UJMR, Conference Special Issue Vol. 9 No. 3. June, 2024, pp.180 - 186

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

https://doi.org/10.47430/ujmr.2493.021

Received: 3rd March, 2024 **Accepted:** 8th June, 2024

Antibacterial Potential of Endophytic Fungi Isolated from *Psidium guajava* (Guava) Leaf against *Escherichia coli* and *Klebsiella pneumoniae*

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Abstract

Antimicrobial resistance has been recognized as a major issue of public health concern and it remains a global threat of the health care system. Endophytic fungi associated with medicinal plants are reported as promising reservoir of novel antibiotics. The study aim was to determine the antibacterial potential of endophytic fungi associated with Psidium gaujava leaf against Escherichia coli and Klebsiella pneumoniae. Leaves of P. guajava were surface sterilized and inoculated on plates of Potato Dextrose Agar and incubated at room temperature. Endophytic fungal isolates that emerged were identified using their macroscopic (cultural) and microscopic characteristics. The endophytes were screen for antibacterial activity on E. coli and K. pneumoniae isolates. Antibacterial activity of endophytic fungi ethyl acetate extracts with antibacterial activity was also evaluated against isolates of E. coli and K. pneumoniae by agar well diffusion technique. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations of the ethyl acetate extracts were also determined. Eight endophytic fungi were isolated from P. guajava leaves and four had antibacterial activity namely: Gonatobotrys sp. P21, Xylaria sp. P11, Fusarium sp. P22 and Trichoderma sp. P25. The extracts exhibited antibacterial activity with zones of inhibition ranging from 11.5 ± 0.5mm to 18.5 ± 1.5mm for E. coli and 12.5 ± 0.5mm to 21.0 ±1.0mm for K. pneumoniae. The MIC was found to be 500mg/mL and 250mg/mL for E. coli and K. pneumoniae respectively. Endophytic fungi isolated from P. gaujava leaf are potential source of novel antibacterial drug since they possess antibacterial activity against isolates of E. coli and K. pneumoniae.

Keywords: Endophytes, fungi, antibacterial, Escherichia coli, Klebsiella pneumoniae

INTRODUCTION

Antimicrobial resistance is a serious health issue that threatens the health care system world-wide. It has become necessary and timely to research for and identify novel therapeutic compound due to the increase in resistance of pathogens to available drugs (Marcellano *et al.*, 2017).

Annually, hundreds of thousands of people die as a result of infections caused by antimicrobial-resistant pathogens. This statistic is predicted to increase to as high as 10 million people by 2050 if interventions are not implemented. The most important intervention is the development of novel antimicrobial compounds (O'Neill, 2016; López-Pérez *et al.*, 2017).

Psidium guajava L., known as Guava in Hausa language, is a plant of medicinal value belonging to the family Myrtaceae. It is a popular medicinal plant used in the treatment of various illnesses in indigenous medicinal systems (Kaneria and Chanda, 2011). For centuries, plants have been a source of bioactive compounds with medicinal value against various diseases. Recent research has focused on plant-associated microorganisms, known as endophytes, revealing that endophytes produce compounds with high therapeutic potential. Endophytes are endosymbiotic microorganisms, including bacteria and fungi, that colonize healthy plants (Gouda *et al.*, 2016). Medicinal plants host certain fungi capable of producing bioactive metabolites (marcellano et al., 2017). The enormous therapeutic potential of endophyte metabolites has been well-

documented, showing activity against microorganisms, viruses, parasites, and tumors (Santiago *et al.*, 2014).

The plant provides nutrients and shelter to the endophytes, while the endophytes produce metabolites that enhance plant fitness by protecting the plant from biotic and abiotic stresses (Gupta *et al.*, 2023). Endophytic fungi are reported to be a reservoir of vast novel antimicrobial compounds. Plant-associated endophytes produce certain metabolites that can induce plant resistance to pathogens (carbungco et al., 2017).

Endophytic fungi associated with medicinal plants such as psidium guajava have been identified as one of the promising sources of new antibiotics to combat antimicrobial resistance (Gupta *et al.*, 2023). They have the ability to secrete a vast repertoire of bioactive compounds with promising therapeutic potentials (Digra and Nonzom, 2023; meshram et al., 2023). The challenge of antibiotic resistance has resulted in increased research on endophytic fungi, especially medicinal plant-associated endophytes, as a source of novel antibiotics (Gupta *et al.*, 2023).

MATERIALS AND METHODS

Collection of Samples

Psidium guajava (guava) leaves were collected within the main (Samaru) Campus of Ahmadu Bello University, Zaria. The leaves were collected in substantial quantity and taken for identification to the Herbarium, Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The plant was identified as psidium guajava and assigned voucher number ABU03253. Subsequently, the leaves were transported to the Department of Microbiology laboratory for further analysis.

Collection of Test Bacterial Isolates

Clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* used as the test bacterial isolates for this study were collected from Main Teaching Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria.

Isolation and identification of Endophytic Fungi

The *Psidium guajava* leaves were rinsed using running tap and then surface sterilized as follows:2 minutes treatment with 70% ethyl alcohol, followed by washing with distilled water and dipping for 2 minutes in 10% sodium hypochlorite. The leaves were washed again with distilled water and cut into small pieces of dimensions 6 mm^2 with the aid of a sterilized blade and forceps. The cut pieces of leaves were inoculated on Potato Dextrose Agar (PDA) medium and incubated for 5 days at 25°C (Savitha *et al.*, 2012). Fungal isolates that emerged from the leaves were identified with the aid of an atlas of mycology based on their macroscopic and microscopic characteristics. Macroscopic characteristics included the fungal mycelia colour and texture as well as reverse of the plate. Microscopic characteristics were observed under the microscope using slide culture technique.

Preliminary Screening of Endophytic Fungi for Antibacterial Activity

To screen the endophytic fungi isolates for antibacterial activity, 0.5 McFarland standardized bacterial isolates were inoculated on Mueller Hinton Agar plates and mycelia plug of 7 days old endophytic fungal isolates were then placed on the surface of the inoculated agar. The plates were then incubated for 24 at 37°C hours and then examined for inhibition zone around the endophytic fungal isolates (Hussaini *et al.*, 2022).

Fermentation Assay of Endophytic Fungi

Endophytic fungal isolates that showed antibacterial activity in the preliminary screening were inoculated into 100 mL of malt extract broth contained in a 150 mL Erlenmeyer flask and incubated in a shaker incubator set at 140 rpm for 14 days at 25°C. Mycelia were removed via filtration after 14 days of fermentation (Santiago *et al.*, 2014).

Preparation of Extract

The fungal metabolites were extracted from the filtrate using the solvent extraction method.

Ethyl acetate was used as the solvent for extraction. The filtrate and ethyl acetate were combined in a separating funnel at a 1:1 ratio and vigorously shaken for 10 minutes. The mixture was then allowed to settle to separate the cell mass and collect the solvent. Ethyl acetate extracts were obtained by evaporating and drying the solvent in a vacuum evaporator. These extracts were dissolved in Dimethyl sulphoxide (DMSO) and stored for further studies (Hussainiet *al.*, 2022).

Evaluation of Antibacterial Activity of Ethyl Acetate Extract

Screening for antibacterial activity was carried out using the agar well diffusion technique. A sterile cotton swab was inserted into 0.5 McFarland standardized inocula of bacterial isolates and inoculated onto the surface of the Mueller Hinton agar medium. The plates were kept for 5 minutes to dry and then wells of 3mm were made into the medium using a sterilized cork borer. Four wells were made 2 inches apart on each plate. Fifty microliter (50µL) aliquot of each test extract was dispensed into appropriate well. Each plate was labeled and incubated at 37°C for 24 hours. The plates were examined for zones of inhibition and measured in millimeters (Biswas *et al.*, 2013)

Determination of MIC and MBC of the Ethyl Acetate Extract of the Endophytic Fungi

The Minimum Inhibitory Concentration (MIC) of the extracts was determined by broth micro-dilution assay. This assay was carried out using 96-well microplates, each well containing 100 µL of MHB. Two-fold serial dilution of the stock extract concentration (2000 µg/mL) was made in MHB to obtain concentrations ranging from 1000to7.8µg/mL. Then10µLof 0.5 McFarland standardized inocula were added to each wells and the plates were incubated for 24 hours at 37°C. The MIC of the extracts was determined after the addition of 30 µL of resazurin solution at concentration of 100 µg/mL and incubation at 37 °C for 2 hour. Bacterial growth changes the resazurin from blue colour to pink colour. Growth inhibition is indicated by a blue colour.The MIC value was the lowest extract concentration which inhibited bacterial growth. To determine the Minimum Bactericidal Concentration (MBC) of the extracts, broth from each well that showed no evidence growth was inoculated on Muller-Hinton agar and incubated for 24 hour at 37 °C. The least concentration that had no bacterial growth after subculturing was recorded as the MBC (Hilario *et al.*, 2017).

RESULTS

A total of eight endophytic fungal isolates were obtained from healthy leaves of psidium guajava (guava). The fungal isolates belong to the genera: *Xylaria* (3; 37.50%), *Aspergillus* (1; 12.50%), *Fusarium* (1; 12.50%), *Gonatobotrys* (1; 12.50%), and *Trichoderma* (2; 25.00%) (Figure 1).

Four out of the eight endophytic fungal isolates exhibited antibacterial activity against *E. coli* and *K. pneumoniae* (Table 1). The isolates that exhibited antibacterial activity are *Xylaria* sp. P11, *Gonatobotrys* sp. P21, *Fusarium* sp. P22, and *Trichoderma* sp. P25.

The ethyl acetate extracts of all four endophytic fungal isolates exhibited antibacterial activity against *E. coli* and *K.* pneumoniae, with zones of inhibition ranging between 11.5 ± 0.5 mm and 21.0 ± 1.0 mm (Table 2; plate I). The highest zone of inhibition (21.0 ± 1.0 mm) was observed for xylaria sp. P11 against *K.* pneumoniae, while the lowest zone of inhibition of 11.5 ± 0.5 mm was observed for trichoderma sp. P25 against *E. coli*.

Minimum Inhibitory concentration values of the extracts against *E. coli* and *K.* pneumoniae were observed to be between 250 µg/mL – 500 µg/mL and 500 µg/mL – 1000 µg/mL respectively. Growth was observed at all concentrations upon sub-culturing to determine MBC (Table 3; Plate II)

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

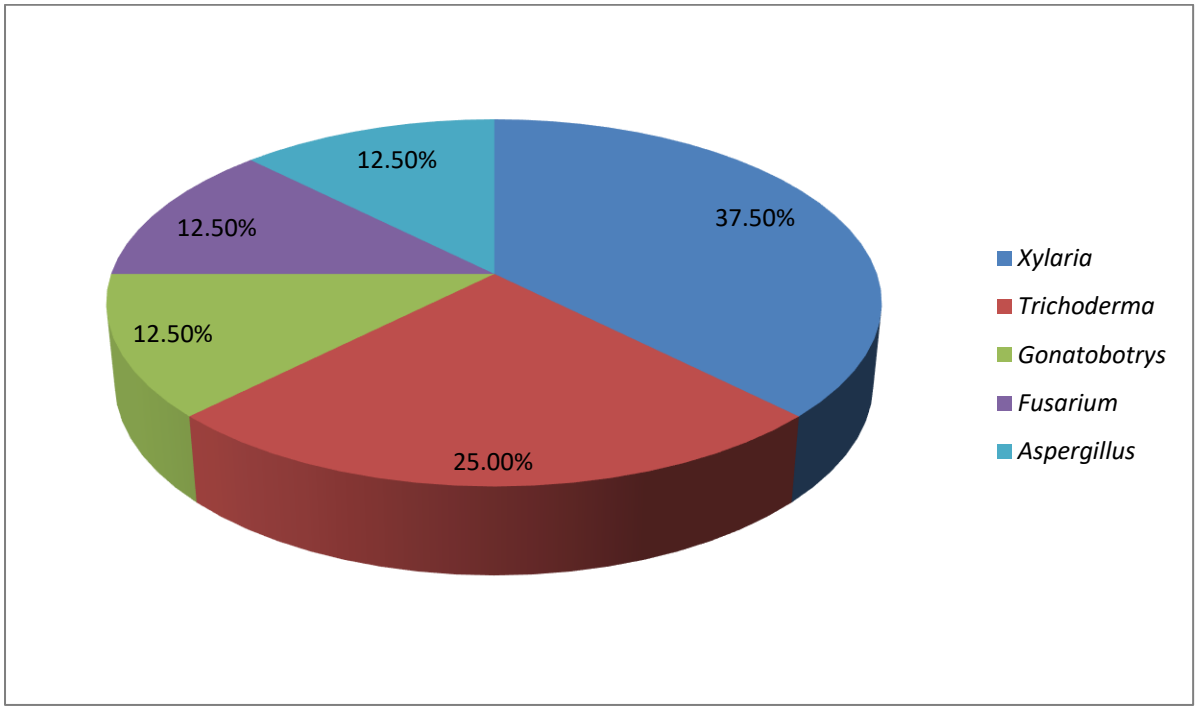

Figure 1: Percentage Distribution of Endophytic Fungi Genera Isolated from *Psidium guajava* leaf

Table 1: Antibacterial activity of endophytic fungal isolates against *E. coli* and *K. pneumoniae*

Isolate code	*E. coli*	*K. pneumoniae*
Xylaria sp. P11	+	+
Tricoderma sp. P12	-	-
Xylaria sp. P13	-	-
Gonatobotrys sp. P21	+	+
Fusarium sp. P22	+	+
Aspergillus sp. P23	-	-
Xylaria sp. P24	-	-
Trichoderma sp. P25	+	+

KEY: + = antibacterial activity; - = no antibacterial activity

Table 2: Antibacterial Activity of Ethyl Acetate Extracts of Endophytic Fungi at 2000µg/mL against *Escherichia coli* and *Klebsiella pneumoniae*

Isolate code	Mean Zone of inhibition (mm) ± SD	
	Escherichia coli	*Klebsiella pneumoniae*
*Xylaria*sp. P11	18.5 ± 1.5	21.0 ±1.0
Fusarium sp. P22	12.0 ± 0.0	15.5 ±0.5
Gonatobotrys sp. P21	18.0 ±2.0	12.5 ±0.5
*Trichoderma*sp. P25	11.5 ±0.5	12.5 ±0.5

Table 3: MIC and MBC values of ethyl acetate extracts of endophytic fungi against *E.coli* and *K.pneumoniae*

	K. pneumoniae		*E. coli*	
Isolate code	**MIC**	**MBC**	**MIC**	**MBC**
*Xylaria*sp. P11	500 µg/ml	-	250 µg/ml	-
Fusarium sp. P22	1000 µg/m	-	500 µg/ml	-
Gonatobotrys sp. P21	1000 µg/ml	-	250 µg/ml	-
*Trichoderma*sp. P25	500 µg/ml	-	250 µg/ml	-

Key: - = Growth was observed at all concentrations

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

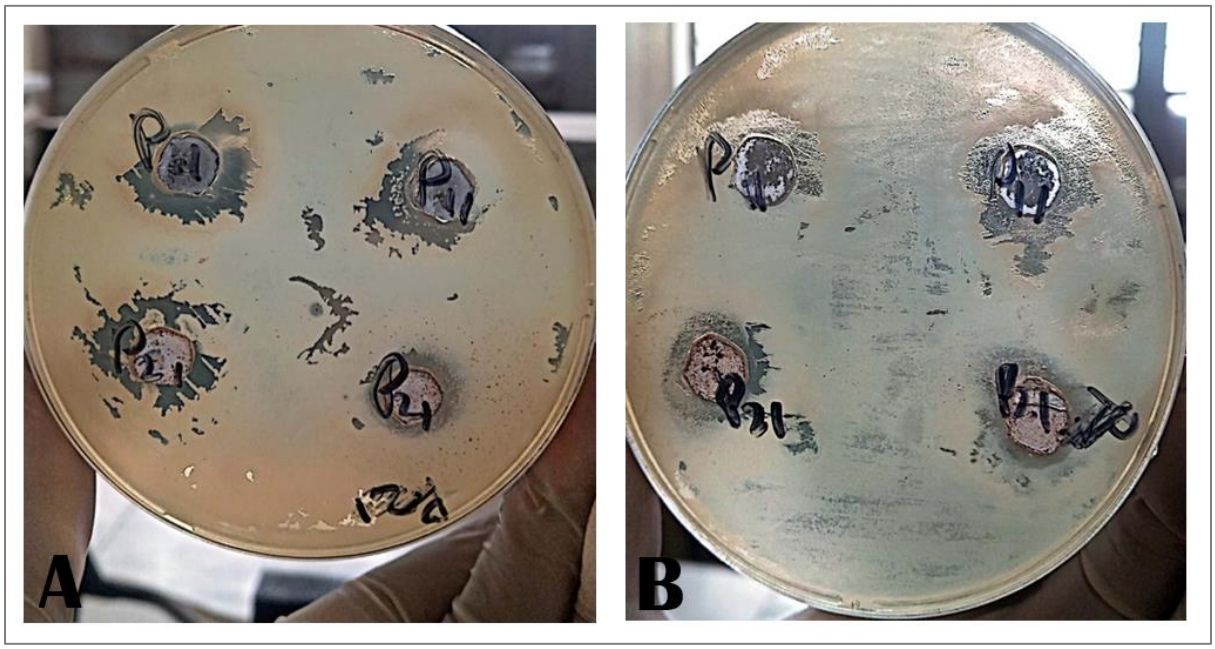

Plate I: Zones of inhibition of extracts against isolates of *E. coli* and *K. pneumoniae* [A= *E. Coli* and B= *K. pneumoniae*

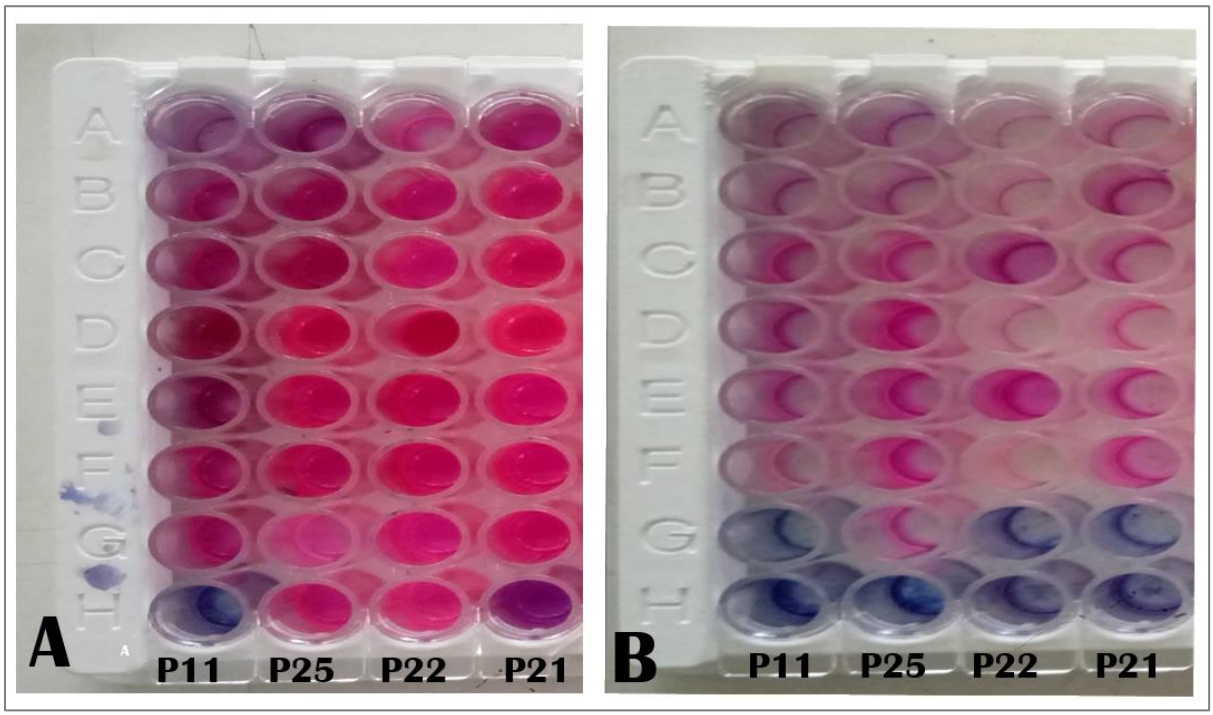

Plate II: MIC of ethyl acetate extract of A=*K. pneumoniae* and B=*E.coli*

DISCUSSION

A total of eight (8) endophytic fungi were isolated from health leaves of *P. guajava* this implies that these isolates are in a mutual relationship with the plant. The edophytic fungal isolates belong to five genera namely *Xylaria, Trichoderma, Fusarium, Aspergillus* and *Gonatobotrys*. Similar to this observation *Xylaria, Fusarium* and *Aspergillus* isolated from leaves of *P. guajava* by Tchamgoue*et al.* (2020). This finding is in contrast to that of Susilawati *et al.* (2018) who isolated four (4) endophytic fungi all belonging to the genus *Aspergillus* from leaves of *P. guajava*. *Xylaria* was observed to be the dominant genus is this study. *Xylaria* is reported to be among the most dominant genera of fungal endophytes because of its ability to adapt to tissue of different plant (Tchamgoue *et al.*, 2020). This is similar to the report of

Tchamgoue *et al*. (2020) where *Xylaria* was observed to be the dominant endophytic fungalgenus.

Four (4) of the fungi isolated, namely xylaria sp. P11, gonatobotrys sp. P21, *Fusarium* sp. P22, and *Trichoderma* sp. P25, exhibited antibacterial activity against the test bacterial isolates. This suggests that the endophytic fungi were capable of producing a variety of secondary metabolites with inhibitory effects against *K. pneumoniae* and *E.* coli.

The ethyl acetate extract of the four (4) endophytic fungi that showed activity during the screening assay exhibited antibacterial activity against the tested isolates. This implies that these four (4) endophytes produced secondary metabolites, which are bioactive compounds that exhibited antibacterial activity against the tested bacteria. These endophytic fungal isolates can be explored as a source of new antibiotics in the fight against antibiotic resistance. Variation was observed in the antibacterial activity of the extracts against *K. pneumoniae* and *E. coli*. The highest zone of inhibition was exhibited by the extract of *Xylaria* sp. P11 against *K. pneumoniae* (21.0 ± 1.0 mm) and *E. coli* (18.5 ± 1.5 mm), while the *Trichoderma* sp. P25 extract exhibited the least zone of inhibition against *K. pneumoniae* (12.5 ± 0.5 mm) and *E.* coli (11.5 ± 0.5 mm). The variation in antibacterial activities exhibited by the endophytic fungal extracts could be linked to differences in the type and quantity of metabolites present in the extracts.

The MIC of ethyl acetate extracts of the endophytic fungi against *E. coli* ranged between 250 µg/mL and 500 µg/mL, while the MIC against *K. pneumoniae* ranged between 500 µg/mL and 1000 µg/mL. Growth was observed at all concentrations, indicating that the extract can only inhibit the growth of the test bacteria at the tested concentrations.

CONCLUSION

Endophytic fungi associated with the leaves of *P.* guajava exhibited antibacterial activity against *K. pneumoniae* and *E. coli*. They produce metabolites that can be exploited as a novel drug for the treatment of infections caused by *K. pneumoniae* and *E. coli*. Future research should focus on the structural elucidation of the bioactive compounds produced and screening for their antibacterial activity against drug-resistant pathogens.

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