RESEARCH ARTICLE

In vitro inhibition of multi-drug resistant Pseudomonas efflux pump by *Xylopia aethiopica* (Dunal) A. Rich

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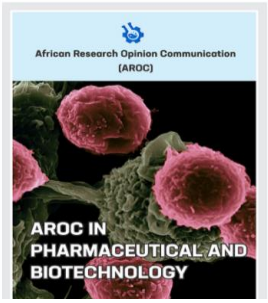

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ABSTRACT

Global health is under constant threat due to antimicrobial drug resistance. Bacterial Infections caused by *Pseudomonas aeruginosa* are of importance because of their antibiotics resistance. This study aimed at evaluating the effects of extracts of *Xylopia aethiopica* (XA) on multidrug-resistant (MDR) *Pseudomonas* isolates. Fresh samples of XA leaf, stem bark and roots were collected from the botanical garden, University of Ibadan, Nigeria. Dried and pulverized samples were extracted with methanol and partitioned into *n*-hexane, dichloromethane and ethyl acetate. Phytochemical screening of the extracts was performed by standard methods. Antimicrobial activity and synergistic interaction were determined using microdilution and checkerboard broth dilution methods, respectively. The results revealed that crude methanol extracts of XA leaf, stem bark and root significantly ($p<0.05$) inhibited the growth of all tested MDR *Pseudomonas* isolates at 10 mg/mL. At 1 mg/mL, the ethyl acetate fraction of the leaf, and dichloromethane fraction of the roots produced clear zones of inhibition of 12 – 20 mm, and minimum inhibitory concentrations (MICs) of 1 µg/mL and 0.5 mg/mL, respectively. The modulation factor (MF) of ciprofloxacin, dichloromethane fraction of XA roots and ethyl acetate fraction of XA leaf were 4, 8, and 4 on MDR isolates E01006, OAU058 and *P. aeruginosa* ATCC 27853, respectively. In all tested isolates, but not E01006 and E01024, the fractional MICs of ciprofloxacin/ethylacetate fraction of XA leaf extract combination was not significantly different ($p>0.05$) compared with ciprofloxacin/verapamil combination. In conclusion, the root and leaf fractions *Xylopia aethiopica* that demonstrated antimicrobial activity against MDR *P. aeruginosa* and synergised with ciprofloxacin have the potential to rejuvenate the antimicrobial activity of ciprofloxacin in MDR *P. aeruginosa*.

Keywords: *P. aeruginosa*; Modulation factor; *Xylopia aethiopica*; Zones of Inhibition; Minimum Inhibitory Concentration

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1.0 Introduction

Antimicrobial resistance occurs when microorganisms are able to withstand the suppressing or lethal ability of antimicrobials [1]. The emergence of infections caused by resistant microbes has led to mortality and morbidity thereby requiring an urgent need to combat microbial resistance [2]. Antibiotic-resistant infections were reported to cause 35,000 deaths among the 2.8 million infected annually in the USA [3].

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen particularly in immune-compromised patients and has been recognized as one of the five main pathogens in nosocomial infections [2]. Pseudomonas species cause a wide range of infections such as urinary tract, respiratory, bone and joint and skin infections. The prevalence of infections caused by MDR *P. aeruginosa* strains is high and varies across the world with a significant risk of increased morbidity and deaths [4,5]. The intrinsic resistance in Gram-negative bacteria and acquired clinical resistance are attributed to the effects of multidrug resistance (MDR) efflux pumps [6].

Multidrug resistant (MDR) is a phenotype in a bacterium that is resistant to three or more classes of antimicrobial drugs [7]. The resistance of *P. aeruginosa* to antibiotics is largely mediated by MexEF-OprN, and MexXY-OprM members of the-resistance-nodulation division (RND) multidrug efflux pumps and contributes to the intrinsic resistance of *P. aeruginosa* [8,9]. Other members of

RND are MexAB-OprM and MexCD-OprJ. Multidrug resistance strains of *P. aeruginosa* extruding antibiotics out of the cell thereby reducing the uptake of antibiotics [7,10]. These important considerations make efflux pumps interesting drug targets for inhibition because the combinations of efflux pump inhibitor (EPI) and antibiotic would lead to increased intracellular drug concentration, enhance potency and less tendency for acquired resistance.

Xylopia aethiopica belongs to the plant family Annonaceae and is cultivated for its fruits in tropical and subtropical zones of West Africa [11]. Preparations from xylopia are used in traditional medicine to treat cough, bronchitis, stomach aches, rheumatism, dysentery, malaria, uterine fibroid, microbial infections and amenorrhea [12-14]. It is therefore hypothesized that *Xylopia aethiopica* extracts possess antibacterial and inhibitory properties against efflux pumps in multidrug-resistant Pseudomonas strains.

2.0 Materials and Methods

2.1 Plant collection, authentication and preparation

The fresh leaves, stem bark and roots of XA were collected from the botanical garden, University of Ibadan and authenticated at the herbarium unit of the Department of Botany, University of Ibadan (voucher number UIH 22754). The plant parts (leaf, stem bark and roots) were size reduced, air-dried and then pulverized using a grinder.

2.2 Plant extraction

Five hundred grams (500g) each of pulverized root, stem, and leaf of XA were weighed into large glass desiccators which were stoppered and 100% methanol added until the separate samples were completely immersed. The set-up was left for 72 hr with periodic agitation. After 72 hr, each of the mixtures was filtered, concentrated using the rotary evaporator and oven-dried at a temperature of 50 - 60^0C until dry. The extracts were weighed and percentage extraction yield was calculated; they were then stored in well-corked universal bottles.

A known weight of each extract was dissolved in 100 mL of distilled water/ methanol in a ratio of 1:3. The extract was then partitioned into n-hexane, dichloromethane, ethyl acetate and methanol fractions and oven-dried. The dried fractions were weighed and the percentage yield calculated.

2.3 Qualitative phytochemical screening

The various fractions (stem, roots and leaf) were qualitatively tested for the presence of secondary metabolites such as tannins, saponins, cardiac glycosides, flavonoids, steroids, alkaloids and anthraquinones. The tests were carried out according to the methods described by Oluremi *et al*. [15].

2.4 Bacterial isolates

The microbial isolates used were clinical isolates whose identities were established by ViTEK (VK2C 18815, Biomerieux, USA) and genome sequencing obtained from the Molecular/Biotechnology Laboratory of the Pharmaceutical Microbiology Department, University of Ibadan. Nine clinical isolates and one ATCC multidrug resistant *Pseudomonas aeruginosa* that were used for this research are; E0124, E0117b, E0102, OAU058, E01006, E0167W, E01052W, E01037, E011011 and ATCC 27853.

2.5 Antimicrobial agents

The eight disc (Abtek) used in this study were ciprofloxacin (CIP) 5µg, Nitrofurantoin (NIT) 300 µg, Cefuroxime (CRX) 30 µg, Gentamicin (GEN) 10 µg, Augmentin (AUG) 30 µg, Ceftazidime (CAZ) 30 µg and Cefixime (CXM) 5 µg.

2.6 Antimicrobial susceptibility screening

The agar diffusion method was employed using the protocol of the Clinical and Laboratory Standards Institute [16]. Twenty milliliters (20 mL) of sterile Mueller Hinton agar prepared in a universal bottle were allowed to cool at 50 °C and inoculated with 0.5 McFarland equivalent MDR samples before they were poured aseptically into petri dishes and allowed to set. Four equidistant wells were aseptically made or placed on the agar using a 6 mm diameter cork borer. Subsequently, 20 µL of 10, 1, 0.8, 0.5, 0.4, 0.2 and 0.1 mg/mL concentrations were then placed in these holes and labeled appropriately. In each case, 0.5 % dimethylsulfoxide (DMSO) was used as a negative control, while the positive controls were ciprofloxacin and gentamicin. In the case of the standard antibiotics, discs were aseptically placed on the set Mueller Hinton agar without wells made in them. The plates were left to stand for 30 minutes before incubation at 37 ℃ for 24 hr after which the zones of inhibitions were measured and recorded.

2.7 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration

The MICs of the MDR isolates were determined using the broth dilution method in a sterile 96-well microtiter plate as described by Oluremi *et al*. [15]. Briefly, 100 µl of Mueller Hinton broth was dispensed into all wells of the microtiter plate. A double strength of the extract was dispensed into column 1 of the plate and properly mixed. A 100 µl of the medium + extracts was transferred into column 2 to make a half dilution of column 1. The procedure was repeated to column 10 and thereafter, the 100 µl of the mixture from column 10 was discarded. Each well was subsequently inoculated with 5 µl of the standardized MDR isolate. Column 11 served as the negative control while column 12 served as the sterility control. The plates were incubated at 37 ℃ for 24 hr. after which the iodonitrotetrazolium (INT) dye was added to view the inhibitory effects of the extracts. A colour change to pink indicated microbial growth. The MIC was determined as the lowest concentration at which the colour of the indicator remained unchanged, representing the absence of bacterial growth.

The MDR isolates that showed no growth on each of the 96-well microtiter MIC plates beginning from the last well in which bacterial growth was observed were streaked on Mueller-Hinton agar and the setup incubated at 37 ℃ for 24 h. The minimum bactericidal concentration was recorded as the concentration that prevented recovery of viable bacteria [17].

2.8 Efflux pump inhibition assay

Studies of inhibitory interactions were performed by a checkerboard broth dilution technique as described by Iman Islamieh *et al*. [18]. Briefly, ciprofloxacin at concentration of $^1/_8$ to $^1/_2$ was added to 50 µL of sterile Mueller-Hinton broth contained in a 96-well microtiter plate and two-fold serial dilutions made in the *x*-axis across the columns. The procedure was repeated for the efflux pump inhibitor (EPI), verapamil or the active fractions of XA on the *y*-axis. An inoculum size of 10^6 CFU/mL of the MDR isolates was added into each well in columns 1-7 and the setup was incubated at 37 ºC for 18 hr. The experiments were performed in duplicates. The lowest possible concentration of the agents with no visible turbidity was recorded as the MIC of the checkerboard.

2.9 Synergistic or antagonistic effects efflux pump inhibitor

The Modulation factor (MF) of the EPI or the fractions on the antibiotic was determined; where the MF is the ratio of the MIC of the antibiotics to the MIC of the antibiotics and the EPI or the factions. MF values greater than 4 fold are significant. The fractional inhibitory concentration (FIC) is calculated as the reciprocal of MF. FIC≤ 0.25 is synergism, 0.25>FIC<2 is indifference and FIC ≥2 is antagonism [18,19].

2.10 Statistical analysis

Data were analysed with statistical package for social sciences (SPSS) software version 16.0 using descriptive statistics and one-way ANOVA at $\alpha_{0.05}$. Mean separation was achieved by Duncan's *post hoc* test.

3.0 Results

3.1 Phytochemical constituents of *Xylopia aethiopica*

The phytochemicals tannins, saponins, flavonoids, terpenoids and steroids were detected in the crude methanol leaf extract of XA as shown in Table 1. Crude methanol extract of the three plant parts used for this study all tested positive for tannins and steroids. The stem bark and root extracts tested positive for tannins, saponins, cardiac glycosides and steroids.

Table 1: Phytochemical constituents of crude methanol leaf, stem bark and roots extracts of *Xylopia aethiopica*

Secondary metabolites	Leaf	Stem	Root
Tannins	++	+	+
Saponins	++	-	+
Cardiac glycosides	-	+	+
Flavonoids	++	-	-
Terpenoids	+	-	-
Steroids	+	+	+
Alkaloids	-	+	-
Anthraquinones	-	-	-

Key: ++ = Abundant; + = scanty; - = Absent

3.2 Antibiogram screening of microbial isolates

The zones of inhibition on the MDR Pseudomonas isolates by the antibiotics were measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. There was no zone of growth inhibition around at least one antibiotic in three different classes of the antimicrobial disc tested on Pseudomonas isolates when read on the CLSI scale (Table 2). The ten Pseudomonas isolates were resistant to either seven or all the eight tested antibiotics with diameter of zones of inhibition less than the breakpoints, except E0117b and *P. aeruginosa* ATCC 27853. Two of the eight Pseudomonas isolates resistant to ciprofloxacin were sensitive to ofloxacin producing diameter of zones of inhibition ≥16 as shown in Table 2.

Table 2: Antibiogram of *P. aeruginosa* isolates

	Zones of inhibition (mm)							
Isolates	**AUG**	**OFL**	**NIT**	**CAZ**	**CXM**	**CRX**	**GEN**	**CIP**
E01024	R	R	R	R	R	R	R	R
E0117b	R	S	R	R	R	R	S	I
E0102	R	R	R	R	R	R	R	S
OAU058	R	R	R	R	R	R	R	R
E01006	R	R	R	R	R	R	R	R
E0167W	R	R	R	R	R	R	R	R
E01052W	R	R	R	R	R	R	R	R
E01037	R	S	R	R	R	R	R	R
E011011	R	R	R	R	R	R	R	R
P. aeruginosa ATCC 27853	R	S	R	R	R	R	S	R

Key: R= Resistant I= Intermediate; S= Susceptible; GEN= Gentamicin (30 µg); CAZ= Ceftazidine (30 µg); CXM= Cefixime (5 µg); OFL= Ofloxacin (5 µg); AUG= Augmentin (30 µg); CIP= Ciprofloxacin (5 µg); NIT= Nitrofurantoin (30 µg)

3.3 Susceptibility of *P. aeruginosa* isolates to *Xylopia aethiopica*

At 10 mg/mL, E0I024, E0117b and E0102 were sensitive to ethyl acetate and DCM fractions of root extract of XA producing zones of inhibition of growth measuring 10 mm, 20 mm and 10 mm respectively. The crude methanol leaves extract of XA inhibited the growth of E0117b and E0102 with the zone of inhibition diameters of 18 mm and 25 mm respectively. The dichloromethane fraction of the leaf extract at concentrations of 1, 0.8, 0.5, 0.4, 0.2 and 0.1 mg/mL inhibited the growth of E0117b with inhibition zones of 20, 14, 12, 10, 8, and 8 mm respectively (Table 3).

Table 3: Susceptibility of *P. aeruginosa* isolates to *Xylopia aethiopica*

	Zones of inhibition (mm)																	
	XRD						XLE						XLM					
Isolates	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
E0117b	20	14	12	10	8	8	12	-	-	-	-	-	15	-	-	-	-	-
E01024	14	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E0102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OAU058	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E01006	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. a ATCC 27853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key: Diameter of cork borer= 6mm; XRD= Xylopia root dichloromethane fraction; XLE= Xylopia leaf ethyl acetate fraction; XLM= Xylopia leaf crude methanol extract; - = No zone of inhibition; 1= 1 mg/mL; 2= 0.8 mg/mL; 3= 0.5 mg/mL; 4= 0.4 mg/mL; 5= 0.2 mg/ mL; 6= 0.1 mg/mL; P. a= *P. aeruginosa*

3.4 Minimum inhibitory concentrations of extract and fraction of *Xylopia aethiopica*

The MIC of dichloromethane (DCM) fraction of XA roots extract on MDR Pseudomonas isolates E01024 and E0117b were 0.5 mg/mL and 0.25 mg/mL, respectively. The ethyl acetate fraction of the leaf extract produced MIC of 1 mg/mL with the MDR Pseudomonas isolate E0117b, but not E01024. The MICs of DCM fraction and ethyl acetate fraction for E01024 and E0117b were significantly higher (p<0.05) compared to the MIC of the standard

antibiotic, ciprofloxacin (5 µg/ml), compared to the MIC of ciprofloxacin alone. The MICs of ciprofloxacin combinations with different fractions of XA were significantly decreased by 2-8 fold (Table 4). In all tested MDR Pseudomonas isolates, the MIC for ciprofloxacin combination with leaf fractions of XA were significantly different (p>0.05) compared to MIC of ciprofloxacin/verapamil combination, but not in MDR *P. aeruginosa* isolates OAU058 and E01024. The modulation factors for ciprofloxacin/ EPI combination in all isolates were ≥4, but not in MDR *P. aeruginosa* isolates OAU058 and E01024 (figure 1). The fractional inhibitory concentrations (FIC) of ciprofloxacin and efflux pump inhibitor (verapamil) for E01024 was significantly different (p<0.05) compared to the FIC of all the tested fractions of XA. The FIC of ciprofloxacin/ verapamil combination and ciprofloxacin/ *Xylopia aethiopica* for E0117b were not significantly different (p>0.05). The FIC of ciprofloxacin/verapamil combination for the MDR Pseudomonas isolates E0102, OAU058 and E01006 compared to the FIC of ciprofloxacin/ *Xylopia aethiopica* were variable as shown in figure 2.

Table 4: Minimum inhibitory concentrations of susceptibility of *P. aeruginosa* isolates using sub-inhibitory concentrations of ciprofloxacin and EPI/ or fractions

	Ciprofloxacin/EPI (µg/mL)				
Isolates	**Ciprofloxacin**	**Verapamil**	**XLE**	**XRD**	**XLM**
E01024	5	0.313	1.25	1.25	1.25
E0117b	>5	0.625	0.625	1.25	1.25
E0102	5	0.625	0.625	>1.25	>1.25
OAU058	2.5	0.313	1.25	0.625	>1.25
E01006	2.5	1.25	1.25	1.25	1.25
P. aeruginosa ATCC 27853	5	1.25	1.25	0.625	1.25

Key: XLE= Xylopia leaf ethyl acetate fraction; XRD= Xylopia root dichloromethane fraction; XLM= Xylopia leaf methanol fraction

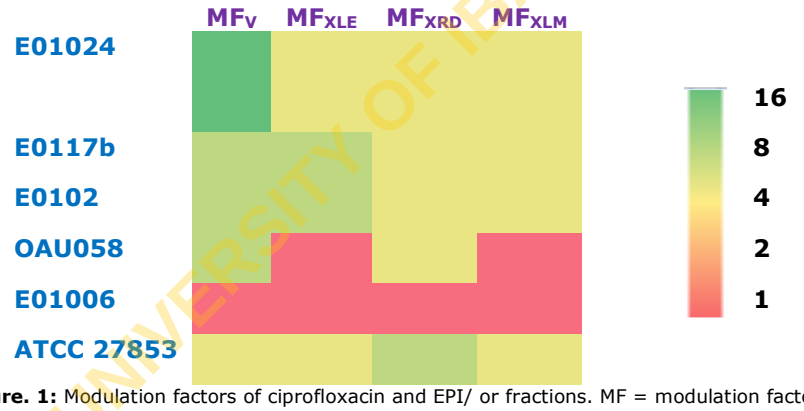

Figure. 1: Modulation factors of ciprofloxacin and EPI/ or fractions. MF = modulation factor; MF values >4fold= significant; MF_V= modulation factor of ciprofloxacin/ verapamil; MF_{XLE}= modulation factor of ciprofloxacin/ Xylopia leaf ethylacetate fraction; MF_{XRD}= modulation factor of ciprofloxacin/ Xylopia root dichloromethane fraction; MF_{XLM}= modulation factor of ciprofloxacin/ Xylopia leaf crude methanol extract

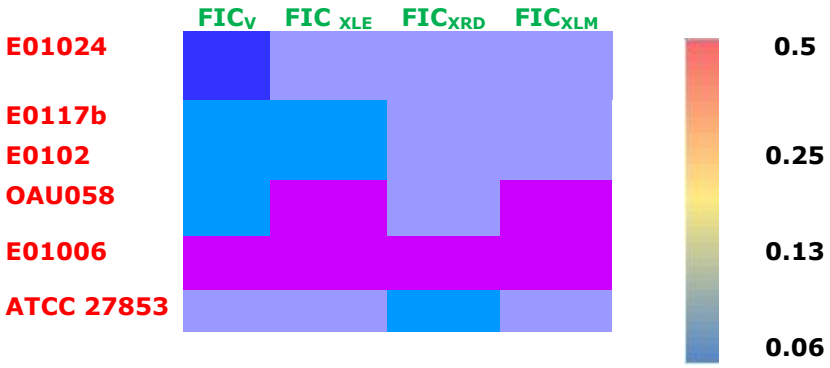

Figure. 2: Fractional inhibitory concentrations (FIC) of ciprofloxacin and EPI/ or fractions. FIC= fractional inhibitory concentration; FIC_V= fractional inhibitory concentration of ciprofloxacin/ verapamil FIC_{XLE}= fractional inhibitory concentration of ciprofloxacin/ Xylopia leaf ethylacetate fraction; FIC_{XRD}= fractional inhibitory concentration of ciprofloxacin/ Xylopia root dichloromethane fraction; FIC_{XLM}= fractional inhibitory concentration of ciprofloxacin/ Xylopia leaf methanol extract; FIC≤ 0.25= synergism, 0.25>FIC<2= indifference and FIC ≥2 is antagonism

4.0 Discussion

This study evaluated the antibacterial effects of the roots, stem barks and leaves of *Xylopia aethiopica* on MDR *Pseudomonas aeruginosa* strains as well as its potential to serve as an efflux pump inhibitor. The highest percentage yield of extract was 2.48 % in the methanol leaf extract. Qualitative phytochemical test detected the presence of tannins, saponins, cardiac glycosides, flavonoids, terpenoids, steroids, alkaloids and anthraquinones. Result from this study to some extent agrees with the previous findings of Aguoru *et al*. [21] who reported the presence of tannins, saponins, flavonoids and steroids in the crude leaf extract of XA. However, crude stem bark methanol extract of XA tested positive for the presence of tannins, steroids and cardiac glycosides in addition to alkaloids reported by Aguoru *et al*. [21]. Although no phytochemical was reported to be present in the crude root extract of XA by Aguoru and co-researchers [21], in this study, trace amounts of tannins, cardiac glycosides, saponins, and steroids were detected in the crude methanol root extract of XA.

Bacterium which is resistant to one antibiotic in three or more classes of antimicrobial drug is classified as multidrug resistant (MDR) [7], and all the Pseudomonas isolates used in this study satisfy these criteria and justifies their use. All the MDR *P. aeruginosa* isolates were sensitive to the crude methanol extracts of leaves, roots and stem bark of XA tested at a concentration of 10 mg/mL. This observation agrees with the findings of Emeh *et al.* [22] who reported that the crude ethanol leaf extract of XA showed some antimicrobial activities against *P. aeruginosa* producing zone growth inhibition of 18 mm at 200 mg/mL. However, only dichloromethane fraction of root extract showed antibacterial activity at concentrations ≤1 mg/mL. This implies that the dichloromethane fraction of root extract XA exhibited superior antibacterial activity against MDR *P. aeruginosa* isolate, E0117b and E01024 compared to the other fractions.

Bacterial membrane proteins that export harmful substances out of the bacterial cell are known as efflux pumps [23]. Overcoming drug efflux by the use of EPIs is a novel strategy to overcoming antibiotic resistance, whilst reducing the rates of resistance development [6]. Efflux pump inhibitors are therapeutic agents with the potential to facilitate the re-introduction of some current antibiotics that have become clinically ineffective [19]. Several agents including verapamil a calcium channel blocker antihypertensive agent that was used in this study as control has been previously employed to investigate potential efflux pump inhibition effects. In this study, all tested MDR Pseudomonas isolates the ethyl actetate leaf fraction, dichloromethane root fraction and leaf methanol extract of XA lowered the MIC of the fluoroquinolone ciprofloxacin by ≥4-fold comparable to the effect of verapamil, but not in OAU058 and E01006. This implies that

the fractions potentiated the activity of ciprofloxacin by a mechanism suggestive of inhibition of efflux pump. Efflux pump inhibitors including those from plant extracts may not have independent antimicrobial properties, but when they are used in combination with standard antibiotics, they suppress microbial efflux pump and as well enhance the efficacy of drugs [24].

The modulation factor of combination of antibiotic and EPI value greater than 4, that is, the ratio of the MIC of the antibiotics to the MIC of antibiotic efflux pump inhibitor combination is regarded as significant [20]. The MF value of combinations of ciprofloxacin and the ethyl actetate leaf fraction, dichloromethane root fraction or methanol leaf extract of *Xylopia aethiopica* were ≥4, suggestive of significant beneficial interaction. In this study, the fractional inhibitory concentrations of combination of ciprofloxacin and ethyl acetate fraction of the leaf extract, dichloromethane fraction of the root and the crude methanol leaf extract of XA were ≤0.25 in all MDR Pseudomonas isolates, but not in OAU058 and E01006, signifying synergism. Fractional inhibitory concentration is a measure of synergism or antagonism of combination of antibiotics and EPI is defined by the scale: FIC≤0.25= synergism, 0.25>FIC<2= indifferent and FIC≥2= antagonism [25,20]. The synergism produced by ethyl actetate leaf fraction, dichloromethane root fraction or methanol leaf extract of XA in combination that was comparable with that of efflux inhibitor, verapamil and ciprofloxacin. The *Xylopia aethiopica* fractions might have inhibited MDR *P. aeruginosa* efflux of ciprofloxacin in a manner that implicates similar mechanism of *P. aeruginosa* efflux pump inhibition by verapamil.

5.0 Conclusion

Xylopia aethiopica used in traditional medicine for the treatment of infectious diseases demonstrated anti-MDR Pseudomonas activity, and could be a potential natural source of efflux inhibitor for use in combination with traditional antibiotics against MDR Pseudomonas strains. Studies to ascertain the active compounds of *Xylopia aethiopica* that are responsible for the inhibition of efflux pump of MDR Pseudomonas are highly recommended.

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