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Antimicrobial Activities of Secondary Metabolites From Fungal Endophytes.

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Abstract: *Some fungal endophytes were isolated from the roots of Ocimum gratissimum, Aloe barbadensis and Carica papaya. Four fungal endophytes, Trichoderma viride, Aspergillus fumigatus, and Aspergillus niger were randomly selected for fermentation and screening for antimicrobial activity against clinical and typed cultures including Staphylococcus aureus, Serratia marcescens and Klebsiella pneumoniae. Agar well diffussion method was utilised in the evaluation of the antimicrobial activity and it was discovered that each of the secondary metabolites showed antibacterial activities against the test bacteria. The result showed good prospects in the continual search for new antibacterial drugs.*
Keywords*: Endophytes, fungi, metabolites, bioactive compounds.*

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I. Introduction

The fungal endophytes remains a potential and widely recognized prolific sources of bioactive secondary metabolites that represent useful leads for the discovery and development of new pharmaceutical bioagents (Dompeipen *et al*., 2011). Endophytes are mutualistic symbiont being harbored in the living tissues of healthy plants without any symptoms and such organisms can be fungi, bacteria, and actinomycetes (Strobel, 2002; Rajagopal *et al*., 2015). They can live there for all or part of their life cycle and when beneficial, such associations can stimulate plant growth, improve the plants ability to withstand environmental stresses and increase disease resistance. (Tejesvi *et al*., 2005). The incidences of acquired resistance by pathogenic organisms against drugs (Motta *et al*., 2004) has necessitated the need to focus on endophytes in the search for novel bioactive natural compounds which can be used as new drugs to replace those against which pathogenic strains have shown resistance. (Tan and Zou, 2001). Large number of structurally diverse and bioactive fungal metabolites have been isolated and characterized and some of these have been used for the development of valuable pharmaceuticals and pesticides **(**Rodriguez *et al*., 2000; Taechowisan *et al*., 2005; Onifade, 2007). However, many more remain unexploited because fungi has enormous biodiversity and it is estimated that a conservative 1.5 million species are recorded worldwide, although many believe the estimate is between 500 thousand to 9.9 million (Hawksworth, 2001). Therefore, as a result of their contribution to the host plant, the endophytes may produce an excess of substances with potential use to agriculture, modern medicine and industry. Novel antibiotics, antidiabetic, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation, culture, purification, and characterization of some endophytes (Strobel et al., 2001; Strobel and Daisy, 2003). The aim of this study was to examine the potential prospects of discovering new drugs which may be effective and useful for treating an array of newly developing diseases in humans, animals and plants.

II. Materials And Methods

2.1Sampling

Plants targeted for sampling were healthy looking, matured and are known to have medicinal properties. Small root pieces was cut from *Ocimum gratissimum, Aloe barbadensis and Carica papaya* with sterilized knife, samples were tagged and placed in separate polythene bags. It was then taken to the laboratory and processed within 24 hours of collection (Tejesvi et. al., 2005).

2.2 Isolation of endophytic fungi

Plants samples were rinsed in water and subsequently surface sterilized in 75% ethanol for 1 min followed by 1.0% sodium hypochlorite (NaOCl) for another 1.5 mins. After immersion in sterile distilled water for rinsing and blotting dry with filter paper, the plant tissues were cut into small pieces and placed on malt extract agar (MEA) and potato dextrose agar (PDA) plates supplemented with 1g/ml of streptomycin to suppress bacterial growth. The Petri dishes were sealed with parafilm and incubated at $27^oC \pm 2^oC$ until fungal growth appeared. The fungal endophytes growing from the plant tissues were picked and subcultured on fresh MEA and PDA to obtain pure culture.

2.3 Identification of endophytes

The fungal isolates were identified based on their morphological and reproductive characters. Colonies were analysed, slides were prepared from cultures using lactophenol cotton blue stain and examined with a microscope (Barnett and Hunter, 1998).

2.4 Inoculation and fermentation

Ten fungal inoculum's discs was transferred into each of fifteen I L Erlenmeyer flasks containing 250ml of fermentation medium. The medium consisted of yeast extract, 0.4%; malt extract, 1.0% and glucose, 0.4%; pH 7.0. All the flasks were incubated at $28^oC \pm 2^oC$ in a rotary shaker (130rpm) for 14 days. During incubation, a flask was removed at random every 48hour and fermented medium processed as described by Onifade (2007).

2.5 Extraction procedure

The broth cultures were passed through 0.45mm filter paper under vacuum to separate the mycelia from the culture broth. The wet mycelia were weighed, blended and extracted with ethyl acetate (1:1w/v). The pH of the broth was noted after which it was extracted with equal volume of ethyl acetate (1:1).The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to dryness in pre-weighed vials after which the metabolites was weighed. This was later diluted with dimethyl sulfoxide (DMSO) and used for the antimicrobial assay.

2.6 Pathogens used for the antimicrobial activity

A total of eight bacterial pathogens were used for the antimicrobial activity. The typed cultures were Bacillus subtilis (NCIB 3610), Serratia marcescence (NCIB 1377), Klebsiella pneumonia (NCIB 4183) and Pseudomonas fluorescense (NCIB 3757) and they were obtained from the Department of Microbiology, Obafemi Awolowo University (OAU), Ile-Ife, Nigeria. The clinical pathogens were also obtained from OAU teaching hospital and the isolates were Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis and Klebsiella pneumonia

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2.7 Antibiotic assay

The antimicrobial activity of the extracts was determined by the agar well diffusion method. Agar plates were inoculated with broth cultures of the test organisms. Five millimeter diameter wells were punched into the agar and filled with 30µL of each extract dilution. The plates were then incubated at appropriate temperatures for 24h after which zones of inhibition was measured.

III. Results

A total of 15 different colonies of endophytes were isolated from the roots of Carica papaya, Aloe barbadensis miller and Ocimum gratissimum (African basil) and they were identified by using the standard taxonomic keys. In this study, a total of three endophytic fungi were randomly selected for fermentation and screening for antimicrobial activity against clinical and typed cultures. The final pH of the fermented culture medium for the various endophytes is shown in Fig. 1 and it was observed that the culture of T viride had the lowest pH at the end of 14 days of fermentation.

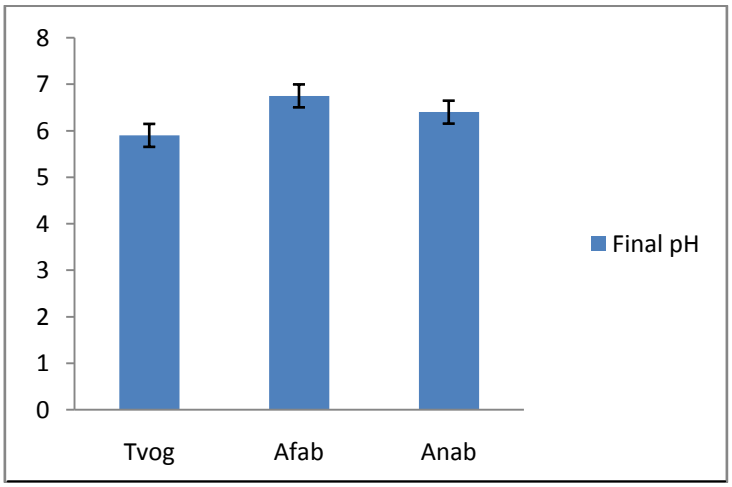

Fig.1 Final pH of the fermented culture medium. Tvog= Trichoderma viride, Afab= Aspergillus fumigatus and Anab= Aspergillus niger.

The effects of fermentation period on the wet mass of the endophytes and dry weight of the culture filtrate extracts are shown in Table. 1-3.

Table 1 Effects of fermentation period on wet mass of *T viride* mycelia and dry weight of culture filtrate extract.

Days	Wet weight of Mycelia (g)	Final volume of filtrate (ml)	Dry weight of filtrate extract (mg)
1	0.00	250	00
2	9.46	228	06
4	18.62	234	08
6	14.57	232	09
8	14.51	229	12
10	20.40	223	14
12	23.58	229	11
14	25.10	235	14

values show mean of three replicates

Table 2 Effects of fermentation period on wet mass of *A fumigatus* mycelia and dry weight of culture filtrate extract.

Days	Wet weight of Mycelia (g)	Final volume of filtrate (ml)	Dry weight of filtrate extract (mg)
1	0.00	250	00
2	2.50	214	30
4	3.50	200	40
6	4.82	191	50
8	4.96	180	55
10	6.20	168	59
12	6.55	160	62
14	8.50	156	68

values show mean of three replicates

Table 3 Effects of fermentation period on wet mass of *A niger* mycelia and dry weight of culture filtrate extract.

Days	Wet weight of Mycelia (g)	Final volume of filtrate (ml)	Dry weight of filtrate extract (mg)
1	0.00	250	00
2	34.00	202	35
4	34.40	198	40
6	35.70	188	49
8	37.00	186	50
10	37.19	185	51
12	39.35	185	51
14	41.56	180	54

values show mean of three replicates

From the tables 1-3, it was observed that *A fumigatus* had the lowest mycelia wet weight while *A niger* had the highest mycelia wet weight. *T viride* produced the lowest secondary metabolites while *A fumigatus* produced the highest secondary metabolites. The antimicrobial activity of the extracts are shown in Table 5 and Table 6. It was observed that *A niger* had the lowest zone of inhibition in both the typed culture and the clinical isolates. Conversely, *T viride* showed the highest zone of inhibition in both the typed culture and the clinical isolates except in *Klebsiella pneumoniae* where it showed a low zone of inhibition.

Table.4 Antimicrobial activity of the extracted secondary metabolites on some clinical isolates.

	Zone of inhibition (mm) n=3			
Fungal specie	*P mirabilis*	*S.aureus*	*K pneumoniae*	*P aeruginosa*
T viridae	13	8.0	4.5	11
A fumigatus	9.0	9.0	6.0	13
A niger	4.5	3.0	4.5	5.0
Tetracycline	18	21	14	13

Table 6 Antimicrobial activity of the extracted secondary metabolites on some typed cultures.

	Zone of inhibition (mm) n=3			
Fungal specie	B. subtilis	*S.marcescence*	*K pneumoniae*	*P fluorescense*
T viridae	11	5.0	3.5	9.0
A fumigatus	8.0	5.0	6.5	6.5
A niger	6.0	2.0	2.0	4.0
Tetracycline	14	13	15	14

IV. Discussion

At the end of the fermentation period, all the growth medium had become acidic with the T viride medium having the lowest pH of 5.9 on day 14. However, the endophytes showed increment in the quantity of the metabolites produced as the fermentation progressed till the last day. All the three selected endophytes screened in this study produced metabolites which were found to have antimicrobial activity against the clinical and the typed cultures used in this study. Generally, T. viride showed a higher level of inhibition more than the others with zones of inhibition measuring 13, 11 and 9 mm in Proteus, Pseudomonas and Bacillus spp . There have been reports on the potentials of Trichoderma spp. to antagonize a wide range of soil borne plant pathogens in addition to their ability to reduce the incidence of diseases caused by these pathogens in a wide range of crops (Monte, 2001). Although, Trichoderma spp has been reported to induce resistance against bacteria like Xanthomonas spp. and Pseudomonas syringae but the most common reason for the death of many microorganisms growing in the vicinity of Trichoderma strains is the starvation and scarcity of limiting nutrients (Waghunde et al., 2016). Aspergillus fumigatus showed a maximum zone of inhibition of 13, 9 and 8 mm against Pseudomonas, Proteus,Staphylococcus and Bacillus spp in this present work. Ratnaweera et al. (2015) reported that A. niger and Fusarium spp showed a promising activities because they were active against three microorganism. Endophytic A. niger showed the highest colonization in the cladodes of O. dillenii while the Fusarium spp. isolated from the pistil of the flowers showed the major biological activity. However, in this study, A. niger showed the lowest zone of inhition compared to all the other two endophytes.

Generally, the result of this study showed that the standard antibiotic, Tetracycline used in this study has a higher zone of inhibition than the metabolites tested but the metabolites are comparable because the metabolites used were crude extracts and promises to show better result when purified. The concentration of the active substance would also play a major role in determining the level of antagonism (Omura, 1992). It has also been reported that better production of bioactive compound can be achieved if the pH of the growth medium is modified and the medium is supplied with good stimulants (Thalavaipandian et al., 2011). Therefore, the result from this study has confirmed the potentials of metabolites from endophytic fungi as a source of bioactive compounds to meet the global challenges of producing better drugs.

V. conclusion

In view of the general need for the discovery of new antibiotics and chemotherapeutic agents that are highly effective and possess low toxicity, endophytic fungi could be a potential source for the production of quality and quantity of bioactive compounds as it has been demonstrated in this study.

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