GSC Biological and Pharmaceutical Sciences

eISSN: 2581-3250 CODEN (USA): GBPSC2

Cross Ref DOI: 10.30574/gscbps

Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Synergistic activities of methanol leave extracts of *Acalypha wilkesiana, Senna alata, Psidium guajava* against selected resistant bacteria isolates

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GSC Biological and Pharmaceutical Sciences, 2021, 16(02), 049–061

Publication history: Received on 27 June 2021; revised on 06 August 2021; accepted on 09 August 2021

Article DOI: https://doi.org/10.30574/gscbps.2021.16.2.0217

Abstract

Resistant strains of bacteria has over the years rendered conventional antibiotics ineffective. Consequently, this has resulted to severe infection, prolonged treatment, high cost of treatment and often times death. This study aimed to identify reliable alternative sources of bioactive agents with activity against resistant *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi*. Methanol extracts of *Acalypha wilkesiana* (MEAW), *Senna alata* (MESA) *and Psidium guajava* (MEPG) were tested alone and in combination against three clinical isolates. Ciprofloxacin was used as the positive control drug. A combination of Microscopic, macroscopic and molecular protocols was used to identify the test isolates. The antibiotic profiles of the isolates *E. coli* (E1), *S. aureus* (S4) and *S. typhi* (St2) indicated MultiDrug-Resisitant status (MDR). All the extracts demonstrated antibacterial activity against the resistant isolates with zones of inhibition that ranged between 3.1 – 25 mm and minimum inhibitory concentration of 12.5 – 200 mg/ml. Amongst the extracts tested, MESA was found to be the most active extract while MEPG was the least active extract. The combination of the different methanol extracts demonstrated synergistic effects against the test organisms with a fractional inhibitory concentration that ranged between 0.06 – 0.8 mg/ml. The observed antibacterial activity may be linked to the presence of some bioactive components such as phenolic compounds and flavonoids present in the extracts. The results of this study suggest *A. wilkesiana, S. alata and P. guajava* may represent reliable sources of important bioactive compounds for new drug development.

Keywords: Bioactive components; Multidrug-resistance; Synergism; *Acalypha wilkesiana; Senna alata; Psidium guajava*; Molecular

1. Introduction

Infections caused by resistant strains of bacteria represent a major public health burden in terms of diseases, deaths, increased expenditure on patient management and infection control measures [1]. These pathogenic bacteria have evolved to gain resistance against several conventional antibiotics due to several reasons such as indiscriminate use of antibiotics [2] thus, the search for newer agents has become more expedient. Traditional medicine systems employs the use of herbal preparations made from selected plants with claimed ethno-medicinal properties in the treatment of various diseases [3]. The use of herbal preparations is a global practice and has remained relevant in the primary health care of the indigenous populations worldwide due to some factors such as cultural significance, accessibility, affordability and safety [4; 5]. The World Health Organization (WHO) estimates that 4 billion people, which is about 80% of the world's population, particularly the underdeveloped countries use herbal medicines for various aspects of

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primary Health care [6; 7]. Also, many countries such as Nigeria still rely on the curative values of herbs and use of medicinal plants for their healing practices [8].

The search for new antimicrobial agents from plants has become more intense due to the continuous spread of multi drug resistance and its attendant challenge [9]. However, some plants have been reported to cause toxic effects resulting from frequent use without available data about their toxicity profile [3; 10]. This has stirred up concerns by researchers with a view to develop new medicines needed to combat the menace of multi antibiotic resistance.

In this study, we evaluated three plants namely *Acalypha wilkesiana* (*Euphorbiaceae*), *Senna alata* (*Fabeaceae*), and *Psidium guajava* Linn. (*Myrtaceae*). They were chosen due to their reported medicinal properties which includes antibacterial [11], anti-diarrhoea [12], anti-inflammatory [13], anti-hyperglycemic [14], and antihypertensive properties [15].

2. Material and methods

2.1. Materials

2.1.1. Sample collection

Prior to collection of specimens, verbal informed consent was obtained from all patients who presented themselves to the hospital with complaint of Urinary tract (UTI) and Typhoid fever infections (TFI).

2.1.2. Cultivation, isolation, purification and identification of test bacteria

These was done according to established standard protocols [16] with slight modifications. Briefly, specimens previously cultured in sterile nutrient broth for 24 hr were subcultured on selected selective media such as MacConkey agar for (*E. coli*), Mannitol salt agar (*S. aureus*), and Salmonella shigella agar (*S. typhi*) at 37°C for 24 hr. Followed by macroscopic, microscopic (Gram stain) and biochemical (confirmatory) tests such as catalase, coagulase, indole and sugar fermentation tests.

2.2. Molecular identification of isolates

2.2.1. DNA isolation and Polymerase chain reaction (PCR)

DNA isolation and amplification were carried out at the Molecular Research Foundation for Students and Scientist Laboratory, Nnamdi Azikiwe University, Awka using standard protocols. Genomic DNA was extracted using Quick-DNATM Miniprep Plus Kit (Zymo Research) according to recommended protocol. The volume of 12.5µl of One Taq Quick-Load 2X Master Mix with Standard buffer (New England Biolabs Inc.); 0.5µl each of forward and reverse primers; 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to a thermalcycler. Amplification conditions for the PCR were first initial denaturation for 30secs at 94°C, followed by 35 cycles of denaturation at 94°C for 20secs, primer annealing at 54°Cfor 45secs and strand extension at 72°C for 1 min. Final extension at 72°C for 5 min on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% Agarose gel and DNA bands were visualized with Ethidium bromide to confirm.

2.2.2. Sequencing

All the PCR products that were sequenced were first cleansed using Exo-SAP-IT (USB, Affymetrix, USA), and 1 ul of the purified product was used as a template for direct sequencing using Big Dye terminator v. 2.0 cycle sequencer, according to manufacturer's instruction. The sequences obtained were blasted in NCBI similarity check platform for proper identification.

2.3. Antimicrobial activity

2.3.1. Test strains

Escherichia coli (E1-E10), *Satphylococcus aureus* (S1-S10) and *Salmonella typhi* (St1-St10), isolated and identified in this study were subject to antibiotic susceptibility testing.

2.3.2. Susceptibility test

The antibiotic profile of the test bacteria were determined using the Kirby-Bauer disk diffusion test. Each of the test bacteria was standardized to 0.5 MacFarland standard. A sterile swab was dipped into the bacteria suspension and used to inoculate each of the isolates on sterile Mueller-Hinton agar (MHA). Using a flamed forcep, each multidisc containing: Meropenum (10 µg), Ofloxacin (5 µg), Ceftriaxone (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), Ampicillin (10 µg), Nalidixic acid (30 µg), Cefuroxime (30 µg), Co-trimaxazole (25 µg) and Chloramphenicol (30 µg) was applied on the inoculated MHA plates. Slight pressure was applied on each multidisc to ensure complete contact of the disc with the agar. Plates were allowed to stand on the bench for 15 min to allow for pre-diffusion then inverted and incubated at 37℃ for 18 – 24 hr. After incubation, inhibition halos indicating susceptibility were measured in millimeters and results were interpreted referencing the standards of Clinical and Laboratory standards Institute [17].

Figure 1 Picture of *Acalypha wilkesiana (a), Senna alata (b)*, and *Psidium guajava (c)*

2.4. Collection and preparation of plant extracts

Leaves of *Acalypha wilkesiana, Senna alata, Psidium guajava* were collected from the botanical garden of Faculty of Pharmaceutical sciences, University of Port Harcourt Nigeria, the plants were identified by Dr Afuoeri of the Department of Pharmacognosy and Phytotherapy, University of Port Harcourt Nigeria and voucher specimens were deposited in the herbarium. The crude extracts from the different leaves were prepared by the marceration method using methanol as the solvent as proposed by Alabi *et al*. [18]; Cimanga *et al*. [19], with slight modifications.

2.5. Phytochemical analysis

Gas chromatography-flame ionization detector (GC-FID) instrument was used for the analysis and quantification of phytochemicals present in the methanol leaf extracts of *Acalypha wilkesiana* (MEAW), *Senna alata* (MESA), *Psidium guajava* (MEPG). A sample of each of the three methanol extracts was placed in 100 µl of Pyridine, after which 20 µl was placed into a vial on the Gas Chromatography machine for phytochemical analysis. The GC-FID phytochemical analysis was performed on a BUCK M910 Gas Chromatograph (GC) (BUCK Scientific, USA), which is furnished with a flame ionization detector (FID). A RESTEK 15 meter MXT-1 column (15m x 250 µm x 0.15µm) was used. The initial injector temperature was 220 ℃with spring injection of 2 µl of sample and a linear velocity of 30/cms, Helium 5.0 Psi was the carrier gas with a flow rate of 40 ml/min. The oven was heated to 280 ℃at a rate of 3 ℃ /min. Phytochemicals were determined by the ratio between the area and mass of internal standard and the area of the identified phytochemicals.

2.6. Agar well diffusion assay

The sensitivity of all the clinical isolates of *E. coli* (E1-E10), *S. aureus* (S1-S10), *S. typhi* (St1-St10) to the methanol leaf extracts of MEAW, MESA, MEPG was determined using the agar well diffusion method as described by Okezie *et al*., [20]. Each of the prepared bacteria suspension equivalent to 0.5 McFarland Standard was spread onto sterile Mueller- Hinton agar plates using a sterile swab stick. A sterile 6 mm diameter cork borer was used to bore 6 wells into the agar medium. The wells were then filled with approximately 0.1 ml of the extract ranging between 12.5 to 200 mg/ml. The plates were allowed to stand on the laboratory bench for 1 hour to allow proper diffusion of the extracts into the medium after which the plates were incubated at 37℃for 24 hours, and then observed for zones of inhibition. A meter rule was used to measure the inhibition zones in millimeters. Ciprofloxacin 5 µg/ml was used as a positive control, while 10% DMSO served as negative control. The experiment was conducted in triplicate and the average value was calculated.

2.7. Synergistic activity

The Checkerboard assay as described by Okore, [21] was employed for the evaluation and measurement of interactive inhibition of synergy between the extracts. This was carried out against each of the representative working isolates (*E. coli* (E1), *S. aureus* (S4), *S. typhi* (St2). Here, the individual MIC were used in preparing the stock solution of each of the

extract after which, the solutions were combined in different ratios, adopting the continuous variation model and then diluted using two folds serial dilution in sterile Pyrex test tubes. An aliquot of 60 µL corresponding to 0.06 mL of each of the serially diluted dilutions was transferred into a corresponding well in a sterile MHA plate previously inoculated with 0.5 McFarland standard of the test organism. The plates were incubated at 37°C for 18-24 hours. The effects of combination were evaluated by calculating the fractional inhibitory concentration (FIC) of each combination.

The sum of the FICs of both extracts gave the FIC index. This can be expressed as in equation 1.1 thus:

$$\text{FIC Index} = \frac{A'}{A''} + \frac{B'}{B''}$$

Where, A' and B' represent Minimal inhibitory concentrations of extracts A and B having inhibitory effects when acting together, while A" and B" stands for the respective MICs of the extracts. The FIC Index is interpreted as synergism if its value is less than 1.0, additivity if it is equal to 1.0, indifference if more than 1.0 and less than 2, and antagonism if more than 2.0.

2.8. Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out using one way analysis of variance (ANOVA) and SPSS 17.0 version statistical software package. The measures were done in triplicate (n = 3).

3. Results and discussion

A total of (30) isolates comprised of [*E. coli* (E1-E10), *S. aureus* (S1-S10), *S. typhi* (St1-St10)] were used for this study. Each of the test bacteria was isolated on their respective selective medium. The macroscopic observation revealed dark pink, yellow, and black colonies on Macconkey agar (MCA), Mannitol salt agar (MSA), and Salmonella shigella agar (SSA) respectively. Microscopic features of isolates on MCA, MSA and SSA were observed to be Gram negative (slender rods); Gram positive (cocci) and Gram negative (rods) respectively, while the biochemical tests conducted showed indole (+ve), citrate (-ve) for *E. coli*; catalase and coagulase (+ve) *S. aureus*; while colonies isolated on SSA were catalase (+ve) and produced H_2S. Morphological and biochemical characteristic of the bacteria isolates were further confirmed by the sequence of 18S rRNA gene of each bacteria that gave a sequence similarity of 100 % for each of the isolate to those accessible in the National Center for Biotechnology Information (NCBI) data base (GenBank). The molecular characterization also provided the accession numbers of CP0486431.3, CP046291.1 and CP026939.1 for *Staphylococcus aureus 4, Salmonella typhi* 2 and *Escherichia coli* 1 respectively.

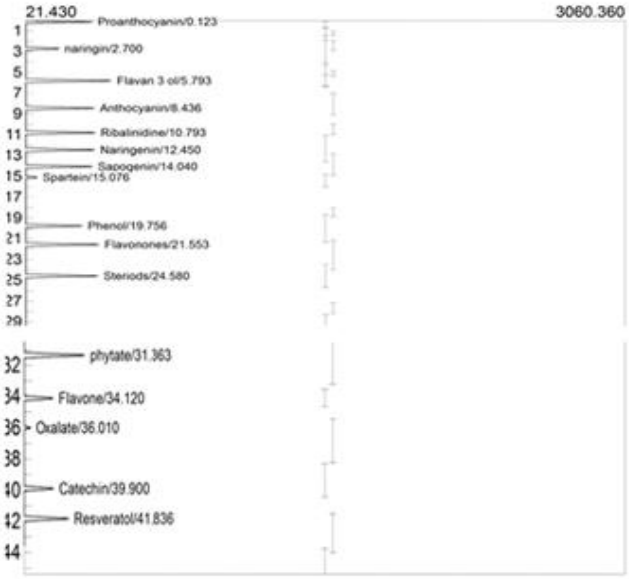
Table 1 Antibiotic Sensitivity Profiles of Selected test isolates

Antibiotics (µg/ml)	Isolates / inhibition zone diameter (mm)		
	E1	**S4**	**St2**
SXT (25)	0±0	0±0	0±0
MEM (10)	0±0	0±0	0±0
E (15)	0±0	0±0	0±0
AMP (10)	0±0	12±0.7	0±0
NA (30)	0±0	0±0	7±0.7
CXM (30)	0±0	0±0	0±0
C (30)	0±0	0±0	0±0
CN (10)	0±0	0±0	0±0
OFX (5)	10±0.7	0±0	0±0
CRO (30)	5±0.7	0±0	0±0
CIP (5)	10±0.7	15±1.4	9±0.7

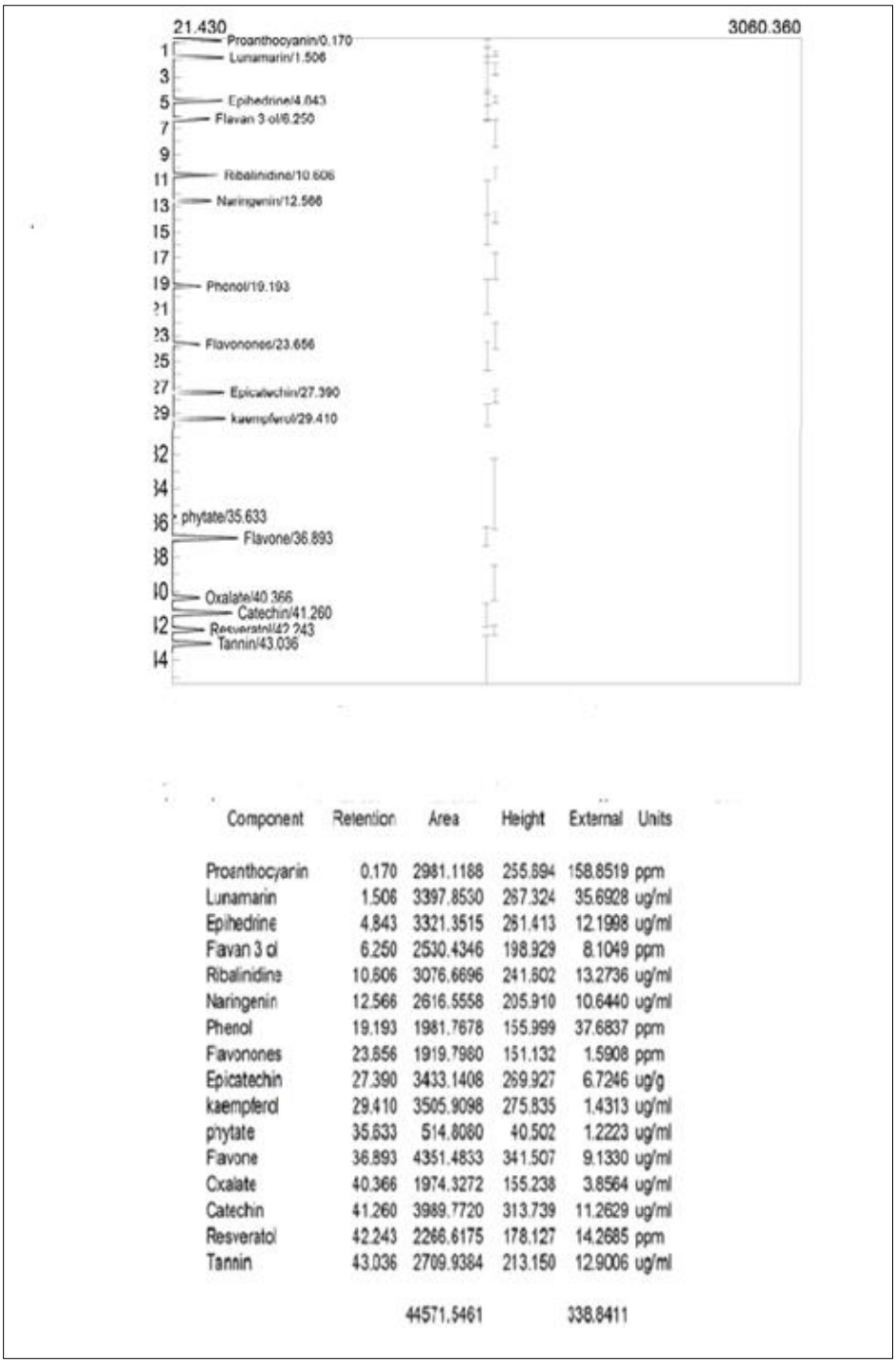
Key: Mem=Meropenem, Ofx=Ofloxacin, Cro=Ceftriaxone, Cn=Gentamicin, E= Erythromycin,

Cip=Ciprofloxacin, Amp= Ampicillin, Na=Nalidixic acid, Cxm= Cefuroxime, Sxt=Cotrimazole, C= Chloramphenicol

The antibiotic profiles of the test isolates *E. coli* (E1), *S. aureus* (S4) and *S. typhi* (St2) is shown on (Table-1). They resisted the antibacterial effects of most of the antibiotics tested including Meropenem**,** Gentamicin, Erythromycin**,** Cefuroxime**,** Chloramphenicol and Co-trimazole, with a 100% resistance while Ofloxacin**,** Ceftriaxone**,** Ciprofloxacin**,** Ampicillin**,** Nalidixic acid inhibited the growth of the test organisms with varying degrees of inhibition zones. The antibacterial activities of the methanol leave extracts of the three different plants demonstrated antibacterial activity at least in vitro Tables (2 – 4). Each extract inhibited at least one Gram positive and Gram negative test bacteria isolates. The MEAW extract inhibited the three test organisms with inhibition zones and MIC that ranged between 4 – 14 mm and 12.5 – 100 mg/mL. Also, the MESA extracts inhibited all the test bacterial isolates producing IZD's and MIC's that ranged between 3.1 – 20 mm and 12.5 – 50 mg/mL respectively. MEPG extract was observed to be the most active against the test organisms, having IZD that ranged between 4.5 – 25 mm. The test organisms also exhibited different degrees of sensitivity to the different extracts as a result of a difference in their cell wall components. The result showed that *S. aureus* (S4) was the most sensitive test organism while, *S. typhi* (St2) was the most resistant test organism.



Component	Retention	Area	Height	External	Units
Proanthocyanin	0.123	3359.9492	360.197	179.0383	ppm
naringin	2.700	2436.2185	191.759	5.3702	ug/ml
Flavan 3 ol	5.793	5819.4134	456.746	18.6394	ppm
Anthocyanin	8.436	4721.4984	371.183	49.4400	ug/ml
Ribalinidine	10.793	4775.5038	375.479	20.6028	ug/ml
Naringenin	12.450	4741.5096	372.761	19.2882	ug/ml
Sapogenin	14.040	4578.3976	359.987	8.2397	ug/ml
Spartein	15.076	1038.4018	81.524	9.8355	ug/ml
Phenol	19.756	3930.5748	309.144	74.7406	ppm
Flavonones	21.553	5025.1523	394.848	4.1640	ppm
Steriods	24.580	4953.9757	389.494	24.5831	ppm
phytate	31.363	4199.9884	329.743	9.9720	ug/ml
Flavone	34.120	2174.5382	171.250	4.5640	ug/ml
Oxalate	36.010	663.9956	52.301	1.2970	ug/ml
Catechin	39.900	2206.4580	173.478	6.2287	ug/ml
Resveratol	41.836	3159.5580	248.559	19.8896	ppm
		57785.1333		455.8931	

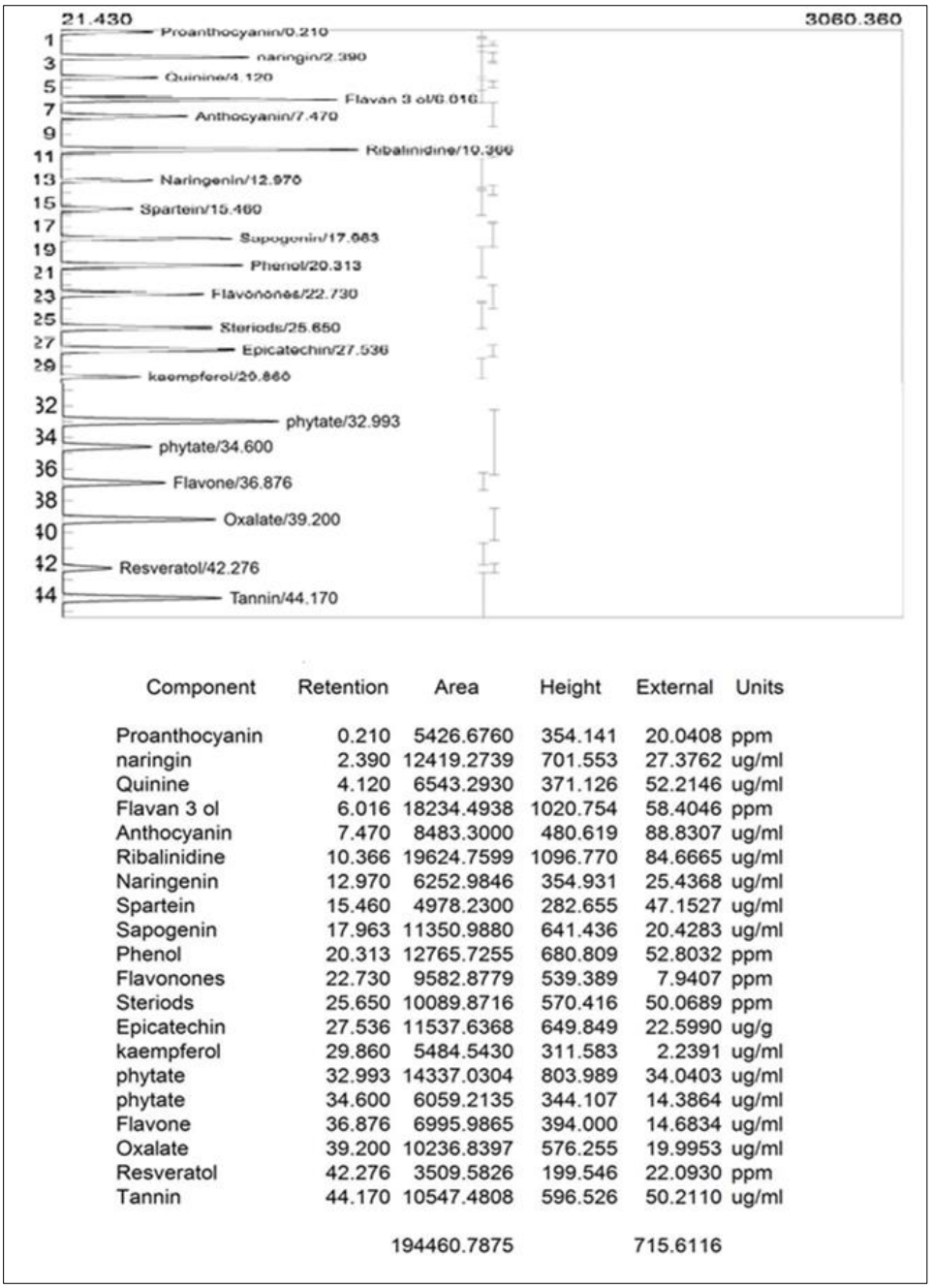


Component	Retention	Area	Height	External	Units
Proanthocyanin	0.170	2981.1188	255.894	158.8519	ppm
Lunamarin	1.506	3397.8530	267.324	35.6928	ug/ml
Epihedrine	4.843	3321.3515	261.413	12.1998	ug/ml
Flavan 3 ol	6.250	2530.4346	198.929	8.1049	ppm
Ribalinidine	10.606	3076.6696	241.602	13.2736	ug/ml
Naringenin	12.566	2616.5558	205.910	10.6440	ug/ml
Phenol	19.193	1981.7678	155.999	37.6837	ppm
Flavonones	23.656	1919.7980	151.132	1.5908	ppm
Epicatechin	27.390	3433.1408	269.927	6.7246	ug/g
kaempferol	29.410	3505.9098	275.835	1.4313	ug/ml
phytate	35.633	514.8080	40.502	1.2223	ug/ml
Flavone	36.893	4351.4833	341.507	9.1330	ug/ml
Oxalate	40.366	1974.3272	155.238	3.8564	ug/ml
Catechin	41.260	3989.7720	313.739	11.2629	ug/ml
Resveratol	42.243	2266.6175	178.127	14.2685	ppm
Tannin	43.036	2709.9384	213.150	12.9006	ug/ml
		44571.5461		338.8411	

Figure 2 The chromatogram of methanol leave extracts of *Acalypha wilkesiana (A), and Senna alata*leaves (B)

The combination of the different methanol extracts demonstrated synergistic effect against the MDR organisms E1, S4 and St2 (Tables 5-10). The FICs' of each synergistic combination ratio was calculated from the checkerboard titre. A marked reduction in the MICs of each synergistic combination in comparison with the individual MICs of each extract when tested alone was recorded. The effects of the extracts tested in combination significantly reduced the MIC values of each extract.

Figures 1, 2 and 3 show the presence of some major bioactive components namely proanthocyanin, naringin, anthocyanin, ribalinidine, naringenin, flavan-3ol, sapogenin, spartein, phenol, flavonones, steroids, phytate, flavone, oxalate, catechin, epicatechin, kaempferol and resveratrol detected in the methanol extracts of the plants studied.



Component	Retention	Area	Height	External	Units
Proanthocyanin	0.210	5426.6760	354.141	20.0408	ppm
naringin	2.390	12419.2739	701.553	27.3762	ug/ml
Quinine	4.120	6543.2930	371.126	52.2146	ug/ml
Flavan 3 ol	6.016	18234.4938	1020.754	58.4046	ppm
Anthocyanin	7.470	8483.3000	480.619	88.8307	ug/ml
Ribalinidine	10.366	19624.7599	1096.770	84.6665	ug/ml
Naringenin	12.970	6252.9846	354.931	25.4368	ug/ml
Spartein	15.460	4978.2300	282.655	47.1527	ug/ml
Sapogenin	17.963	11350.9880	641.436	20.4283	ug/ml
Phenol	20.313	12765.7255	680.809	52.8032	ppm
Flavonones	22.730	9582.8779	539.389	7.9407	ppm
Steriods	25.650	10089.8716	570.416	50.0689	ppm
Epicatechin	27.536	11537.6368	649.849	22.5990	ug/g
kaempferol	29.860	5484.5430	311.583	2.2391	ug/ml
phytate	32.993	14337.0304	803.989	34.0403	ug/ml
phytate	34.600	6059.2135	344.107	14.3864	ug/ml
Flavone	36.876	6995.9865	394.000	14.6834	ug/ml
Oxalate	39.200	10236.8397	576.255	19.9953	ug/ml
Resveratol	42.276	3509.5826	199.546	22.0930	ppm
Tannin	44.170	10547.4808	596.526	50.2110	ug/ml
		194460.7875		715.6116	

Figure 3 The chromatogram of methanol leave extract of *Psidium guajava* leaves

Table 2 Antibacterial Activity of Methanol Leaf Extract of *Acalypha wilkesiana* (MEAW) at different concentrations of the extract

Mean Zone of inhibition in (mm)								
Isolate no	**Isolate**	***Acalypha wilkesiana* (mg/ml)**					**Ciprofloxacin**	**DMSO**
		200	100	50	25	12.5		
E1	*E. coli*	10±0.03	9±0.03	7±1.83	4±0.02	-	26±2.50	-
S4	*S. aureus*	14±0.00	10.2±0.21	6.3±2.87	5.4±0.12	5.4±0.12	24±1.34	-
St2	*S typhi*	10±2.97	8.2±2.11	-	-	-	32±2.24	-

Key: (MEAW) Methanol leave extract of *Acalypha wilkesiana*, E1-E10 =Escherichia coli, S1-S10= *Staphyloccoccus aureus*, St1- St10= *Salmonella typhi*

Table 3 Antibacterial Activity of Methanol Leaf Extract of *Senna alata* (MESA) at different concentration of the extract

Mean Zone of inhibition in (mm)								
Isolate no	**Isolate**	***Senna alata* (mg/ml)**					**Ciprofloxacin**	**DMSO**
		200	100	50	25	12.5		
E1	*E. coli*	14±0.03	10±0.04	8±1.21	7.6±0.50	3.1±0.00	26±2.50	-
S4	*S. aureus*	20±2.12	16.1±1.14	10±0.01	8.1±0.87	4.3±0.06	24±1.34	-
St2	*S typhi*	12±0.01	10±0.12	10±2.01	-	-	32±2.24	-

Key: MESA= Methanol leave extract of *Senna alata,* E1-E10 =*Escherichia coli*, S1-S10= *Staphyloccoccus aureus*, St1- St10= *Salmonella typhi*

Table 4 Antibacterial Activity of Methanol Leaf Extract of *Psidium guajava* (MEPG) at different concentration of the extract

Mean Zone of inhibition in (mm)								
Isolate no	**Isolate**	***Psidium guajava* (mg/ml)**					**Ciprofloxacin**	**DMSO**
		200	100	50	25	12.5		
E1	*E. coli*	14.1±0.50	11±0.01	9±0.02	7.1±0.10	-	26±2.50	-
S4	*S. aureus*	16±0.03	14±0.23	10±0.02	8.1±0.05	4.5±0.01	24±1.34	-
St2	*S typhi*	10±0.0	-	-	-	-	37±1.72	-

Key: MEPG= Methanol leave extract of *Psidium guajava*, E1-E10 =Escherichia coli, S1-S10= *Staphyloccoccus aureus*, St1- St10= *Salmonella typhi*

Table 5 Results of the Combination Effect of MEPG and MESA Extracts against the *E. coli* (E1) Isolate Sensitive to the Preliminary Evaluation

Tubes	**Combination ratios**	**W1**	**W2**	**W3**	**W4**	**W5**	**FIC index**	**Remarks**
T1	10:0	*0.5:0	0.25.5:0	0.13:0	0.06:0	0.03.13:0	-	
T2	9:1	0.45: 0.05	*0.23:0.03	0.11:0.01	0.06:0.01	0.03:0.003	0.69	SYN
T3	8:2	0.4:0.1	0.2:0. 05	0.1:0.03	*0.05:0.01	0.03:0.01	0.18	SYN
T4	7:3	0.35:0.15	0.18:0.08	0.09:0.04	*0.04:0.02	0.02:0.01	0.23	SYN
T5	6:4	0.3:0.2	0.15:0.1	0.08:0. 05	*0.04:0.0.3	0.02:0.01	0.31	SYN
T6	5:5	0.25:0.25	0.13:0.13	0.06:0.06	*0.03:0.03	0.02:0.02	0.29	SYN
T7	4:6	0.2:0.3	0.1:0.15	0.05:0.08	*0.03:0.04	0. 01:0.02	0.37	SYN
T8	3:7	0.15:0.35	0.08:0.1 8	0.04:0.09	*0.02:0. 04	0.01:0.02	0.35	SYN
T9	2:8	0.1: 0.4	0.05.0.2	0.03:0.1	0.01:0. 05	0.01:0.03	0.8	SYN
T10	1:9	*0.05:0.45	0.03:0.23	0.01:0.11	0.01:0. 06	0.003:0.03	3.56	ATA
T11	0:10	0:0.5	0:0.25	*0:0.13	0:0.06	0:0.03	-	-

Key: W= well/hole. Asterisk Figures*: MIC of the most effective combination; FIC Index < 1.0 is synergism (SYN); FIC Index = 1.0 is additivity (ADD); FIC Index > 1.0 is indifference (IND); FIC Index >2.0 is antagonism (ATA)

Table 6 Results of the combination effect of MEPG and MESA extracts against *S. aureus* (S4) isolate sensitive to the preliminary evaluation

Tubes	Combination ratios	W1	W2	W3	W4	W5	FIC index	Remarks
T1	10:0	1:0	0.5:0	0.25:0	0.13:0	0.06:0	-	
T2	9:1	0.9: 0.1	0.45:0.05	0.23:0.03	0.11:0.01	0.06:0.001	-	-
T3	8:2	*0.8:0.2	0.4:0. 1	0.2:0.05	0.1:0.03	0.05:0.01	1.2	IND
T4	7:3	*0.7:0.3	0.35:0.15	0.18:0.08	0.09:0.04	0.04:0.02	1.3	IND
T5	6:4	0.6:0.4	*0.3:0.2	0.15:0. 1	0.08:0.05	0.04:0.03	0.7	SYN
T6	5:5	0.5:0.5	0.25:0.25	0.13:0.13	0.06:0.06	0.03:0.03	-	-
T7	4:6	0.4:0.6	0.2:0.3	0.1:0.15	0.05:0.08	0. 03:0.04	-	-
T8	3:7	0.3:0.7	0.15:0.35	0.08:0.18	0.04:0. 09	0.02:0.04	-	-
T9	2:8	0.2: 0.8	0.1.0.4	0.05:0.2	0.03:0. 1	0.01:0.05	-	-
T10	1:9	0.1:0.9	0.05:0.45	0.03:0.23	0.01:0. 11	0.1:0.06	-	-
T11	0:10	0:0.1	0:0.5	0:0.25	0:0.13	0:0.06	-	-

Key: W= well/hole. Asterisk Figures*: MIC of the most effective combination; FIC Index < 1.0 is synergism (SYN); FIC Index = 1.0 is additivity (ADD); FIC Index > 1.0 is indifference (IND); FIC Index >2.0 is antagonism (ATA)

Table 7 Results of the combination effect of MEAW and MESA extracts against the *S. typhi* (St2) isolate sensitive to the preliminary evaluation

Tubes	Combination ratios	W1	W2	W3	W4	W5	FIC index	Remarks
T1	10:0	0.5:0	0.25:0	0.13:0	0.06:0	0.03:0	-	
T2	9:1	0.45:0.05	*0.23:0.03	0.11:0.01	0.06:0.01	0.03:0.003	0.52	SYN
T3	8:2	*0.4:0.1	0.2:0.05	0.1:0.03	0.05:0.01	0.03:0.01	1	ADD
T4	7:3	*0.35:0.15	0.18:0.08	0.09:0.04	0.04:0.02	0.02:0.01	1	ADD
T5	6:4	*0.3:0.2	0.15:0.1	0.08:0.05	0.04:0.03	0.02:0.01	1	ADD
T6	5:5	*0.25:0.25	0.13:0.13	0.06:0.06	0.03:0.03	0.02:0.02	1	ADD
T7	4:6	0.2:0.3	0.1:0.15	0.05:0.08	0.03:0.04	0. 01:0.02	-	-
T8	3:7	0.15:0.35	0.08:0.18	0.04:0.09	0.02:0. 04	0.01:0.02	-	-
T9	2:8	0.1: 0.4	0.05.0.2	0.03:0.1	0.01:0.05	0.01:0.03	-	-
T10	1:9	0.05:0.45	0.03:0.23	0.01:0.11	0.01:0.06	0.003:0.03	-	-
T11	0:10	0:0.5	0:0.25	0:0.13	0:0.06	0:0.03	-	-

Key: W= well/hole. Asterisk Figures*: MIC of the most effective combination; FIC Index < 1.0 is synergism (SYN); FIC Index = 1.0 is additivity (ADD); FIC Index > 1.0 is indifference (IND); FIC Index >2.0 is antagonism (ATA)

Table 8 Results of the combination effect of MEPG and MEAW extracts against the *S. typhi* (St2) isolate sensitive to the preliminary evaluation

Tubes	Combination ratios	W1	W2	W3	W4	W5	FIC index	Remarks
T1	10:0	0.5:0	0.25:0	0.13:0	0.06:0	0.03:0	-	
T2	9:1	0.45:0.05	0.23:0.03	0.11:0.01	0.06:0.01	0.03:0.003	-	-
T3	8:2	0.4:0.1	0.2:0.05	0.1:0.03	0.05:0.01	0.03:0.01	-	-
T4	7:3	*0.35:0.15	0.18:0.08	0.09:0.04	0.04:0.02	0.02:0.01	1	ADD
T5	6:4	*0.3:0.2	0.15:0.1	0.08:0.05	0.04:0.03	0.02:0.01	1	ADD
T6	5:5	0.25:0.25	0.13:0.13	0.06:0.06	0.03:0.03	0.02:0.02	-	-
T7	4:6	*0.2:0.3	0.1:0.15	0.05:0.08	0.03:0.04	0. 01:0.02	1	ADD
T8	3:7	0.15:0.35	0.08:0.18	0.04:0.09	0.02:0. 04	0.01:0.02	-	-
T9	2:8	0.1: 0.4	0.05.0.2	0.03:0.1	0.01:0.05	0.01:0.03	-	-
T10	1:9	*0.05:0.45	0.03:0.23	0.01:0.11	0.01:0.06	0.003:0.03	1	ADD
T11	0:10	0:0.5	0:0.25	0:0.13	0:0.06	0:0.03	-	-

Key: W= well/hole. Asterisk Figures*: MIC of the most effective combination; FIC Index < 1.0 is synergism (SYN); FIC Index = 1.0 is additivity (ADD); FIC Index > 1.0 is indifference (IND); FIC Index >2.0 is antagonism (ATA)

Table 9 Results of the combination effect of MEAW and MESA extracts against the *E. coli* (E1) isolate sensitive to the preliminary evaluation

Tubes	Combination ratios	W1	W2	W3	W4	W5	FIC index	Remarks
T1	10:0	0.5:0	0.25:0	0.13:0	0.06:0	0.03:0	-	
T2	9:1	0.45:0.1	0.23:0.05	0.11:0.03	0.06:0.01	0.03:0.01	-	-
T3	8:2	0.4:0.2	0.2:0.1	0.1:0.05	0.05:0.03	0.03:0.01	-	-
T4	7:3	0.35:0.3	0.18:0.15	0.09:0.08	0.04:0.04	0.02:0.02	-	-
T5	6:4	0.3:0.4	0.15:0.2	0.08:0.1	*0.04:0.05	0.02:0.03	0.13	SYN
T6	5:5	0.25:0.5	0.13:0.25	0.06:0.13	0.03:0.06	0.02:0.03	-	-
T7	4:6	*0.2:0.6	0.1:0.3	0.05:0.15	0.03:0.08	0. 01:0.04	1	ADD
T8	3:7	*0.15:0.7	0.08:0.35	0.04:0.18	*0.02:0. 09	0.01:0.04	0.06	SYN
T9	2:8	0.1: 0.8	0.05.0.4	*0.03:0.2	0.01:0.01	0.01:0.05	0.26	SYN
T10	1:9	0.05:0.09	*0.03:0.45	0.01:0.23	0.01:0.11	0.003:0.06	0.51	SYN
T11	0:10	0:1	0:0.5	0:0.25	0:0.13	0:0.06	-	-

Key: W= well/hole. Asterisk Figures*: MIC of the most effective combination; FIC Index < 1.0 is synergism (SYN); FIC Index = 1.0 is additivity (ADD); FIC Index > 1.0 is indifference (IND); FIC Index >2.0 is antagonism (ATA)

Table 10 Results of the combination effect of MEAW and MESA extracts against the *S. aureus* (S4) isolate sensitive to the preliminary evaluation

Tubes	Combination ratios	W1	W2	W3	W4	W5	FIC index	Remarks
T1	10:0	0.5:0	0.25:0	0.13:0	0.06:0	0.03:0	-	
T2	9:1	0.45:0.1	*0.23:0.05	0.11:0.03	0.06:0.01	0.03:0.01	0.56	SYN
T3	8:2	*0.4:0.2	0.2:0.1	0.1:0.05	0.05:0.03	0.03:0.01	1.2	IND
T4	7:3	*0.35:0.3	0.18:0.15	0.09:0.08	0.04:0.04	0.02:0.02	1.2	IND
T5	6:4	0.3:0.4	*0.15:0.2	0.08:0.1	0.04:0.05	0.02:0.03	0.7	SYN
T6	5:5	0.25:0.5	*0.13:0.25	0.06:0.13	0.03:0.06	0.02:0.03	0.7	SYN
T7	4:6	0.2:0.6	0.1:0.3	0.05:0.15	0.03:0.08	0. 01:0.04	-	-
T8	3:7	0.15:0.7	0.08:0.35	0.04:0.18	0.02:0. 09	0.01:0.04	-	-
T9	2:8	0.1: 0.8	0.05.0.4	0.03:0.2	0.01:0.01	0.01:0.05	-	-
T10	1:9	0.05:0.09	0.03:0.45	0.01:0.23	0.01:0.11	0.003:0.06	-	-
T11	0:10	0:1	0:0.5	0:0.25	0:0.13	0:0.06	-	-

Key: W= well/hole. Asterisk Figures*: MIC of the most effective combination; FIC Index < 1.0 is synergism (SYN); FIC Index = 1.0 is additivity (ADD); FIC Index > 1.0 is indifference (IND); FIC Index >2.0 is antagonism (ATA)

4. Discussion

In this study, the clinical strains used such as *S. aureus, E. coli* and *S. typhi* were identified based on their reactions to selected biochemical tests: catalase and coagulase; indole and citrate; and hydrogen sulphide production respectively. The Blast outcome of each of the sequence gave a 100% sequence similarity score and accession number CP048643.1, CP046291.1 and CP026939.1 for *S. aureus* (S4), *S. typhi* (St2) and *E. coli* (E1) respectively.

The sensitivity of each of the test organisms *S. aureus* (S4), *S. typhi* (St2) and *E. coli* (E1) to conventional antibiotics was observed to be multidrug resistance. This was based on their observed resistance to most of the antibiotics to which they were exposed to. Ciprofloxacin was the only antibiotic that inhibited the three test organisms. The methanol extracts of MEAW, MESA and MEPG demonstrated inhibitory potentials against all the test organisms, with varying inhibition zones. A maximum inhibitory zone of 14±0.03, 20±2.12 and 12±0.01 mm was observed against E1, S4 and St2 respectively. Also, different MICs were recorded for the different test organism. MEAW had an MIC of 25, 50 mg/mL for E1, S4 and St2 respectively. Also, MIC of MESA was 12.5 for E1, S4 and St2 respectively while MEPG had an MIC of 25, 12.5 and 200 mg/ml against E1, S4 and St2 respectively. Amongst the test organisms, St2 was observed to be the most resistant bacteria, while S4 was the most sensitive test organism used in this work. The activity demonstrated by the methanol extracts in this work can be said to be broad spectrum, inhibiting both Gram positive and Gram-negative organisms. Similarly, Joseph and Priya, [22], Aliyu *et al*. [23], Gotep *et al*. [24], Doughari and Okafor, [25], reported a broad-spectrum activity against a panel of both Gram positive (*Staphylococcus aureus*) and Gram negative (*Salmonella typhi* and *Escherichia coli*) bacteria. Higher sensitivity shown by the Gram-positive test bacteria to the extracts in this work may be attributed to inhibition of cell wall development by the extracts. Gram positive bacteria have a mesh-like peptidoglycan layer which was more accessible to permeation by the extracts. The negative control (DMSO) exhibited no inhibitory activity against any of the test organisms.

The combination of the different methanol extracts at specific ratios worked synergistically against the MDR *S. aureus* (S4), *S. typhi* (St2) and *E. coli* (E1). Combination of MEPG and MESA demonstrated synergistic effects against E1 at all the combined ratios. Whereas, a combination of MEPG with MEAW had no synergistic effect against St2. Furthermore, a significant decrease in MIC values was observed in the combinations that produced synergistic effect. The FICI of the various combined methanol extracts that produced synergistic effects ranged between 0.06 – 0.8 mg/mL. The results strongly suggest a possible synergistic potential of the bioactive compounds in the methanol extracts of the plants under study. Thus, may be developed into new medicines for treatment against infectious caused by MDR organisms. However, some combinations were observed to be antagonistic. The phytoconstituents that may be responsible for the recorded activities were detected using GCFID. The GCFID chromatogram revealed the presence of important biologically active

compounds such as proanthocyanin, naringin, anthocyanin, naringenin, flavan-3-ol, sapogenin, phenol, flavonones, catechin, epicatechin, kaempferol and resveratrol.

Several other biological activities including antibacterial and radical scavenging have been reported for flavonoids such as naringin and naringenin [26], catechin & epicatechin [27]. Also, phenols with antibacterial activities have been identified. Activity against *Salmonella typhimurium* and MRSA have been reported [29]. While Flavonoids have multiple cellular targets including DNA synthesis and have been reported to have broad spectrum activity [26].

5. Conclusion

In conclusion, MEAW, MESA and MEPG demonstrated antibacterial activity against the multidrug resistant *E. coli, S. aureus* and *S. typhi* tested in this work. Also, the combined effects produced synergistic activity against the tested organisms at very low concentrations. The recorded activities may be suggestive of the presence of biologically active phytoconstituents in the extracts previously reported to have varying biological activities including antibacterial activities. Thus, the recorded activity in this work provides additional data on the potential use and development of these plants' bioactive compounds into newer medicines and also these plants can be relied on as reservoir for the detected biologically active compounds.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

This was obtained from the Research Ethics Committee, University of Port Harcourt Teaching Hospital, Rivers State, Nigeria. (UPTH/ADM/90/S.I/VOL.XI/712).

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