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In Vitro Antibacterial Testing Of Fruit And Leaf Extracts Of *Physalis angulata* (L) On Multidrug Resistant Bacterial Isolates From HIV Patients

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Abstract: ***This study was aimed at investigating the antibacterial activity of Physalis angulata (L) against multidrug resistant (MDR) bacterial isolates from the blood of HIV patients attending a tertiary healthcare institution in Ondo State, Nigeria. A total of 293 bacterial species were isolated from the blood of the recruited HIV patients. The 16S rDNA technique was used for the molecular characterization of these isolates sequel to culturomic characterization. Drug resistance patterns were established using impregnated antibiotic discs in the Kirby Bauer's method. The MDR bacterial species were Proteus mirabilis CYPM, Salmonella typhi Ty21, Salmonella typhimurium L-3553, Enterobacter aerogenes CAV1320 and Escherichia coli O103:H2. The antibacterial efficacy of the leaf and fruit extracts of Physalis angulata (L) on these organisms was evaluated using agar well diffusion method. The extracts were prepared by hot continuous extraction using Soxhlet apparatus with methanol and n-Hexane as extracting solvents. The phytochemical screening of their bioactive constituents was conducted, the Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations of these extracts were also determined. The structural elucidation of bioactive components of the most effective extracts was carried out by Gas Chromatography/Mass Spectrophotometry. Methanolic extracts of fruits and leaves of P. angulata, were highly effective against Proteus mirabilis CYPM, that displayed the highest antibiotic resistance while the n-hexane extract of P. angulata fruits was highly effective against S. typhi Ty21. P. angulata is a promising candidate of antibacterial effectiveness against MDR bacterial isolates, encountered in HIV Patients and other immunocompromised individuals. The structural elucidation of its extracts also points towards the possession of chemical compounds effective for anticancer therapy.***

Keywords: Antibacterial, inhibition, isolates, MDR, antibiotics.

I. INTRODUCTION

The menace of drug resistance in the treatment of infectious diseases is of serious public health concern all over the world (WHO, 2016). Antibiotics are basically chemicals produced from certain microorganisms through fermentation to inhibit or eliminate other bacteria (Atta, 2015). Generally, antibiotics used in the treatment of bacterial infections are categorized into broad spectrum and narrow spectrum antibiotics (Acar, 1997). Bacterial pathogens genetically build resistance against antibiotics due to prolonged exposure to these antibiotics (Roberts, 1996). These aetiological agents of infections resist treatment by antibiotics through various techniques such as; expulsion of antimicrobials by efflux pumps, modification of porin channels against entry of the antimicrobial agent, inactivation of antibiotics and alteration of the antibiotic-binding sites in the cell (Dever and Dermody, 1991). The increasing rate of drug resistance to various groups

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of antibiotics has rendered the conventional treatment of infections with known antibiotics less effective (WHO, 2016). Antibiotic resistance increases the rate of morbidity and mortality in individuals with immunocompromised state of health (WHO, 2016). There is however, the need for investigating into alternative therapy of ethnobotanical origin, with plants of folkloric claim to health benefits and disease control. Drug resistance occurs in bacteria with specific and generic peculiarities; there is usually emergence of resistant strains of bacterial species to previously effective antibiotics, likewise there are genera of bacteria that are profoundly resistant to certain groups of antibiotics. Resistance of a pathogen towards multiple groups of antibiotics (three or more) is tagged multidrug resistance. This research seeks to investigate and validate the proof of *Physalis angulata* (L), a medicinal plant in folklore medicine as a potent source of alternative therapy in immunocompromised individuals with multidrug resistant bacterial pathogens isolated from the blood of Human Immunodeficiency Virus (HIV) patients attending the Federal Medical Centre Owo, Ondo State as a case study.

II. MATERIALS AND METHODS

Multidrug resistant (MDR) bacterial isolates were determined by antibiogram profiling of all bacterial isolates from blood samples of HIV patients attending the HIV Clinic at the Federal Medical Centre, Owo, Ondo State. All bacterial isolates were subjected to culturomic techniques and subsequently biochemical characterization. BOX-PCR Fingerprinting was adopted for identification of bacterial isolates to the molecular level using the 16S rDNA technique. Antibiogram was conducted by disc diffusion test adopting the Kirby Bauer's principle. Eight groups of antibiotics were used which were commercially available in antibiotic-impregnated discs. These are; Sulfamethoxazole (Cotrimoxazole - 25µg), Beta lactam (Cloxacillin - 5µg, Augmentin - 30µg, Amoxycillin - 25µg), Macrolides (Erythromycin - 5µg), Aminoglycosides (Gentamicin - 10µg, Streptomycin - 10µg), Quinolones (Ofloxacin - 5µg, Nalidixic Acid - 30µg), Nitrofurantoin - 200µg, Tetracycline - 10µg, Chloramphenicol -10µg.

Broth cultures of each isolate were prepared and incubated for 18 hours at 37^{o}C. Each of these cultures were standardized and compared with 0.5 Mcfarland's standard solution turbidity. Fresh Mueller Hinton agar prepared according to manufacturer's instruction was allowed to cool down to 45^{o}C. About 15ml of this medium was aseptically poured into each sterile Petri dish required for the test and labeled appropriately. The medium was allowed to set; about 1ml of the inoculated broth culture of each isolate was aseptically introduced to the surface of corresponding MHA plates and spread with a sterile spreader. This inoculation was done in duplicates. The plates were left to stay for about two minutes before placing the impregnated antibiotic discs on the surface of each inoculated MHA plate. The plates were inverted and incubated at 37^{o}C for 24hrs. Bacterial isolates with zones of inhibition of diameters up to 17mm around an antibiotic disc were regarded as being susceptible to such an antibiotic, intermediate at 14-16mm and resistant at diameters less than 13mm (Hudzicki, 2013).

The DNA extraction, amplification and gel electrophoresis of amplicons were conducted by adopting the method of Barraquio *et al*. (2008) as follows; the Fruits and leaves of *Physalis angulata* (L) were collected in Ibule soro community of Ondo state and identified with standard appropriate references (Agyakwa and Akobundu, 1998). These plant parts were air-dried in the microbiology laboratory for a period of three weeks, they were blended after drying. Extraction of bioactive components from blended Plant parts was conducted by hot continuous extraction using two different solvents (n-Hexane and Methanol). The extraction process was done with the aid of a Soxhlet apparatus (Wang and Weller, 2006).

The phytochemical screening (Qualitative and Quantitative) was carried out according to the method of Ravi *et al*. (2012) and structural elucidation of the plant extracts tested was carried out according to procedures of Lluveras *et al*., 2010.

The plant extracts were reconstituted using 30%$^{v}/_{v}$ Dimethyl Sulfoxide. The plant extracts were sterilised (by filtration) using autoclavable sterile injection filters of 0.22µm pore size. Agar well diffusion test was conducted adopting the method of CLSI, 2012 with little modifications. Seven various concentrations of each of the extracts were used ranging from 0.10 - 0.60g (0.1, 0.2, 0.3, 0.4, 0.5, 0.55, 0.60 g/ml).

This was done by inoculating the bacteriological culture medium (Mueller Hinton Agar) with 1ml of the test organism of within 24hrs after being made to 0.5 Mcfarland solution standard. The culture medium was bored with a sterile cork borer, a bacteriological culture plate containing a test microorganism with a control well in the middle of the plate for DMSO solution only was prepared. Viability check was also run on each tested microbial isolate by culturing in a plate of Mueller Hinton agar to validate recovery of isolates. Incubation of culture plates were done at 37^{o}C for 24hrs. The various antibacterial actions of the various extracts in different concentrations were observed by the zones of inhibition around each agar well and recorded.

The Minimum inhibitory Concentration (MIC) was determined by using the method specified by EUCAST (2003). One millilitre of each concentration of the antimicrobial was added to a freshly prepared nutrient broth, with one millilitre from an overnight culture of each test bacterium equivalent to 0.5 Mcfarland's standard solution. A test-tube containing a blank nutrient broth medium (uninoculated) was prepared and set as the negative control. A broth culture was also prepared by inoculating the test bacterial isolate for each batch to ascertain viability of the bacterial isolates. All the broth media (both inoculated and uninoculated) were incubated at 37^{o}C for 24 hours. The lowest concentration at which there was no visible growth of bacterial isolates was recorded as the Minimum Inhibitory Concentration (MIC) for each bacterial isolate.

The determination of Minimum Bactericidal Concentration (MBC) was carried out according to the method of Buller *et al.* (2014). One milliliter of broth was taken from the least concentration with no visible growth to the highest concentration in the series and plated on corresponding

uninoculated Mueller Hinton agar plates. The least concentration with no growth on the agar media was recorded as the minimum bactericidal concentration for each set of antimicrobials. Data analysis was carried out using the Statistical Package for Social Sciences Version 16.0 (SPSS Inc., Chicago, IL). The Chi-square (X^2) test and New Duncan Multiple Range Test were used to determine significant differences and effects.

III. RESULTS

The molecular characterization revealed that there were 9 genera of bacterial species found in the study (Figure 1). There were five MDR strains of bacteria in the study, namely; *Escherichia coli* O103:H2, *Salmonella typhi* Ty21, *Salmonella typhimurium* L-3553, *Proteus mirabilis* CYPM, *Enterobacter aerogenes* CAV1320 (Table 1). *Proteus mirabilis* CYPM was the isolate which displayed highest level of multidrug resistance (Table 1). Methanolic extract of *P. angulata* fruits was found to have the highest antibacterial effect on tested MDR isolates with relatively lower MIC values than other extracts (Figure 2 and Table 2).

Tables 3 and 4 present the quantitative and qualitative phytochemical screening outcome of extracts. Phenols, Tannins, Flavonoids and Alkaloids were notably present in the effective extracts (Table 4). The structural elucidation of potent antibacterial *Physalis angulata* extracts by Gas Chromatography / Mass Spectrophotometry was analysed, captured and reported in chromatograms. A total of Fourteen compounds were separated from the two extracts (Table 5).

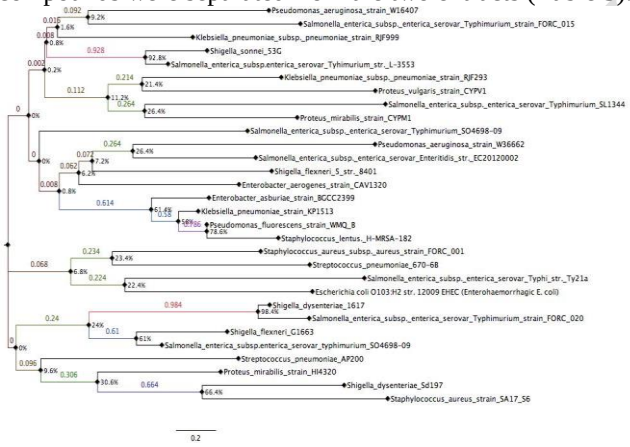
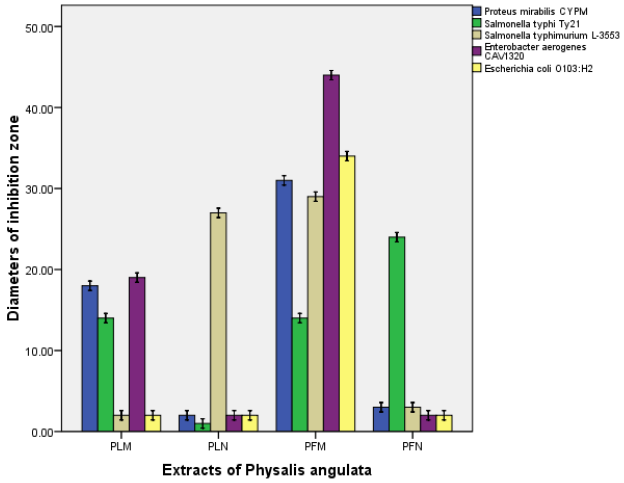


Figure 1: Phylogenetic tree of the isolated bacterial species from blood samples of studied HIV Patients at the FMC Owo

Serial Number	Bacterial isolates	Antibiotic class resisted	Resistance type
1	***Escherichia coli* O103:H2**	**SBM**	**Multidrug Resistance**
2	*Klebsiella pneumoniae* KP1513	Nil	No resistance.
3	***Salmonella typhi* Ty21**	**SBAM**	**Multidrug Resistance**
4	***Salmonella typhimurium* L-3553**	**SBM**	**Multidrug Resistance**
5	*Staphylococcus aureus* FORC001	BM	Drug resistance
6	*Streptococcus pneumoniae* (AP200)	BM	Drug resistance
7	*Pseudomonas aeruginosa* W16407	SB	Drug resistance
8	*Shigella dysenteriae*	Nil	No resistance
9	***Proteus mirabilis* CYPM**	**SBAMC**	**Multidrug Resistance**
10	***Enterobacter aerogenes* CAV1320.**	**SBMC**	**Multidrug Resistance**

Key: S - Sulfonamides. B - Beta-lactam. A – Aminoglycosides. M – Macrolides. C – Chloramphenicol.

Table 1: Resistance levels of the microbial isolates to tested antibiotic classes



Key: PLM (P. angulata Leaves methanolic extract), PLN (P. angulata Leaves n-hexane extract), PFM (P. angulata fruits methanolic extract), PFN (P. angulata fruits n-hexane extract).

Figure 2: Growth inhibition patterns of Physalis angulata fruit and leaf extracts against multidrug resistant bacterial isolates

	Test isolates	*Proteus mirabilis* CYPM.		*Salmonella typhi* Ty21		*Salmonella typhimurium* L-3553		*Enterobacter aerogenes* CAV1320		*Escherichia coli* O103:H2	
S/N	**Plant Extracts**	**MIC (g/ml)**	**MBC (g/ml)**	**MIC (g/ml)**	**MBC (g/ml)**	**MIC (g/ml)**	**MBC (g/ml)**	**MIC (g/ml)**	**MBC (g/ml)**	**MIC (g/ml)**	**MBC (g/ml)**
1	**PLM**	0.30	0.40	0.30	0.50	0.50	0.55	0.30	0.40	0.50	0.55
2	**PLN**	0.55	0.60	0.60	0.60	0.20	0.20	0.50	0.55	0.55	0.60
3	**PFM**	0.20	0.20	0.40	0.50	0.20	0.20	0.10	0.10	0.20	0.30
4	**PFN**	0.55	0.60	0.30	0.30	0.55	0.60	0.60	0.60	0.55	0.60

Key: PLM - Physalis angulata Leaves Methanolic extract, PLN - Physalis angulata Leaves n-Hexane extract, PFM - Physalis angulata Fruits Methanolic extract, PFN – Physalis angulata Fruits n - Hexane extract.

Table 2: The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of test bacterial isolates

S/No	Plant Extracts	Steroids	Phenols	Tannins	Alkaloids	Saponins	Flavonoids	Terpenoids	Cardiac glycosides	Glycosides
1.	**PLM**	+	+	+	+++	+	+	-	-	+
2.	**PLN**	-	+	+	++	++	+	-	-	-
3.	**PFM**	+	+	+	+++	-	+	-	-	-
4.	**PFN**	-	+	-	++	-	+	-	-	++

Key: - Negative. + Present. ++ Moderately Present. +++ Strongly Present.

Key: PLM - Physalis angulata Leaves Methanolic extract, PLN - Physalis angulata Leaves n-Hexane extract, PFM - Physalis angulata Fruits Methanolic extract, PFN - Physalis angulata Fruits n - Hexane extract.

Table 3: Qualitative Phytochemical screening

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S/No.	Plant Extracts	Phenols	Tannins	Flavonoids	Alkaloids	Saponins
1.	PLM	0.32 ± 0.002[d]	0.15±0.122[bc]	0.09±0.001[c]	236.06±0.052[c]	63.51±0.001[b]
2.	PLN	0.06 ±0.003[a]	0.05±0.001[ab]	0.05±0.001[b]	176.41±0.090[b]	63.51±0.001[c]
3.	PFM	0.24 ±0.002[c]	0.20±0.002[c]	0.04±0.001[a]	240.03±0.007[d]	-
4.	PFN	0.083±0.001[b]	-	0.05±0.001[ab]	150.64±0.080[a]	-

Mean values with the same alphabets as superscripts along the columns are not significantly different.
Key: PLM - Physalis angulata Leaves Methanolic extract, PLN - Physalis angulata Leaves n-Hexane extract, PFM - Physalis angulata Fruits Methanolic extract, PFN - Physalis angulata Fruits n - Hexane extract.

Table 4: Quantitative Screening of phytochemicals

S/No	Peak time (min)	Compound	Molecular Weight	Molecular formular	Activity
			PLM		
1.	5.558	1-Cyclohexenylacetic acid	140.182g/mol	$C_8H_{12}O_2$	Inactive
2.	5.794	Piperidine-2-carboxylic acid	129.159g/mol	$C_6H_{11}NO_2$	Inactive
3.	6.776	1,3,5,7 – Cyclooctatetraene	104.152g/mol	C_8H_8	Inactive
4.	6.925	4-Methyleneproline	127.143g/mol	$C_6H_9NO_2$	unspecified
5.	7.891	6-Methyl-2-heptyne	110.2g/mol	C_8H_{14}	
6.	8.410	Butanoic acid	88.106g/mol	$C_4H_8O_2$	Anticancer.
7.	8.653	Acetylcholine bromide	226.114g/mol	$C_7H_{16}BrNO_2$	neurotransmission
8.	9.549	L- Valine, methyl ester	131.175g/mol	$C_6H_{13}NO_2$	unspecified
9.	10.114	1-Pyrrolidinylacetonitrile	110.16g/mol	$C_6H_{10}N_2$	unspecified
10.	12.384	2-Piperidinemethanol	115.176g/mol	$C_6H_{13}NO$	Inactive
11.	13.123	2-Cyclopenten-1-one	82.102g/mol	C_5H_6O	Antitumour
12.	13.453	6,6- Dimethylhepta-2,4-diene	124.227g/mol	C_9H_{16}	unspecified
13.	14.372	Cyclohexanecarbonyl chloride	146.614g/mol	$C_7H_{11}ClO$	unspecified
			PFM		
14.	39.854	9,12 – Octadecadienic acid (z,z)	280.452g/mol	$C_{18}H_{32}O_2$	Biosynthesis of prostaglandins and cell membranes

Key: PLM - Physalis angulata leaf Methanolic extract
PFM - Physalis angulata fruit methanolic extract

Table 5: Structural elucidation of bioactive constituents from P. angulata

IV. DISCUSSION

HIV patients are constantly placed on treatment with Sulfonamides for its antifolate properties. The most frequently resisted groups of antibiotics in the study include Sulfonamides, Beta-lactam antibiotics and Macrolides. The mechanisms of action of these antibiotics have been observed to involve inhibition of protein syntheses and enzymatic reaction in bacterial cells either by blocking the action of transpeptidases in the cell wall synthesis (beta-lactams) or by binding to the 50S subunit of the ribosomes (Macrolides) (Soares *et al.*, 2012).

In this study *Proteus mirabilis* CYPM displayed the highest level of multidrug resistance to antibiotics. MDR strains of *Proteus mirabilis* like many other multi drug resistant Enterobacteriaceae are extended-spectrum Beta-lactamase producers, they also have affinity for integron mediated determinants of drug resistance, this in addition with the constant administration of sulfonamides to this group of patients provides for the low potency of conventional antibiotics to *Proteus mirabilis* CYPM and other MDR bacterial isolates from this study. This agrees with the findings of Mokracka *et al.* (2012).

Methanolic extract of *P. angulata* fruits was found to have the highest antibacterial effect on tested MDR isolates. Phenols, Tannins, Flavonoids and Alkaloids were notably present in the effective methanolic extracts. The polarity of the solvent - methanol as against n-Hexane could be responsible for the substantial extraction of bioactive components in the plant samples used. This agrees with the findings of Zhang (2015). Tannins have been reputed to play a significant role in inhibition of cell wall synthesis. Phenols and Flavonoids also are observed to have antimicrobial efficacy for plants protection against microbial attack. This reckons with a report from Mujeeb *et al.* (2014). This validates the proof of high antibacterial effectiveness noticed in the methanolic extracts from leaves and fruits of *P. angulata*.

The structural elucidation revealed the presence of chemical compounds of anticancer and antitumorigenic potencies. This agrees with the findings of Januario *et al.* (2002). This provides additional information about its other therapeutic usefulness for which it can be harnessed.

V. CONCLUSION

The indiscriminate use of antibiotics without prescription by a physician is therefore discouraged as it is a major way of propagating antibiotic resistance in bacteria, which in turn increases the morbidity and mortality rates in immunosuppressed individuals such as people living with HIV.

This research unveils that antimicrobials from *Physalis angulata* L, possess a promise of higher antibacterial efficacy against notable MDR bacteria in HIV Patients than most conventional antibiotics. The therapeutic potentials of *Physalis angulata* could also be explored beyond antimicrobial potency to anticancer and antitumourigenic fronts, due to the possession of chemical compounds revealed from its separation by Gas Chromatography/ Mass Spectrophotometry.

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