Magna Scientia Advanced Research and Reviews

eISSN: 2582-9394

Cross Ref DOI: 10.30574/msarr

Journal homepage: https://magnascientiapub.com/journals/msarr/

(RESEARCH ARTICLE)

Check for updates

Synergistic action of endophytic fungi secondary metabolites against multidrug-resistant strains of *Staphylococcus aureus*

Ugochukwu Moses Okezie [1, *], Makwochukwu Augustine Okoyeocha [1], Somtochukwu Jeffery Chimbiko [1], Nnamdi Leonard Ekeoma [1], Chukwuemeka John Okezie [2], Chinoso Kenneth Ibeagwa [3], Onyeka Chinwuba Obidiegwu [3], Nnamdi Markben Adione [3], Festus Basden Chiedu Okoye [3] and Charles Okechukwu Esimone [1]

[1] *Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria. PMB 50522.*

[2] *Univine Medical Diagnostic Laboratory, Igbo-Elerin, Lagos State, Nigeria.*

[3] *Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria. PMB 50522.*

Magna Scientia Advanced Research and Reviews, 2023, 09(01), 052–060

Publication history: Received on 01 August 2023; revised on 13 September 2023; accepted on 15 September 2023

Article DOI: https://doi.org/10.30574/msarr.2023.9.1.0127

Abstract

The global threat to treatment of infections associated with multidrug-resistant microorganisms as well as side effects associated with combination therapy calls for the urgent development of new medicines to address these challenges. Endophytic fungi are efficient bio synthesizers and offer a wide array of these reliable bioactive compounds. The present study is aimed at evaluating the effect of combined extracts of endophytic fungi isolated from the leaves of *Musa paradisiaca* against multidrug-resistant *Staphylococcus aureus* (MRSA) strains. Screening for multidrug resistance was carried out using the Kirby-Bauer disc diffusion assay followed by a preliminary assay of endophytic fungal extracts bioactivity using agar diffusion assay against selected MRSA. A synergistic study using checkerboard assay technique was performed, then extracts were subjected to quantitative evaluation of secondary metabolites using Gas Chromatography with Flame Ionization Detector. Axenic cultures of five endophytic fungi from *M. paradisiaca* (EMp1-Emp5) were isolated from the sampled leaves. All the endophytic fungal extracts inhibited the growth of all the multi-drug resistant *Staphylococcus aureus* at 0.5 mg/mL. Varying inhibition zones that were concentration-dependent were observed. The combined effects of *EMp3* and *EMp5* extracts at 9:1; 8:2; 4:6; 3:7; and 2:8 combination ratios produced synergistic effects against *S. aureus* (S18) with very low Fractional inhibitory Concentrations of 0.03:0.01, 0.01:0.01, 0.006:0.02, 0.01:0.04 and 0.01:0.1 mg/mL respectively. Several bioactive secondary metabolites such as anthocyanin, (-) epicatechin, naringenin, resveratrol, catechin, rutin, phenol were produced by the fungal endophytes. Our findings further affirm fungal endophytes associated with *M. paradisiaca* leaves as producers of natural antibacterial agents with synergistic potentials.

Keywords: Antimicrobial Activity; Endophytic fungus; Multidrug-resistant *Staphylococcus aureus*; *Musa paradisiaca*; Secondary metabolites; Synergistic activity

1. Introduction

The joy of the advent of antibiotics is threatened to be cut short by the emergence of antibiotic-resistant strains of infectious microorganisms, especially bacteria. This brought about increased activity-guided development of newer pharmaceuticals against these resistant pathogens.

* Corresponding author: OKEZIE UGOCHUKWU MOSES

One of these resistant strains is the Methicillin-resistant *Staphylococcus aureus* (MRSA). They have been implicated as the causative agent for certain minor skin infections, life-threatening infections, such as; pneumonia, meningitis, postoperative infection, septicemia, and toxic shock syndrome [1]. MRSA is now responsible for more deaths per year in the United States than HIV [2]. The added healthcare costs for fighting MRSA infections are in the billions of dollars per year worldwide. Plus, the danger of not having effective antimicrobial therapy available is imminent, necessitating the development of a potent antibiotic. The quest to develop an active medicinal principle has cut across many paths, but the most used path remains the route from a natural product.

Natural products (plants, animals, etc.) are the primary source of active medicinal principle, it is the backbone of traditional medicine, and this has been the basis of its exploration and exploitation in the development of modern medicine [3]. Most studies have attributed this group of microbes as major producers of secondary metabolites that are of biological importance and a potential source of natural lead agents for exploitation in the pharmaceutical industry [4, 5, 6, 7]. Natural products and health has come a long way, but the advent of drug-resistant makes it imperative that efforts are intensified in the quest for new drugs to tackle the resistant strains.

In the present study, five endophytic fungi were isolated from healthy leaves of the medicinal plant *Musa paradisiaca,* and their metabolic products were extracted with ethyl acetate and each crude extract was screened for its antibacterial activity against multidrug-resistant *Staphylococcus aureus*.

2. Material and methods

2.1. Cultivation, isolation, Fermentation and extraction of secondary metabolites

Axenic cultures of each of the unidentified (*EMp*1, *EMp*2, *EMp*3, *EMp*4, *EMp*5) endophytic fungus isolated from the leaves of *Musa paradisiaca* (Figure 1) were grown on Malt Extract Agar (MEA) following the cultivation, isolation, and purification procedures described by [6]. Then, each was subjected to solid-state fermentation in a 1L Erlenmeyer flask containing autoclaved rice medium (100 g of rice and 200 mL of distilled water) [6]. Each fermentation flasks was inoculated with agar blocks containing the fungus and incubated under static conditions at 28°C for 21 days [6]. After fermentation, the secondary metabolites were extracted using 500 mL ethyl acetate and then concentrated under a vacuum at a reduced temperature of 40 °C using a rotary evaporator [7].

2.2. Test isolates

Pure cultures of nineteen (19) *Staphylococcus aureus* strains (*S*1-*S*19) for the *in-vitro* antimicrobial assay were provided by the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka-Nigeria and were re-identified combining macroscopic, microscopic (Gram staining technique), and specific biochemical tests such as catalase, oxidase, starch hydrolysis and indole tests [8].

2.3. Antibiotics susceptibility testing

The susceptibility tests were performed following the method M2A6 disc diffusion method as recommended by the National Committee for Clinical Laboratory Standards [9] using Mueller Hinton agar.

Standard microbial suspension corresponding to 0.5 McFarland turbidity standard of each test organism was made in 3 ml sterile nutrient broth inoculating with a single colony. The suspensions were incubated for 3 hour at 37 °C to allow for the growth of the test organism till the density was equivalent to the turbidity of 0.5 McFarland.

The standardized bacteria suspensions were swabbed onto sterile Mueller Hinton Agar (MHA) plates using sterile swabs and the multi discs: ceftazidime (30 µg; microgram); cefuroxime (30 µg); gentamicin (10 µg); ceftriaxone (30 µg); erythromycin (5 µg); cloxacillin (5 µg); ofloxacin (5 µg); augmentin (30 µg), (Abtek, UK) were aseptically placed on the inoculated plates and pressed firmly onto the agar plate for complete contact. The Plates were inverted and left on the workbench for 30 min to allow for pre-diffusion of antibiotics into the agar then incubated at 37°C for 18-24 h. The susceptibility of each isolate to each antibiotic was shown by a clear zone of growth inhibition and this was measured in millimeters using a meter rule and the diameter of the zones of inhibition was then interpreted using a standard chart [9, 10]. Each isolate was tested in triplicate.

2.4. Preliminary bioassay

The antimicrobial assay for each of the fungi crude extract (*EMp*1-*EMp*5) was carried out using the agar well diffusion assay as described by [6]. The antimicrobial activities of the endophytic fungi extracts were tested against three (3)

multidrug-resistant *Staphylococcus aureus* strains previously confirmed through the antibiotic sensitivity profiling. They include *Staphylococcus aureus 1* (*S14*), *Staphylococcus aureus 2* (*S17*), *and Staphylococcus aureus 3* (*S18*). A 0.5 McFarland standard bacterial suspensions of each of the multidrug-resistant test strains was prepared and these formed the bacteria stock solutions used in the agar well diffusion assays as outlined below.

2.5. Agar well diffusion assay

Briefly in the method, "the media i.e Mueller-Hinton Agar (MHA) was prepared and treated according to the manufacturer's specification. The sterile MHA plates were inoculated with the test culture by seeding method. Here, 0.1 mL of each of the previously standardized cultures was transferred into an empty sterile plate and 20 mL of the molten agar that has been cooled to 50 °C was added and then mixed thoroughly by swirling clock wisely and anti-clockwise to ensure even distribution of the organism. This was done to obtain uniformity of each inoculum. A sterile cork borer was used to make five wells (8 mm in diameter) on each of the MHA plates. Aliquots of 80 µl (microliter) of each extract (*EMp*1-*EMp*5) dilutions, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL (milligram per milliliter) for each of the extracts were applied in each of the wells in the culture plates previously seeded with the test organisms. Ciprofloxacin (10 µg/mL) and DMSO served as the positive and negative controls respectively. The cultures were left on the bench for 15 min, for pre-diffusion to occur before they were incubated at 37°C for 18-24 h to allow for the growth of microorganisms. The antimicrobial potential for each endophytic fungal extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). This was performed in triplicates and the antimicrobial potential was expressed as an average of the inhibition zone diameter" [6].

2.6. Synergistic study

Checkerboard assay was employed for the evaluation of interactive inhibition of the extracts of *EMp*3 and *EMp*5 against the most resistant *Staphylococcus aureus* (*S18*) in the preliminary evaluation [11]. First, "separate solutions of the endophytic fungal extracts were prepared in DMSO, each solution containing twice its Minimum Inhibitory Concentration (MIC). Thereafter, the solutions were combined in different ratios, adopting the continuous variations model. Each combination was then diluted using two-fold serial dilution up to 5 dilutions in sterile Pyrex test tubes. An aliquot of 80 µL corresponding to 0.08 mL of each of the serially diluted dilutions was transferred into a corresponding well (8 mm) in a sterile MHA plate previously seeded with 0.5 McFarland standard of the test organism. The plates were incubated at 37°C for 18-24 h. The fractional inhibitory concentration (FIC) of each combination ratio was determined using equation 1.1" [11].

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

Where, A' and B' represent minimal concentrations of extracts A and B having inhibitory effects when acting together, while A" and B" stand 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 antagonism if more than 2.0.

2.7. Detection of phytochemicals, (Quantitative evaluation of secondary metabolites present in the extract) by GC-FID

2.7.1. Sample preparation

For each endophytic fungal extract (*EMp1-EMp5)*, 20 g was weighed and transferred into a test tube and 15 ml of hexane was added. The test tube was allowed to react in a water bath at 60°C for 60 min. After the reaction time, the reaction product contained in the test tube was transferred to a separator funnel. The tube was washed successively with 20 ml of ethanol, 10 ml of cold water, 10 ml of hot water, and 3 ml of hexane, which were all transferred to the funnel. These solutions were combined and washed three times with 10 ml of 10%v/v ethanol aqueous solution. The solution are dried with anhydrous sodium sulfate and the solvent was evaporated. The sample was solubilized in 1000 µl of hexane of which 200 µl was transferred to a vial for further analysis [12].

The analysis of secondary metabolites was performed on a BUCK M910 Gas chromatography equipped with a flame ionization detector. A RESTEK 15 meter MXT-1 column (15 m x 250 um x 0.15um) was used. The injector temperature was 280 °C with a splitless injection of 2 µl of sample and a linear velocity of 30 cms-1, Helium 5.0 pa.s was the carrier gas with a flow rate of 40 mlmin-1. The oven operated initially at 2000 c, then heated to 3300 c at a rate of 30 c min-1 and was kept at this temperature for 5 min. the detector operated at a temperature of 3200. Phytochemicals were determined by the ratio between the area and mass of internal standard and the area of the identified phytochemicals, the concentration of the different phytochemicals were expressed/quantified in µg/g [12].

2.8. Statistical analysis

The results were expressed as mean ± standard deviation. All measurements were done in triplicate (n = 3)

3. Results and discussion

Table 1 Antibiotic susceptibility profiles

	Isolate code	Antibiotics (µg) / Inhibition Zone Diameter (mm)							
		CAZ (30)	CRX (30)	GEN (10)	CTR (30)	ERY (5)	CXC (5)	OFL (5)	AUG (30)
S6	*S1*	11±0	0±0	19±0.7	0±0	0±0	0±0	19±0	0±0
S7	*S2*	0±0	0±0	25±0.8	0±0	0±0	0±0	30±0.8	0±0
S11	*S3*	0±0	0±0	19±0.4	0±0	0±0	0±0	22±0	0±0
S15	*S4*	0±0	0±0	17±0.7	0±0	16±0	0±0	12±0	0±0
S18	*S5*	0±0	0±0	8±0.5	0±0	11±0.7	0±0	15±0.7	0±0
S22	*S6*	0±0	0±0	20±0.9	15±0	0±0	0±0	20±0	0±0
S24	*S7*	0±0	0±0	20±0.7	0±0	0±0	0±0	20±0.7	0±0
S31	*S8*	0±0	0±0	22±0.7	0±0	12±0.9	0±0	20±0.8	0±0
S45	*S9*	0±0	0±0	19±0	0±0	17±0.8	0±0	25±0	0±0
S49	*S10*	0±0	0±0	18±0.6	23±0.4	0±0	0±0	20±0.6	0±0
S61	*S11*	0±0	0±0	16±0.7	0±0	0±0	0±0	21±0	0±0
S63	*S12**	0±0	0±0	9±0.8	0±0	0±0	0±0	21±0	0±0
S70	*S13*	0±0	0±0	24±0	0±0	0±0	0±0	2±0	0±0
S74	*S14*	0±0	0±0	10±0.7	0±0	0±0	0±0	11±0	0±0
S75	*S15*	0±0	0±0	18±0	0±0	0±0	0±0	10±0.7	0±0
S77	*S16*	0±0	0±0	11±0.7	0±0	16±0	0±0	12±0.8	0±0
S80	*S17**	0±0	0±0	10±0	0±0	0±0	0±0	9±0	0±0
S93	*S18**	0±0	0±0	10±0	0±0	0±0	0±0	10±0	0±0
S94	*S19*	11±0	9±0.8	21±0	18±0	0±0	0±0	22±0	9±0

Key: CAZ: ceftazidime; CRX: cefuroxime; GEN: gentamicin; CTR: ceftriaxone; ERY: erythromycin; CXC: cloxacillin; OFL: ofloxacin; AUG: augmentin

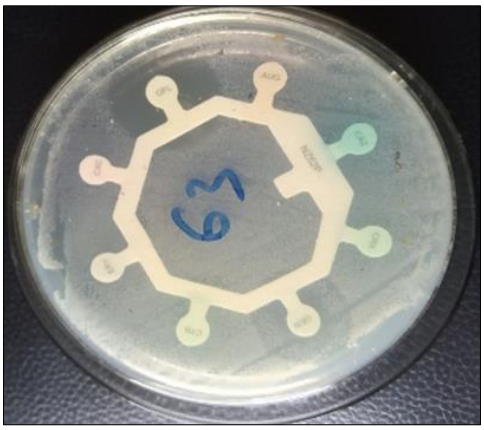

Figure 1 Multidrug resistance pattern of *Staphylococcus aureus (S12)* to selected antibiotics

Table 2 (a) Antibacterial effect of extract *emp1* against the mdr-*S. aureus*

Test organism	Concentration (mg/mL) / Inhibition Zone Diameter (mm)						
	1	0.5	0.25	0.13	0.06	DMSO	Cipro
S12	5±0	3±0	2±0.7	2±0.7	0±0	0±0	13±0
S17	3±0	2±0.7	2±0	2±0.7	0±0	0±0	13±0
S18	5±0	4±0	2±0	0±0	0±0	0±0	12±0

Key: *EMp1*: Endophytic fungus 1 from *M. paradisiaca*; S: *Staphylococcus aureus;* DMSO: negative; Ciprofloxacin 10 mg/ml: Positive control

Table 2 (b) Antibacterial effect of extract *emp2* against the mdr-*S. aureus*

Test organism	Concentration (mg/mL) / Inhibition Zone Diameter (mm)						
	1	0.5	0.25	0.13	0.06	DMSO	Cipro
S12	6±0	5±0	4±0.7	2±0	0±0	0±0	13±0
S17	5±0	3±0	2±0	2±0	0±0	0±0	13±0
S18	6±0.7	5±0.7	4±0	4±0	4±0.7	0±0	12±0

Key: *EMp2*: Endophytic fungus 2 from *M. paradisiaca*; S: *Staphylococcus aureus;* DMSO: negative; Ciprofloxacin 5mg/ml: Positive control

Table 2 (c) Antibacterial effect of extract *emp3* against the mdr-*S. aureus*

Test organism	Concentration (mg/mL) / Inhibition Zone Diameter (mm)						
	1	0.5	0.25	0.13	0.06	DMSO	Cipro
S12	6±0	5±0.7	4±0.7	2±0	0±0	0±0	13±0
S17	4±0	3±0	0±0	0±0	0±0	0±0	13±0
S18	5±0	4±0.7	3±0.7	3±0.7	0±0	0±0	10±0

Key: *EMp3*: Endophytic fungus 3 from *M. paradisiaca*; S: *Staphylococcus aureus;* DMSO: negative; Ciprofloxacin 5 mg/ml: Positive control

Table 2 (d) Antibacterial effect of extract *emp4* against the mdr-*S. aureus*

Test organism	Concentration (mg/mL) / Inhibition Zone Diameter (mm)						
	1	0.5	0.25	0.13	0.06	DMSO	Cipro
S12	6±0	4±0	0±0	0±0	0±0	0±0	13±0
S17	3±0.7	3±0	2±0.7	2±0.7	0±0	0±0	13±0
S18	4±0.7	4±0	3±0	0±0	0±0	0±0	12±0

Key: *EMp4*: Endophytic fungus 4 from *M. paradisiaca*; S: *Staphylococcus aureus;* DMSO: negative; Ciprofloxacin 5 mg/ml: Positive control

Table 2 (e) Antibacterial effect of extract *emp5* against the mdr-*S. aureus*

Test organism	Concentration (mg/mL) / Inhibition Zone Diameter (mm)						
	1	**0.5**	**0.25**	**0.13**	**0.06**	**DMSO**	**Cipro**
S12	6±0	5±0	0±0	0±0	0±0	0±0	13±0
S17	6±0	5±0	5±0.7	4±0	0±0	0±0	13±0
S18	8±0	5±0	4±0	0±0	0±0	0±0	12±0

Key: *EMp5*: Endophytic fungus 5 from *M. paradiasca*; S: *Staphylococcus aureus;* DMSO: negative; Ciprofloxacin 5mg/ml: Positive control

Table 3 Synergistic effect of *emp*3 and *emp*5 fungal extracts against multidrug-resistant *Staphylococcus aureus* (*s18*)

Combination ratio	MICa	MICb	FIC Index	Remark
9:1	0.03	0.01	0.58	SYN
8:2	0.01	0.01	0.24	SYN
7:3	0.09	0.008	2.12	ATA
6:4	0.05	0.04	1.05	IND
5:5	0.07	0.13	2.17	ATA
4:6	0.006	0.02	0.25	SYN
3:7	0.01	0.04	0.47	SYN
2:8	0.01	0.1	0.93	SYN
1:9	0.001	0.03	O.23	ATA

Key: MICa: MIC of the most effective combination of *EMp3*: MICb: MIC of the most effective combination of *EMp5*: 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 4 Quantitative determination of the secondary metabolites present in the endophytic fungal extracts

Secondary metabolites	Concentration of secondary metabolites (µg/mL)				
	EMp1	***EMp2***	***EMp3***	***EMp4***	***EMp5***
Proanthocyanin	0.0977	-	-	1.8014	-
Naringin	2.6073	-	1.7791	3.3621	2.9457
Anthocyanin	27.4182	4.1867	-	28.0677	-
Sapogenin	8.8729	4.987	32.3649	10.2977	-
Phenol	38.4845	11.5591	34.4970	97.8189	44.3596
Flavonones	8.2861	-	-	9.1885	-
Steroids	9.0728	-	4.6026	10.0338	12.0374
Epicatechin	17.8974	-	-	18.9908	-
Kaempferol	1.9517	-	7.7897	2.4070	4.6261
Flavone	9.0068	1.5407	-	2.8991	2.1337
Naringenin	2.2154	-	1.2595	2.4049	0.9089
Resveratol	2.4966	13.0016	-	3.3606	-

Tannin	15.8736	7.9873	12.4117	15.8309	11.1164
Rutin	-	3.8897	1.7863	-	3.0526
Phytate	-	0.0085	1.7430	1.9079	2.2971
Catechin	9.6756	17.897	38.0966	-	49.7357
Flavan-3-ol	-	-	-	10.7395	-
Total	192.6802	47.9379	158.1092	265.8639	148.6186

EMp: Endophytic fungus isolated from *Musa paradisiaca* leaves

Table 5 Lists of major constituents identified in the extracts of endophytic fungi isolates

Identified compound	**Reported biological activity**	**References**
Naringin	Antibacterial	13
Quinine	Antioxidant, Antimicrobial	14
Sparteine	Anti-inflammatory. Anticonvulsant, Antiarrhythmic	15
(-) Epicatechin	Antimicrobial, Antioxidant, Anticancer	16
Kaempferol	Antitumor, Antioxidant, Anti-inflammatory	17, 13
Naringenin	Antimicrobial (anti-Staphylococcal), Antioxidant	18
Resveratrol	Anticancer, Antimicrobial	18
Catechin	Antimicrobial, Antioxidant, Anticancer	16, 17, 19,
Rutin	Anti-inflammatory	13

Following the cultivation of the healthy plant samples of *M. paradisiaca*, five (5) axenic endophytic (EMp1-EMp5) fungi were isolated and purified on MEA. Antibiotic sensitivity profiling of the nineteen *S. aureus* revealed three (*Staphylococcus aureus* 1 (S12), *Staphylococcus aureus* 2 (S17), and *Staphylococcus aureus* 3 (S18).) to be multidrug-resistant (Table 1).

The *S. aureus* strains used in this study responded differently to the selected test antibiotics used in Table 1. Varying degrees of resistance by the different strains to the antibiotics were recorded: ceftazidime: 17/19 (89.5 %); cefuroxime: 18/19 (94.7 %); ceftriaxone: 16/19 (84.2 %); erythromycin: 14/19 (73.7 %); cloxacillin: 19/19 (100 %); augmentin: 18/19 (94.7 %) Table 1. While all the strains resisted the effect of cloxacillin. However, total (100 %) susceptibility of all the strains was observed for gentamicin and ofloxacin respectively Table 1. Taking a critical observation of the sensitivities of the different strains, S12, S17, and, S18 were the most resistant strains. Thus, were selected for the preliminary antibacterial evaluation of endophytic fungal extracts.

The preliminary antibacterial assay showed that all the extracts demonstrated good activity against all the multi-drug resistant *Staphylococcus aureus* (Table 2a-e). The activities varied among the endophytic fungal extract as well as the sensitivities to the extracts by the resistant strains Table 2(a-e). Also, the recorded antibacterial activities of each of the endophytic fungal extracts were observed to be concentration-dependent. The EMp1 extract at a concentration of 1 mg/mL produced inhibition zones of 5, 3, and 5 mm against the multidrug-resistant isolates S12, S17, and S18 respectively. At 1 mg/mL EMp2 extract produced inhibition zones of 6, 4, and 5 mm against S12, S17, and S18 respectively. Also, EMp3, EMp4, and EMp5 demonstrated a good antibacterial effect with inhibitions zones of 6, 6, 5 mm against S12; 3, 6, 3 mm against S17 and 4, 8, 5 mm against S18.

The EMp1 fungal extract was the most active amongst the extracts tested inhibiting the resistant strains with minimum inhibitory concentrations that ranged between 0.06 – 0.13 mg/mL. Similarly, [1] and [20] recorded minimum inhibitory concentrations of endophytic fungi extract of 0.032 – 0.25 and 0.032 – 0.512 mg/mL respectively against some multidrug-resistant S. aureus.

3.1. Synergistic study

A combination of EMp3 and EMp5 extracts against the most resistant *Staphylococcus aureus* (S18) produced synergistic effects (Table 3) observed for some combination ratios. The result showed that the EMp3 and Mp5 inhibited the resistant organism at very low concentrations

The combined effect of EMp3 and EMp5 against multidrug-resistant *Staphylococcus aureus* (S18) was synergistic. The FICIs that were synergistic are presented in Table 3. The combination of EMp3 and EMp5 fungal extracts in the ratios 9:1; 8:2; 4:6; 3:7; and 2:8, produced synergistic effect with MIC's of 0.03:0.01, 0.01:0.01, 0.006:0.02, 0.01:0.04 and 0.01:0.1 mg/mL respectively. The combination of both endophytic fungal extracts had synergistic effects against the multidrug-resistant *S. aureus* at very low (MIC) concentrations. However, antagonistic effects were observed at combination ratios of 7:3; 5:5, and 1:9.

Overall, synergism was the most recorded effect when both endophytic fungal extracts were combined against the multidrug-resistant *S. aureus*. Thus, confirmed the chemical compatibility, potentiation, and synergistic effects of the secondary metabolites present in the endophytic fungal extracts combined.

The Quantitative determination of the secondary metabolites present in the endophytic fungal extracts (EMp1-EMp5) identified some bioactive secondary metabolites that may have produced the micro-biostatic effects recorded (Table 4 & 5). These include flavonoids: anthocyanin [13], (-) epicatechin [16], naringenin [18], resveratrol [18], catechin [16, 17, 19], rutin [13], and phenol reported to have several biological activities such as antibacterial [6, 21, 13], antifungal [13], and antioxidant [22]. The identified bioactive secondary metabolites when isolated, purified, and developed into new combination chemotherapeutic (medicines) agents may yield a positive outcome when used in the treatment of infections caused by multidrug-resistant *S. aureus*.

4. Conclusion

Endophytic fungi isolated from the leaves of *M. paradisiaca* have expressed their metabolic capabilities through the biosynthesis of some interesting secondary metabolites with synergistic potentials against pathogenic strains and proven to be reliable producers of these bioactive secondary metabolites. Our study therefore showed that fungal endophytes from *M. paradisiaca* are producers of chemically compatible bioactive compounds capable of producing synergistic effects against disease-causing resistant microorganisms, and also prove that bioprospecting bioactive compounds of endophytic fungi associated with medicinal plants provides a huge opportunity of isolating chemically compatible chemotherapeutic agents.

Compliance with ethical standards

Acknowledgments

Authors are grateful to Late Prof. O.A. Arinze and Mrs. Udoka R Okoyeocha for funding this research and the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Anambra State, Nigeria, for the support provided.

Disclosure of conflict of interest

The authors declare no conflict of interest.

References

[1] Arivudainambi E, Anand T.D, Shanmugaiah V, Karunakaran C, Rajendran A: Novel bioactive metabolites producing endophytic fungus Colletotrichum gloeosporioides against multidrug-resistant Staphylococcus aureus. FEMS Immunol Med Microbiol. 2011; 61: 340-345.

[2] Raygaert W: In book: Microbial pathogens and strategies for combating them: science, technology and education. Formatex Research Center 2013; 1:(1)

[3] Yuan H, Ma Q, Ye L, Piao G: The Traditional Medicine and Modern Medicine from Natural Products. Molecules 2016; 21(5): 559.

[4] Selim, K., El-beih, A., Abdel-rahman, T., & El-diwany, A: Biology of Endophytic Fungi. Current Research in Environmental and Applied Mycology 2012; 2(1), 31–82.

[5] Okezie UM, Eze PM, Ajaghaku DL, Okoye FBC, Esimone CO: Isolation and screening of secondary metabolites from endophytic fungi of Vernonia amygdalina and Carica papaya for their cytotoxic activity. Planta Med 2015; 81 - PM_177.

[6] Okezie U.M, Eze P.M, Okoye F.B.C, Ikegbunam M.N, Ugwu M.C, Esimone C.O: Secondary Metabolites from an endophytic fungus of Vernonia amygdalina. African Journal of Pharmaceutical Research and Development 2017; Vol. 9 No.1:pp. 24-26

[7] Okezie UM, Okoli OA, Ajaegbu EE, Okoye FBC, Esimone CO: Antioxidant Activities of Extracts of Endophytic Fungi Isolated from Healthy Leaves of Carica Papaya. International Journal of Research Studies in Biosciences (IJRSB) 2021; 8(8): 48-56

[8] Cheesbrough: District Laboratory Practice in Tropical Countries, Second Edition Part II, Cambridge University press 2009; Pg 62-70.

[9] Clinical and Laboratory Standard Institute (CLSI): Performance standards for antimicrobial susceptibility testing; Twenty-sixth informational supplement. CLSI document M100-S26. Wayne, PA: Clinical and Laboratory Standards Institute 2016.

[10] Ekwealor PA, Ugwu MC, Ezeobi I, Amalukwe G,Ugwu BC, Okezie U, Stanley C, and Charles Esimone CO: Antimicrobial Evaluation of Bacterial Isolates from Urine Specimen of Patients with Complaints of Urinary Tract Infections in Awka Nigeria. Hindawi Publishing Corporation International Journal of Microbiology 2016

[11] Igwe JO, Okezie UM. Ikegbunam MN, and Esimone CO: Synergistic activities of methanol leaf extracts of Acalypha wilkesiana, Senna alata, and Psidium guajava against selected resistant bacteria isolates. GSC Biological and Pharmaceutical Sciences 2021; 16(02), 049-061

[12] Kelly DA, Nelson R: Characterization and quantification by gas chromatography of flavonoids. J. Braz. Chem. Soc 2014; Vol 25.

[13] Cushnie TPT and Lamb AJ: Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 2005; 26: 343-356.

[14] Pranay G. and Puspal DE: Spectrum of biological properties of Cinchona alkaloids: A brief review. Journal of Pharmacognosy and Phytochemistry 2017; 6(4): 162-166

[15] Villalpando-vargas, F. and Medina-ceja, L: Sparteine as an anti-convulusant drug: Evidence and possible mechanism of action. Seizure: European Journal of Epilepsy 2016; 39: 49-55.

[16] Bahri-Sahloul CR, Fredi RB, Boughalleb N, Shrina J, Saguem S, Hilbert JL, Trotin F, Ammar S, Bouzid S, nad Harzallah-Skhiri F; Phenolic Composition and Antioxidant and Antimicrobial Activities of Extracts Obtained from Crataegus azarolus L. var. aronia (Wild). Batt. Ovaries. Hindawi Publishing Corporation Journal of Botany 2014.

[17] Kumar S and Pandey AK: "Chemistry and Biological Activities of Flavonoids: An Overview". The Scientific World Journal 2013

[18] Bahare Salehi B, Fokou PVT, Sharif-Rad M, Zucca P, Pezzani R, Martins N, and Sharifi-Rad J: The Therapeutic Potential of Naringenin: A review of Clinical Trials. Pharmaceuticals (Basel) 2019. 12(1): 11.

[19] Stapleton PD, Shah S, Anderson JC, Hara Y, Hamilton-Miller JMT, Taylor PW: Modulation of beta-lactam resistance in Staphylococcus aureus by catechins and gallates. Int. J. Antimicrob. Agents 2004; 23:462-7.

[20] Phongpaichit, S., Rungjindamai, N., Rukachaisirikul, V., and Sakavoroi, J: Antimicrobial activity in cultures of endophytic fungi isolated from Garcinia species. FEMS Immunol Med Microbiol. 2006; 48(3):367-72.

[21] Mishra A., Sharma A.K., Kumar S., Saxena A.K., Pandey A.K. Bauhinia variegate leaf extracts exhibit considerable antibacterial, antioxidant, and anticancer activities. Biomed. Res. Int. 2013. 3:915436.

[22] Kelly EH, Anthony RT, and Dennis JB: "Flavonoids antioxidants: chemistry, metabolism and structure-activity relationship. "Journal of Nutritional Biochemistry 2002. Vol. 13(10): 572-584