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Studies on Endophytic *Fusarium* Species Isolated from Medicinal Plants

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Abstract

The seasonal availability and possible extinction of some medicinal plants necessitated the search for sustainable source of bioactive compounds. The study evaluated the bioactivity potentials of endophytic *Fusarium* species isolated from two medicinal plants, *Senna alata* L, and *Mitracarpus hirtus* L (DC), using *in vitro* methods. The isolation of *Fusarium* involved the surface-sterilization of plant leaves followed by inoculation on potato dextrose agar supplemented with 5 mL/L of gentamicin, incubation at 28±2°C for 3–7 days, and fungal identification using standard microbiological methods. Mycelial plug from seven-day old pure culture of endophytic fungi was inoculated into 50 ml potato dextrose broth, incubated for 21 days at 28±2°C for the secondary metabolites production. The resultant broth culture was filtered to obtain a cell-free filtrate. The antimicrobial activity of the filtrate was determined against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* and *Bacillus* species using agar-well diffusion technique. Further antimicrobial assay was carried out against *S. aureus* using broth dilution method by measuring the optical density at one-hour interval. The enzyme activity of the endophytes and the phytochemical analysis of the secondary metabolites were determined. The isolated endophytic fungi from the plants were identified as *Fusarium* species. The cell-free filtrate (secondary metabolite) inhibited the growth of *S. aureus* showing its activity within 2-5 h of incubation when compared to the control. Endophytic fungi isolated from *M. hirtus* produced terpenoid, flavonoid and tannin while isolate from *S. alata* produced flavonoid and tannin. The production of amylase was significantly high in isolates from *Mitracapus* (MSW) but moderate in isolates from *Senna* (CAG), while proteinase was produced in low quantity by both isolates. None of the isolates produced laccase. The study has shown that *Fusarium* species isolated form *S. alata* and *M. hirtus* produced secondary metabolites with potential bioactivities.

Keywords: Fungal endophyte, *Fusarium* species, bioactive compound, antimicrobial activity, terpenoid, medicinal plant.

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Introduction

The use of plants for medicinal purposes is an age long practice. Medicinal plants, although promising source of bioactive compounds, are associated with the problems of species extinction and environmental degradation which necessitate the search for bioactive compounds from other natural sources. One of the novel natural sources of bioactive compounds is endophytic fungi. Endophytic fungi are the eukaryotic microbes inhabiting the living tissues of the plant through establishment of symbiotic or mutualistic relationship with their host plants.

Medicinally important bioactive compounds have been sourced from fungal endophytes (Ancheeva *et al.*, 2019). Bioactive compounds produced from fungal endophytes have demonstrated bactericidal, fungicidal, cytotoxic, and enzymatic activities (Sudha *et al*., 2016). Taxol (paclitaxel), a highly-selling multimillion dollar anticancer drug, was obtained from a fungal endophyte, *Taxomyces andreanae*, inhabiting the yew plant, *Taxus brevifolia* (Stierle *et al*., 1993; Tripathi *et al*., 2024). Other anticancer drug like podophyllotoxin, camptothecin were natural product of fungal endophyte isolated from medicinal plants (Omeje *et al*., 2017; Uzma *et al.,* 2018).

Fungal endophytes from medicinal plants remain the potent and widely recognized prolific source of bioactive secondary metabolites, a hidden treasure worth exploring for novel drug discovery and development (Sibanda *et al*., 2018; Saha *et al*., 2019).

The emergence of new infections and increased resistance of pathogens to antibiotics has necessitated the search for novel bioactive natural compounds from medicinal plants.*Senna alata* (formerly known as *Cassia alata*), and *Mitracarpus hirtus* (formerly known as *Mitracarpus scaber*) (Figure 1) are well-known medicinal plants in Nigeria used in for treatment of various diseases. These plants have wide spectrum of pharmacological activities including antimicrobial activity (Abere *et al*., 2007; Varghese *et al*., 2013). Studies have shown that these plants have bioactive metabolites such as flavonoids, terpenoids, tannins, and saponins (Ekalu, 2020; Oladeji, 2020).

Endophytic fungi are endowed with the special potential to produce the same or similar compounds as their host plants, and other bioactive compounds within a short time with guaranteed reproducibility and sustainability (Stierle *et al*., 1993; Kaul *et al*., 2012; Soltani & Hosseyni, 2015). A common endophyte, *Fusarium* species have been reported as capable of producing diverse bioactive compounds for the promotion of human health (Huang *et al*., 2009; Zhang *et al*., 2016; Singh *et al*., 2023). Information on the endophytic fungal diversity and their secondary metabolites abound in research reports from developed countries but sparse in Nigeria, a country with enormous species of medicinal plants.

Based on the aforementioned, it is deemed necessary to study endophytic *Fusarium* species isolated from two common medicinal plants, *Senna alata* and *Mitracarpus hirtus*, identify the secondary metabolites and ascertain their bioactivities.

Mitracarpus hirtus

Senna alata

Figure 1: Picture of *Mitracarpus hirtus* and *Senna alata*

Materials and Methods

Sample collection

Matured leaves of *Mitracarpus hirtus*, and *Senna alata* were collected from Botanical Garden in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka (UNN) in October 2020. The plants were authenticated and voucher specimen (Voucher number- *Mitracarpus hirtus* (L.) DC. UNN/13139; *Senna alata* (L.) Roxb. UNN/13140) deposited in the University of Nigeria Herbarium domiciled in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. Sample of the leaves were cut using sterilized cutter.

Isolation of endophytic fungi

Plant leaves were washed in sterile distilled water and subsequently surface sterilized in 70% ethanol for 2 minutes followed by 4% sodium hypochlorite (NaOCl) for 90 seconds. Surface sterilization was done to remove the epiphytes while only the endophytes remained. The surface sterile plant material was rinsed thrice in distilled water (1 minute for each rinse). The surface-sterile leaves were dried on sterile filter paper to remove excess water, then cut into small pieces (about 10 mm x 10 mm), and cultured on potato dextrose agar (PDA) supplemented with gentamycin (5 mL/L) to suppress bacterial growth. The culture plates were sealed with paraffin and incubated at 28 ±2ºC for 3 – 7 days until the fungal growth appeared. The fungal endophytes growing from the plant's tissues were picked and sub-cultured on a fresh PDA slant to obtain pure culture for identification and other assays. The efficacy of the surface sterilization was ascertained by making an imprint of the surface-sterile leaf on sterile PDA plate and incubated.

Identification of isolated endophytes

The fungal isolates were identified based on their macroscopic and microscopic morphological characteristics. Fungal colonies were analyzed, slides cultures were prepared from pure cultures, stained using lactophenol cotton blue stain, and examined under the microscope using x40 objective lens (Barnett and Hunter, 1998). The colony appearance and microscopy were used to identify the isolates by comparing them with pictures found in mycology books.

Production of secondary metabolites using isolated endophytic fungi

Three mycelial plugs (8 mm in diameter) from seven-day old pure culture of endophytic fungi were inoculated into conical flasks each containing 50 ml of fermentation medium of potato dextrose broth. All flasks were incubated at room temperature for 21 days. The control inoculum flask contained no fungal plug. During incubation period, the inoculum flasks were shaken at interval to obtain homogeneity of the bioactive compound produced. The crude fermentation broth was filtered through membrane filtration. The cell-free filtrate was used for the antimicrobial assay and phytochemical analysis.

Determination of antibiotics susceptibility of the test bacterial pathogens

A total of four bacterial pathogens were used for the antimicrobial susceptibility assay. They include *Staphylococcus aureus, Escherichia coli*, *Salmonella,* and *Bacillus* species. They were collected from Microbiology Department, University of Nigeria Nsukka. The bacterial pathogens were standardized using 0.5 McFarland turbidity

equivalent. A total of four 18-24 h-old culture of *Staphylococcus aureus, Bacillus* sp.*, Escherichia coli*, and *Salmonella* sp.*,* were used for the antimicrobial activity. The standardized test organisms were inoculated on the Muller-Hinton agar (MHA) and a standard antibiotic disc (gentamicin 10 µg) placed on the agar surface and then incubated at 37ºC for 18 – 24 hours to ascertain the antimicrobial susceptibility of each bacterial pathogen to standard antibiotic.

Determination of antibacterial activity of endophytic fungal culture filtrate

The antimicrobial activity of the extracts was determined by the agar-well diffusion method. Sterile Mueller-Hinton agar (MHA) plates were inoculated with standardized actively growing cultures of the test bacteria. Four-millimeter diameter wells were punched in the inoculated Mueller-Hinton agar plate, well-spaced, labelled, and filled with 20 microliters of corresponding fungal filtrate. Agar-well containing 0.2 mL gentamicin served as the positive control. The MHA culture plates were put in the refrigeration for one hour to promote diffusion of the metabolites into the agar. The MHA plates were incubated at 37ºC for 18 – 24 hours after which the zones of inhibition were measured. The test bacterium that recorded the highest inhibition zone diameter as well as the filtrate was selected for further analysis.

Extraction of bioactive metabolite from fungal filtrate using ethyl acetate

Each of the cell-free filtrate (200 ml) produced by endophytic fungus was mixed with 50 ml ethyl acetate and centrifuged for 10 minutes at 1500 rpm. The upper layer (supernatant) was decanted into a sterile container and the extraction process repeated thrice. The supernatants from each filtrate were pooled respectively, and subjected to evaporation at 45ºC using the rotary evaporator. The resultant extracts were put in a dryer for complete removal of ethyl acetate. The dried extracts from CAG and MSW were stored in the refrigerator for further use.

Effect of extract of fungal filtrates on growth of S. aureus

Three milliliters of standardized inoculum of *S. aureus* were added to 27 ml of sterile Mueller-Hinton broth (MHB), mixed thoroughly, and then 1 ml of the resultant suspension was dispensed into small sterile tubes. The extract of fungal filtrate (secondary metabolites) was dissolved in 0.2% DMSO to get a resultant concentration of 100 µg/ml of *Mitracarpus hirtus* (MSW) and *Senna alata* (CAG) respectively. One milliliter of the dissolved extract was added to 1 mL of the cell suspension (50 µg/mL), incubated at 37ºC. The spectrophotometer (wavelength set at 600 nm) was used to measure the optical density of the bacterial population at time 0, and every one-hour interval for 7 h. The cell suspension in test tube to which 1 ml of 0.2% DMSO was added served as the control. The optical density of the bacterial cells grown in the control experiment and extracts (MSW and CAG)) were recorded.

Preliminary tests for phytochemicals produced in the endophytic fungal cell-free filtrate

Qualitative and quantitative phytochemical analytical methods were used to determine the presence and quantity of alkaloids, glycosides, saponins, tannins, terpenoid, and steroids in the fungal-derived cell-free filtrate containing the secondary metabolites (Harborne 1973; Trease and Evans 1989).

The quantitative phytochemical analyses were done using the method of Harborne (1973). The concentration of the various phytochemical constituents was calculated thus:

$$Concentration\ (mg/100\ g)\ =$$

$$\frac{Absorbance\ of\ the\ sample\ \times\ dilution\ factor}{Slope\ of\ the\ standard}\ 1$$

Screening of endophytic fungi for enzyme production

Endophytic *Fusarium* isolates were screened for ability to produce amylase, proteinase and laccase. The assay was based on the degradation of starch to give a clear zone for amylase activity, degradation of gelatin to give clear zone for proteolytic activity, and oxidation of alpha-naphthol to give blue coloration for laccase activity (Sunitha *et al*., 2013; Desire *et al*., 2014). The fungal plug (4 mm in diameter) was cut out from a 7-day old culture and placed carefully into the 4 mm well made in the agar plate containing substrate for enzyme assay. The control was sterile agar plate that contained the desired substrate but incubated without the fungus. The enzyme activity was graded according to the diameter of halo around the fungal colony, namely- high (+++), moderate (++) and low (+). The analysis was done in duplicate.

Laccase activity

The fungus was inoculated on the glucose yeast extract peptone agar medium supplemented with 0.05g of α-naphthol and incubated for 5 days. The zone surrounding the fungal colony will change from colorless to blue, if laccase was produced (Deire *et al*., 2014).

Amylase activity

The fungus was inoculated on the glucose yeast extract peptone agar medium supplemented with 0.2% of starch and incubated for 5 days**.** The culture plate was flooded with a reagent solution (1% iodine and 2% potassium iodide). A clear zone around the fungal colony indicated production of amylase (Desire *et al*., 2014).

Proteolytic activity

The test was carried out on the glucose yeast extract peptone agar medium supplemented with 0.4% gelatin (pH 6.0). Sterilized gelatin was added to the sterile agar medium, mixed and dispensed into petri dishes. The agar plate was inoculated with the endophytic fungus and incubated for 5-7 days. A clear zone indicates the degradation of gelatin. After incubation, the culture plate was covered with saturated ammonium sulphate, leading to formation of precipitate. Thus, the culture plate turned opaque, and improved the visibility of the halo/clear zone around the fungal colony. The clear zone around the fungal colony indicates production of proteinase.

Statistical analysis

The data collected were presented as mean ± standard deviation and analyzed to determine statistical significance by independent samples t-test, one-way analysis of variance (ANOVA), and least-significant difference (LSD) where applicable, using the Statistical Package of the Social Sciences (SPSS) version 23. The level of significance was set at $P < 0.05$.

Results

Identification of endophytic fungi

The endophytic fungi isolated from the leaves of *Mitracarpus hirtus* and *Senna alata* were identified morphologically as *Fusarium* species (Table 1, Figures 2a-2d).

Antibacterial activities of the secondary metabolites of endophytic fungi

The secondary metabolites produced by the endophytic fungi significantly inhibited the growth of *S. aureus,* with the inhibition zone diameter (IZD) of 13 mm while little zone of inhibition was observed for *Bacillus, Escherichia coli,* and *Salmonella* sp. (Table 2).

The secondary metabolites had an inhibitory effect on the growth of *S. aureus* between 2-5 h of incubation (Figure 2)
Constituents of the secondary metabolites produced by endophytic fungi

The phytoconstituent produced by MSW include terpenoids, tannins, saponins, flavonoids, and steroids, while the secondary metabolites detected from CAG were tannins, flavonoids, steroids and tannins (Tables 3 and 4).

Activity of enzymes produced by endophytic fungal isolates
The analysis of the endophytic fungi isolated from the leaves of *M. scaber* and *C. alata* showed that production of amylase was significantly high in the endophytic fungus from *M. scaber* (MSW) but moderate in the endophytic fungus from *C. alata* (CAG). Proteinase was found to be produced by CAG and MSW but in low quantity. None of the endophytes produced laccase (Table 5).

Table 1: Macroscopic and microscopic characteristics of endophytic fungal isolates

Macroscopic appearance of endophytic fungi on PDA	Microscopy using slide culture technique	Probable name of isolate
Colony: Woolly to cottony flat and spreading (Figure 2a) Colour: White with Tan/brown reverse (Figure 2b).	Hyaline septate hypha, long conidiophores, cylindrical phialides, macroconidia and microconidia in chains (Figure 2c and 2d)	*Fusarium* species

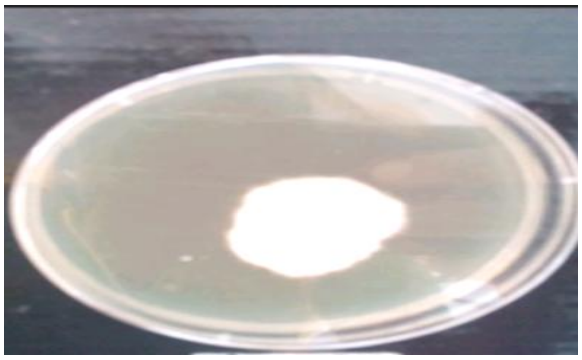

Figure 2a: White Colony of fungi endophyte on potato dextrose agar

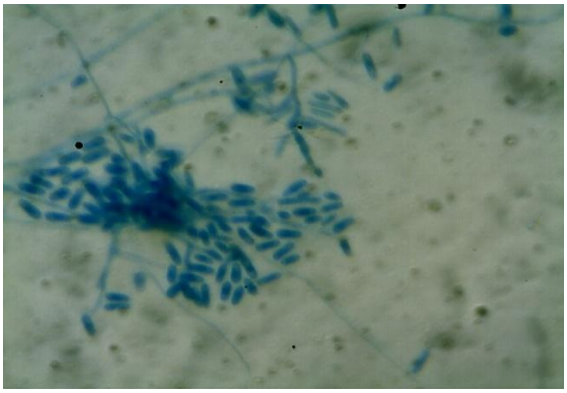

Figure 2c: *Fusarium* from *M. hirtus*

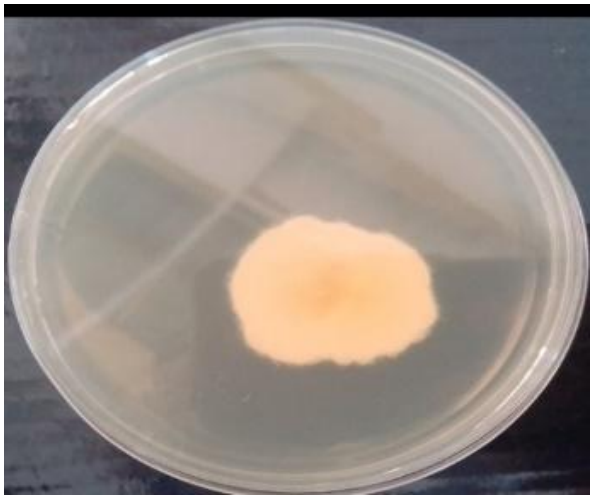

Figure 2b: Reverse of the colony on potato dextrose agar

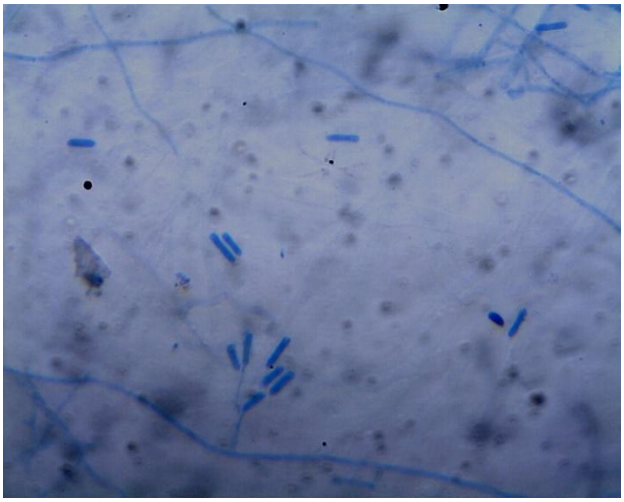

Figure 2d: *Fusarium* from *S. alata*

Figure 2a-d: Macroscopic and microscopic characteristics of *Fusarium* isolates

Table 2: Antibacterial activity of endophytic *Fusarium* culture filtrate

Bacteria	Mean Inhibition Zone Diameter (IZD) measured in millimeter			
	MSW	CAG	+Ctrl	- Ctrl
S. aureus	13.2±0.42*	11.4±0.28	26.3±0.28*	0
Bacillus sp.	9.0±0.42	8.6±0.28	22.1±0.14*	0
E. coli	8.05±0.21	8.15±0.49	18.1±0.28*	0
Salmonella sp.	8.1±0.28	8.0±0.14	18.0±0.28*	0

Diameter of corkborer = 4 mm; MSW= Endophyte isolated from *Mitracapus hirtus* CAG=Isolate from *Senna alata;* +ctrl = Positive control, gentamicin; -ctrl = Negative control, nutrient broth. *P < 0.05

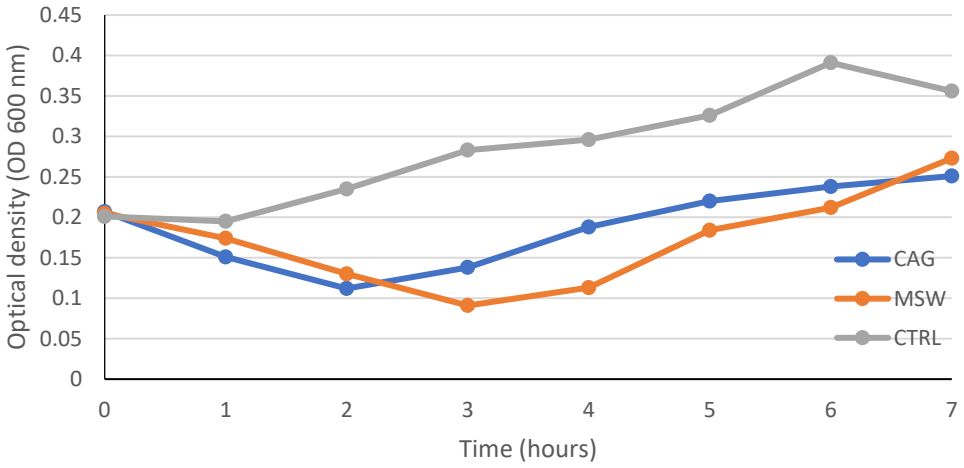

Figure 2: Effect of the fungal extracts produced by endophytic *Fusarium* isolates of *M. scaber* and *C. alata* on growth of *Staphylococcus aureus*. CTRL = control, cell suspensiom grown in nutrient broth containing 0.2% DMSO; MSW= Extract of endophytic fungi from *M. hirtus*; CAG= Extract of endophytic fungi from *S .alata*; Concentration of crude extract used = 50 µg/mL.

Table 3: Phytochemical composition of the secondary metabolites produced by endophytic *Fusarium* species, MSW and CAG

Metabolites	Relative abundance	
	MSW	CAG
Tannins	+	++
Saponins	+	-
Alkaloids	-	-
Flavonoids	+	+
Terpenoids	+++	-
Steroids	++	+

+++ = Abundantly present**;** ++ = Moderately present**;** + = Present in low concentration**;** -- = Absent**;** MSW = Cell-free filtrate produced by endophytic fungi from *Mitracaptus hirtus;* CAG; Cell-free filtrate produced by endophytic fungi from *Senna alata*.

Table 4: Amount of terpenoids, flavonoids, and tannins produced by endophytic *Fusarium* species

Phytochemicals	MSW (mg/100g)	CAG (mg/100g)
Terpenoid	22.09 ± 1.82	Not determined
Flavonoid	0.27 ± 0.16	1.71 ± 0.72
Tannin	2.32 ± 0.46	2.33 ± 0.38

Results are expressed in Mean ± SD (n=3)

Table 5: Enzyme activity of endophytic *Fusarium* isolated from *C. alata* and *M. scaber*

Enzyme assayed	Zone of enzyme activity measured (mm) Plate A	Plate B	Average	Remark
Amylase				
Mitracarpus hirtus (MSW)	28.5	31.5	30.0±2.09*	+++
Senna alata (CAG)	16.5	18.5	17.5±1.41*	++
Protease				
Mitracarpus hirtus (MSW)	7.5	6.8	7.15±0.49	+
Senna alata (CAG)	6.5	7.4	6.95±0.64	+
Laccase				
Mitracarpus hirtus (MSW)	0	0	0	-
Senna alata (CAG)	0	0	0	-

*P < 0.05; +++ = high; ++ = moderate; + = low; - = not detected or unable to produce. Enzyme activity was graded according to the diameter of halo around the fungal colony.

Discussion

The white mycelial colonies isolated from surface-sterilized leaves of *Senna alata* and *Mitracarpus hirtus* were phenotypically identified as *Fusarium* species. *Fusarium* species has been reported as one of the endophytes inhabiting medicinal plants (Zhang *et al*., 2016; Caicedo *et al.,* 2019; He *et al*., 2023). The cell-free filtrate containing the secondary metabolites produced by the endophytic *Fusarium* isolates from the two medicinal plants had inhibitory effect on the growth of *S. aureus* but insignificant inhibition zone diameter (<10 mm) for other test pathogens. Studies have reported the activity of *Mitracarpus scaber* against *S. aureus* and other pathogens (Ekpendu *et al*., 1994; Abere *et. al*., 2007; Musa *et al*., 2021). In the same vein, Mordi *et al*. (2016) and Ehiowemwenguan *et al*. (2014) documented the antimicrobial potential of *Senna alata* which justified the folkloric use of such plant in treatment of various skin diseases. In the present study, the zone of inhibition observed for *E. coli*, *Salmonella* and *Bacillus* species by the fungal-produced filtrate might be due to the low concentration of metabolites in the filtrate used for the antimicrobial susceptibility study. However, the present study showed the potential of endophytic fungal filtrate to inhibit *S. aureus* growth at low concentration.

The phytochemical analysis of the endophytic fungal cell-free filtrates produced after 21 days fermentation of *Fusarium* revealed the presence of bioactive compounds.

Report has shown that endophytic fungi can produce important pharmacologically-active metabolites related to compounds found in plants. Singh *et al*. (2021) reported the production of plethora of biochemicals by *Fusarium proliferatum* isolated from a medicinal plant, *Cissus quadrangularis*. Fungal metabolites have application in healthcare, agriculture and environment (Wadhwa *et al*., 2024).

Terpenoids produced in high concentration by fungal isolates may have potential applications in pharmaceutical, food and cosmetics industries. Terpenoids are potential geroprotectors, and have been reported to delay aging, extend lifespan, and useful in prevention and treatment of chronic diseases (Proshkina *et al*., 2020; Negi *et al*., 2020; Yang *et al*., 2020; Kim *et al*., 2020). Fungal terpenoids have potential, application in treatment of skin diseases and cosmetics (Trepa *et al*., 2024; Mittu *et al*., 2024). Terpenoids also promote oral health (Arzani *et al*., 2025) and useful in agriculture for weed control (Shaaban *et al*., 2025). Flavonoids are plant-derived polyphenols known to exhibit anticarcinogenic, anti-inflammatory, antioxidant, and anti-viral properties (Yang *et al*., 2020; Hamsalakshmi *et al*., 2022). Ma *et al*. (2023) documented the use of flavonoids in the prevention and treatment of skin cancer, premature aging, skin disorders, and promotion of wound healing. Pei *et al*. (2020) also indicated that the consumption of flavonoids improves gut health. Flavonoids are mainly sourced from fruits, legumes, nuts, tea, and vegetables. The present study has shown that endophytic fungi can serve as an alternative source of flavonoids.

Tannins are antinutrients associated with both beneficial and adverse health effects. Tannins are used as an anti-diarrhea, promotes vascular health, and immediate relief from skin ulcer and dysentery. They possess anti-inflammatory, anti-cancer, anti-helminthic, antimicrobial and wound-healing properties (Sharma *et al*., 2021). The protein precipitating property of tannin is also useful in controlling hemorrhage and they are applied to wounds as protective coatings (Chung *et al*., 1998). Steroids are useful in antenatal care, in alleviation of respiratory problems and severe hemorrhage (Myles, 2011). Fungi are promising source of steroidal drugs associated with numerous bioactivities (Karpova *et al*., 2016); Lindsay *et al*. 2023). Thus, the presence of these bioactive metabolites in the culture filtrate of endophytic *Fusarium* is an indication that endophytic fungi can be a potential source of essential phytochemicals

with applications in agriculture, cosmetics, and pharmaceutical industries.

The results of the present study are in agreement with that of Hawar (2022) and Raghav *et al*. (2022) who reported that endophytic fungi isolated from medicinal plants are potential producers of extracellular enzymes such as amylase, protease, *et cetera*, with therapeutic and biotechnological applications. The enzymes, amylase and protease can be used in medicine for treating cancer and digestive disorders, food industry, for preparation of animal feed, and in detergent, paper and textile industries. Protease are useful for the treatment of blood clotting, diabetes and wounds as well as biofilm degradation (Bezerra *et al*., 2021; Matias *et al*., 2021; Hawar 2022).

Conclusion

The study has shown that endophytic *Fusarium* species isolated from the leaves of *S. alata* and *M. hirtus* produced important secondary metabolites which inhibited the growth of *Staphylococcus aureus*. The isolated fungal endophytes also produced enzymes such as amylase, and proteases which may be useful in cosmetics and pharmaceutical industries.

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Conflict of interests

The authors declare that there is no known conflict of interest.

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