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ISOLATION AND EVALUATION OF *In VITRO* ANTIBACTERIAL POTENTIAL OF ENDOPHYTIC FUNGI FROM THE LEAVES OF *Psidium guajava* (Guava)

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ABSTRACT
Endophytic microorganisms colonize the various living tissues of many plants and establish a mutualistic relationship without causing any apparent symptoms of diseases. Endophytic fungi represent an excellent source of antimicrobials and their natural products have proved effective in the control of a wide range of pathogenic microorganisms like bacteria, fungi, viruses and protozoa. Medicinal plants have played a vital role in the search for novel bioactive groups of endophytic fungi with the assumption that their antimicrobial properties are the consequences of metabolic molecules produced by their own endophytic community. Psidium guajava is a small medicinal tree that can live for several years ranging from 30 to 50 years. The various parts of this plant are traditionally used to treat many ailments. In this study, six (6) fungal endophytes were isolated from the leaves of P. guajava by direct incubating the prepared leaves on sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) plates for 6-10 days at 28 ^{o}C as well as streak plate technique. Evaluation of antibacterial activity of crude metabolites of the fungi was conducted using agar well diffusion method. The fungal isolates were identified to be Rhizopus arrhizus, Candida albicans, Epidermophytum floccosum, Aspergillus niger, Mucor circinelloides and Lichtheimia corymbifera. The antibacterial activity of the crude metabolites of these fungi assessed on three (3) bacterial isolates, including Escherichia coli, Proteus mirabilis and Klebsiella pneumoniae revealed clear zones of growth inhibition of the fungi against all the test bacteria. In conclusion, six (6) endophytic fungi were successfully isolated from the leaves of P. Guajava and their antibacterial evaluation revealed in vitro antibacterial activity against the test organisms.
Keywords: Endophytic fungi, Psidium guajava, secondary metabolites, antibacterial activity

INTRODUCTION

Endophytic microorganisms are those organisms that reside in the various living tissues (stem, leaf, root, fruit, seed, etc.) of many plants and establish a mutualistic relationship without causing any obvious symptoms of diseases (Sandhu *et al.,* 2014; Strobel and Daisy, 2003). The endophytes play several physiological and ecological roles in their host plants as well as protection against infectious agents and adverse conditions through the secretion of certain bioactive secondary metabolites. There is an It has been established that Hypericin ($C_{30}H_{16}O_8$), a naphthodianthrone derivative and Emodin ($C_{15}H_{10}O_5$), the key precursor of hypericin were produced by an enzdophytic increased interest in the search for the potentialities of endophytic fungi with respect to the production of bioactive metabolites such as pestaloside, taxol, torreyanic acid and enzymes (Xylanase**,** Asparaginase)(Tintjer and Rudgers, 2006; The antana *et al*., 2007; Radji *et al*., 2011).

Endophytic fungi represent an excellent source of antimicrobials and their natural products have proven effectiveness in the control of a wide range of pathogenic microorganisms like bacteria, fungi, viruses and protozoa. fungi isolated from a medicinal plant with a proven antimicrobial activity on various bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella*

enteric and Escherichia coli, as well as fungal organisms like *Candida albicans* and *Aspergillus niger* (Kusari and Spiteller, 2011). PolyketideCitrinin compound which showed a strong antibacterial activity was synthesized by another endophytic fungus known as *Penicilliumj anthinellum* isolated from the fruits of *Melia azedarach* (Marinho *et al*., 2005). Medicinal plants have played a vital role in the search for novel bioactive groups of endophytic fungi with the assumption that their antimicrobial properties are the consequences of metabolic molecules produced by their own endophytic community (Santos *et al*., 2015; Kaul *et al*., 2012; Kusari *et al*.,2013). In spites of this potential, a repertoire of medicinal plants should be studied for their endophytic composition (Santos *et al*., 2015). *Psidium guajava* is a small medical tree that is native to South America and Tropical areas of the world and it can live for several years ranging from 30 to 50 years. The various parts (fruits, leaves, bark and roots) of this plant are traditionally used to treat many ailments including dysentery, diarrhea, malaria fever, typhoid fever, stomach ache, respiratory and gastrointestinal disorders, antispasmodic, anti-inflammatory, management of hypertension, obesity and in the control of diabetes mellitus. Due to the tremendous medicinal properties of *P. guajava*, this research aimed at isolation and evaluation of antimicrobial activity of endophytic fungi from the leaves of this plant.

MATERIALS AND METHODS

Collection and Authentication of samples

Healthy leaves of matured *Psidium guajava* were collected in a sterile plastic bag and authenticated at the Herbarium of Biological Sciences department of Gombe State University where a voucher number was provided (075).

Preparation of media

All media (Potato dextrose agar, Sabroud dextrose agar, MacConkey agar, Nutrient agar and Mueller Hinton agar) were prepared according to the manufacturers' instructions.

Isolation of fungal endophytes

Endophytic fungi were isolated according to the method of Sandhu *et al*., 2014 with little modification. Leaves of the *P. guajava* were washed in running tap water to remove excess dust and sterilized the surfaces by soaking them in Mercuric chloride (1%) solution for 30 second. The leaves were further rinsed using sterile distilled water and then immersed into 50ml of 70% ethanol for 10 minutes followed by washing with sterile distilled water. Leave segments of approximately 2×2 mm were aseptically cut to expose the interior tissues of the leaves on the culture plates. The plates were incubated for 6-10 days at 28 ºC for the appearance of fungal growth. Aliquots of the fungal growth from the periphery were transferred onto fresh media plates until pure cultures of the fungal colonies were obtained.

Identification of fungal organisms

The fungi were identified using culture and microscopic characteristics such as shape, size, texture, colour, pattern and arrangement of mycelium, conidial arrangement and types of spore with reference to fungal atlas(Watanabe, 2002)and similar report (Kidd *et al*., 2016).

Source of test organisms

The test organisms of *Escherichia coli, Proteus mirabilis and Klebsiella pneumoniae* were obtained from Microbiology laboratory, Department of Microbiology, Gombe State University.

Preparation of standard Inoculum

The test organisms were cultured on MacConkey and nutrient agar plates and incubated for 24 hours at 37 ^{o}C. Discrete colonies of each test organism were picked using a sterile wire loop from an overnight culture and emulsified in sterile distilled water contained in a test-tube. Turbidity of the suspension was matched to 0.5 McFaland standards (Cheesebrough, 2000; Garba *et al*., 2018; Yusha'u *et al*., 2008).

Antibacterial susceptibility test

Agar well diffusion method was used to assess the antibacterial activity of the endophytic fungi. The fungi were cultured in 10 ml of potato dextrose broth (PDB) contained in test tubes and incubated for 7, 14 and 21 days at 28^{o}C. Wells of approximately 6 mm diameter were aseptically cut using a sterile cork borer in Mueller Hinton agar plates seeded with the test organisms. Aliquots of 100µl of the fungal broth culture were supplied into the wells and incubated for 24hoursat 37^{o}C. The plates were observed for zones of growth inhibition (Sandhu *et al*., 2014).

RESULTS

Isolation and identification of endophytic fungi

A total of six (6) different fungal species which include *Rhizopus arrhizus, Candida albicans, Epidermophytum floccosum, Aspergillus niger, Mucor circinelloides* and *Lichtheimia corymbifera* were successfully isolated from the leaves of *P. guajava* based on their cultural characteristics and microscopic appearances (Table 1).

Table 1: Fungal endophytes isolated from the leaves of *Psidium guajava*

Media	Code	Characteristics	Endophytic Fungi
SDA	PgLs1	White cottony colonies which changed to brownish grey or blackish-grey.	*Rhizopus arrhizus*
SDA	PgLs2	White to cream-coloured colonies that are smooth with glabrous conidial heads.	*Candida albicans*
SDA	PgLs3	Slow growing, greenish-brown colonies with a suede-like surface, raised and folded in the centre. Microscopic morphology showed characteristic smooth, thin-walled macroconidia.	*Epidermophytum floccosum*
PDA	PgLp1	Colonies with a compact basal felt covered by a dense layer dark-brown to black conidial heads.	*Aspergillus niger*
PDA	PgLp2	Colonies appeared as floccose, pale greyish-brown and grow poorly. Sporangiophores are hyaline and mostly sympodially branched with long erect branches and shorter circinate (coiled).	*Mucor circinelloides*
PDA	PgLp3	Fast growing colonies, floccose, white at first becoming pale grey. Sporangiophores faintly pigmented and simple arising in groups with a flat periphery and submerged fringe of growth.	*Lichtheimia corymbifera*
PDA	PgLp4	Colonies are white cottony at first becoming brownish grey to blackish-grey.	*Rhizopus arrhizus*

Key: SDA= Sabouraud Dextrose Agar, PDA= Potato Dextrose Agar

Antibacterial potential of endophytic fungi

The overall results of the antibacterial testing showed that the fungal metabolites were active against the test bacterial isolates. The fungal species exhibited varying degrees of growth inhibition zones on the bacteria after seven (7) days, fourteen (14) days and twenty (21) days of incubation as presented in Tables 2, 3 and 4, respectively. However, the fungal metabolites showed higher antibacterial activity after 14 days of incubation followed by 21 days of incubation and then 7 days of incubation. Moreover, among the six (6) fungal isolates, *E. floccosum* and *R. arrhizus* were found to be the most and the least active species, respectively, against all the test bacteria at the entire periods of incubation based on the zones of growth inhibition produced.

Table 2: Antibacterial activity of crude fungal broth cultures after 7 days of incubation

Endophytic Fungi	Test Organisms/ Zones of growth inhibition (mm)		
	E. coli	*K. pneumoniae*	*P. mirabilis*
A. niger	13	14	12
C. albicans	09	12	08
E. floccosum	16	17	16
L. corymbifera	12	11	13
M. circinelloides	13	10	09
R. arrhizus	08	06	07

Table 3: Antibacterial activity of crude fungal broth cultures after 14 days of incubation

Endophytic Fungi	Test Organisms/ Zones of growth inhibition (mm)		
	E. coli	*K. pneumoniae*	*P. mirabilis*
A. niger	19	18	20
C. albicans	14	17	12
E. floccosum	21	22	21
L. corymbifera	18	17	19
M. circinelloides	16	17	14
R. arrhizus	10	08	11

Table 4: Antibacterial activity of crude fungal broth cultures after 21 days of incubation

Endophytic Fungi	Test Organisms/ Zones of growth inhibition (mm)		
	E. coli	*K. pneumoniae*	*P. mirabilis*
A.niger	14	15	16
C. albicans	13	16	10
E.floccosum	18	19	17
L. corymbifera	13	14	15
M. circinelloides	14	15	12
R. arrhizus	09	07	08

DISCUSSION

Several endophytic fungal species constitute an ecological niche within the inner tissues of plants with ultimate mutualistic relationship. These endophytes have very good potential applications in plants development and control of pathogens (Sandhu *et al*., 2014). One of the most significant characteristics of endophytic microorganisms such as fungi and bacteria is directly associated to their ability of producing an array of bioactive substances effective in protecting the plant against pathogens (Inuwa *et al*., 2017; Santos *et al*., 2015; Tan and Zou, 2001; Strobel, 2003). Previous studies have established that endophytic fungi synthesise natural compounds which act as inhibitors of numerous plant and animal pathogens (Santos *et al*., 2015; Gunatilaka, 2006; Zhao *et al*., 2011).

The ability of plants endophytic microorganisms to produce biologically active secondary metabolites which are thought to be responsible for the medicinal properties of plants has intrigued many researchers to scrutinize the various endophytic microbiota for potent antimicrobial alternatives (Kaul *et al*., 2012; Kusari *et al*., 2013). A conspicuous example could be traced back to the discovery of taxol (an anticancer agent) extracted from *Taxus brevifolia* Nutt. as well as its endophyte, *Taxomy cesandreanae* (Stierle *et al*., 1993).

Some of the fungal endophytes isolated in this study (Table 1) are comparable to other species reported from different plants such as *Sceletium tortuosum* (Manganyi *et al*., 2019), *Suaeda maritime* and *S. monoica* (Kalyanasundaram *et al*., 2015), *Sonneratia griffithii* (Handayani *et al*., 2017) and *Mentha piperita* (Chowdhary and Kaushik, 2018). This suggests the biodiversity of similar fungal endophytes colonizing different plants tissues. Moreover, the fungi isolated from the leaves of *P. guajava* in our study have demonstrated a strong antibacterial activity against all the test bacterial isolates based on the zones of growth inhibition observed in Tables 2, 3 and 4, which corroborate with similar findings by several researchers such as Santos *et al*., 2015, Sandhu *et al*., 2014, Gong and Guo, 2009 and Lin *et al*., 2007.

CONCLUSION

In conclusion, six (6) endophytic fungi were successfully isolated from the leaves of *Psidium guajava*. The fungi produced wide zones of growth inhibition against all the test organisms which suggested their potential antibacterial activity. Further research is needed to assess the actual bioactive molecules produced by these fungi.

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