ethanol and methanol extracts of root of *Terminalia glaucescens,* stem-bark of *Mangifera indica* and leaves of *Mitracarpus villosus* on CRE isolates recovered from urine and blood samples of humans. The methanol extract of *Mitracarpus villosus* was the most effective compared to the methanol extracts of *T. glaucescens* and *M. indica* by exerting antibacterial activity on 34.8% of the isolates including *E. coli, E. aerogenes* and *P. mirabilis*. The methanol extract of *T. glaucescens* was effective on 17.4% of the isolates including *K. aerogenes* and *P. mirabilis*; and *M. indica* was potent on 21.7% of the isolates *E. aerogenes* and *P. mirabilis*.

The ethanol extract of *M. indica* showed the highest antibacterial activity compared to extracts of *T. glaucescens* and *Mitracarpus villosus* by exerting potency on 65.2% of the isolates. Both the ethanol and methanol extracts of the plants used in this study have been reported to show antibacterial efficacy against gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella* sp. and a range of gram positive bacteria (6, 7, 8, 9). *T. glaucescens* has been studied to show considerable levels of activity against *K. pneumoniae, E. coli* and *P. mirabilis* (22, 23, 24), which is also validated in this study.

Lower concentrations of the methanol extracts of all the plants were less effective against the test isolates compared to the ethanol extracts which could be attributed to the efficacy of solvents. Ethanol extracts of *Terminalia glaucescens* and *Mitracarpus villosus* showed remarkable activity on the carbapenem-resistant *Klebsiella pneumoniae* with MIC at ≤ 0.1 mg/ml. Gbadamosi and Ogunsuyi (24) reported that the ethanolic extract of the root of *Terminalia glaucescens* showed high potency and against multidrug resistant *Escherichia coli* at a concentration of 100 mg/ml which also conform with the findings of this study. The low MIC values recorded for plant extracts show their high efficacy as bacteriostatic agents. Despite the low MIC values recorded, none of the concentrations of the plants extracts showed bactericidal effect on any of the isolates.

There were variations in the phytochemical contents of the ethanol and methanol extracts of each plant which could be responsible for the different level of potency exhibited. The presence of phytochemicals has likewise been studied to vary depending on the

type of extraction and the solvent used. However, all the extracts contained alkaloids, flavonoids and tannins. Alkaloids are alkaline chemical substances with high ammonia content which act as stimulants; thus effective in the treatment of respiratory and gastrointestinal diseases (26). Also, flavonoids possess anti-oxidative and anti-inflammatory properties; thus effective in the protection of the blood capillaries (26). The acidic nature of tannins as well as the presence of gallic and epigallic acids has been studied to be effective as antiseptics. The antibacterial activities exhibited by the different extracts of the plants used in this study can therefore be attributed to the presence of these different phytochemicals.

Conclusion

The plants used in this study have shown explorable bacteriostatic efficacy against extensive/ pan-drug resistant Enterobacteriaceae isolates. Higher concentrations of the extracts may be required to exert bactericidal actions on the CRE isolates. The quantitative phytochemical analysis will also be necessary in determining the most abundant phytoconstituent that may be responsible for the inhibitory activity exhibited by the extracts.

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