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Screening of Secondary Metabolites and Antagonistic Activity of Endophytic Fungi of Selected Medicinal Plants in Western Kenya

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ABSTRACT

Endophytes mimic biochemical pathways of their host plants to synthesize secondary metabolites with antimicrobial properties. Anti-phytopathogenic activities and secondary metabolites of fungal endophytes from some medicinal plants have been reported. However, there is scanty information on the antifungal properties and secondary metabolites of *Carissa edulis, Microglossa pyrifolia* and *Stegonataenia araliacea.* Roots, stems and leaves of the three plants were collected from western Kenya, processed and cultured on potato dextrose agar to recover endophytic fungi. Antagonistic activity of endophytic fungi against *Cercosporae zeae maydis* and *Fusarium verticillioides* was determined by dual culture technique. Secondary metabolites were determined from endophytic fungi ethyl acetate extracts using standard protocols. Fifteen fungal endophytes recovered from the three plants had varied growth inhibition percentage against *C. zeae maydis* and *F. verticillioides.* Mean percentage inhibition of the isolates against the pathogens was not significantly ($p>0.05$) different but the highest mean inhibition percentage against *Cercosporae zeae maydis* and *Fusarium verticillioides* was from DSTS2 (72.67%) and DSRR2 (73%) respectively. Tannins and alkaloids were present in all fungal extracts while saponins were absent. The inhibitory effects of endophytic fungi against *Cercosporae zeae maydis* and *Fusarium verticilloides* are attributed to their ability to synthesis secondary metabolites with antimicrobial properties. Fungal endophytes from these medicinal plants should be exploited for the management of fungal pathogens of maize.

Keywords: Endophytes, Antagonistic, Econdary metabolites, *Fusarium verticillioides*, *Cercosporae zeae maydis*, Medicinal plants

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INTRODUCTION

Endophytes, including bacterial, fungal, and actinomycete species, quietly inhabit healthy plant tissues, both intercellularly and intracellularly, without causing any visible signs of disease (Musyimi et al. 2021; Emitaro et al. 2022). This relationship benefits both the endophytes and their host plants, as they engage in a symbiotic exchange of nutrients (Li et al. 2022; Mejia et al. 2009). Endophytes enhance the plant's nutrient uptake, fostering robust growth and development. They are

widespread, colonizing virtually all plant species and tissues, with their presence observed across a broad spectrum of plants studied to date (Saaikkonen et al. 2010). Their association with plants can be either obligate or facultative, yet they pose no harm to their host plants.

Endophytes from medicinal plants inhibit the growth of plant pathogens a trait mirrored from the host plant due to their ability to synthesize secondary metabolites. Recent studies have delved into the colonization patterns of vegetative tissues by endophytes and their impact on plant growth, showcasing their antagonistic effects against plant pathogens like *Xanthomonas campestris pv. Musacearum* (Emitaro et al. 2021). However, there remains a gap in our understanding regarding the antagonistic potential of endophytic fungi residing within *Carissa edulis, Microglossa pyrifolia*, and *Steganotaenia araliacea* against plant pathogens such as *Cercosporae zeae maydis* and *Fusarium verticillioides,* which are notorious for causing maize loss. These pathogens are transmitted through seeds and pose significant threats, leading to the deterioration of grain quality, decreased germination rates, and diminished vigor (Agrios, 2005). Despite the availability of various natural and synthetic products to combat fungal pathogens, the rise of resistant fungi underscores the necessity of discovering novel sources of antifungal compounds.

Research has demonstrated that the biological functions of endophytes can be attributed to specific secondary metabolites that they produce (Ogbe et al. 2020). These metabolites encompass a wide range of compounds including alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavonoids, peptides, and phenols, as found in fungal extracts (Zhao *et al.* 2010). Such bioactive compounds hold significant value across pharmaceuticals, environmental applications, agriculture, and industries (Egamberdieva *et al.* 2017). However, limited information is available regarding the secondary metabolites produced by endophytic fungi residing within *Carissa edulis, Microglossa pyrifolia*, and *Steganotaenia araliacea* plants. Such knowledge gaps are particularly significant in identifying antagonistic activities against pathogens like *Cercosporae zeae maydis* and *Fusarium verticillioides* which continue to threaten maize yields and compromise food security. Therefore, there is a pressing need for further research to elucidate the secondary metabolites of endophytic fungi associated with these medicinal plants. Therefore, the objective of this study was to investigate the antagonistic abilities of endophytic fungi isolated from the three indigenous Kenyan medicinal plants—*Carissa edulis, Microglossa pyrifolia*, and *Steganotaenia araliacea* and the variety of secondary metabolites production by these endophytes.

MATERIALS AND METHODS

Study site, sampling and processing

Plants material of *Carissa edulis, Microglossa pyrifolia* and *Steganotaenia araliacea* were collected from parts of Kakamega, western Kenya, 0° 17' 3.19'' N and 34° 45' 8.24'' E. Leaves, stems and roots were collected in different cloth bags. Identification of plants was done at Maseno Botanical Garden. Laboratory experiments were carried out at JOOUST microbiology laboratory. Maize for pathogens *Cercosporae zeae maydis* and *Fusarium verticillioides* were isolated from maize fields showing symptoms of the pathogens around Jaramogi Oginga Odinga University of Science and Technology.

Endophytic Fungi Isolation

Isolation of fungal endophytes was carried out according to the procedure developed by Mahadevamurthy et al. (2016). Roots, stems, and leaves of *Carissa edulis, Microglossa pyrifolia,* and *Steganotaenia araliacea* were separately obtained from cloth bags and transported in a cooler box to the laboratory for identification. Roots were washed with running tap water to remove soil attached to them. Stems, roots, and leaves of each plant species were cut separately and dipped in 70% ethanol for 3 minutes then washed with 4% fresh sodium hypochlorite solution for about 5 minutes and finally washed five times with sterilized distilled water. Fungal endophytes were isolated by plating 3-5cm pieces of each plant part separately on Potato dextrose agar plates amended with streptomycin (1.0 g/L) to inhibit bacterial growth. Plates were sealed with parafilm and incubated at 25 ± 2°C for 7 days in a completely randomized design. The endophytic fungi colonies that emerged from the plant host were picked with sterile fine-tip needles and were subcultured on fresh PDA plates without antibiotic to get pure cultures for identification and subsequent procedure.

Isolation of fungal plant pathogens

Diseased maize leaves with specific symptoms of *Cercosporae zeae maydis* and *Fusarium verticillioides* were identified based on field identification manual (CIMMYT 2004), collected and processed according to Khaiyam et al. (2017). Samples were washed thoroughly under running tap water, surface sterilized with 4% NaOCl, rinsed several times with distilled water and blotted to dry. Leaf samples were cut into pieces of approximately 4 cm and placed on sterile moist blotter in a sterile petri dish and incubated at 25°C for 5 days to allow the pathogen to develop and sporulate in growth cabinets under a 12 hour fluorescent light/dark regime. Plant sections were then examined under a binocular microscope Leica DM 500 for the presence of conidia. Conidia were picked with an isolation needle and plated on PDA in triplicates to obtain pure cultures. Plates were incubated at 25°C for 5-7 days and hyphal tips from the advancing colony margins were transferred onto PDA with isolating needle as pure culture and kept at 4°C.

Morphological characterization of *Cercosporae zeae maydis* colonies on PDA; the mycelial colour was grey, light, brown corn silk and white on top and dark grey, brown, corn silk, and grey on the bottom. Morphological characterization of *Fusarium verticillioides*; the colour of the colony varied from white to purple and the type of mycelium from aerial to compact. On PDA, isolates formed aerial mycelia and produced pigmentation that varied from dark violet or dark magenta on PDA media (Leslie and Summerell, 2008).

Pathogenicity* test of *Cercosporae zeae maydis* and *Fusarium verticillioides

This was done according to Shemnkande *et al.* (2023). Five local maize seeds "rachar" were hand sown in nine 30 cm pots containing sterilized soil and kept in greenhouse. Di-ammonium phosphate fertilizer was applied during planting at the rate of 1.5 g/pot. Calcium ammonium nitrate was applied at the second leaf (V2) growth stage at the rate of 2.5 g/pot. Experimental pots were arranged in a randomized complete block design. Fungal isolate was inoculated on fresh PDA and incubated in darkness for 9 days to induce sporulation. Conidial suspension was prepared by adding 5ml of sterile distilled water onto fresh cultures, then straining suspension between two layers of cheesecloth, and conidia concentration was adjusted to 2×10^4 conidia/ml in a spectrophotometer at 460 nm. Four plants were inoculated in triplicates at the sixth leaf (V6) growth stage by spraying the conidia suspension of the pathogens using a hand sprayer until runoff. Inoculated plants were covered with transparent plastic bags for 5 days for maximum humidity.

Growth inhibition potential of endophytic fungal isolates against Cercosporae zeae maydis and Fusarium verticillioides

The growth inhibition activity of isolated endophytic fungi against fungal pathogens was determined using dual culture method (Katoch and Pull, 2017). Discs of isolated endophyte and pathogen measuring 0.5mm were co-cultured at two equidistant opposite ends of PDA plates, sealed with parafilm and incubated at 25±2°C for 7 days. Plates inoculated with fungal pathogen disc at the centre without endophyte served as control. The experiment was replicated thrice with plates arranged in a completely randomized design. Radial growth of pathogenic fungi in the presence and absence of the endophyte was measured after 7 days, and growth inhibition percentage was calculated using the formular

Growth inhibition% = CDC-CDT/CDC *100
Where CDC – represents the colony radial growth of the pathogen in mm on the control plate
CDT- represents the colony radial growth of the pathogen in mm in the treatment plates.

Extraction of Endophytic Fungal Metabolites

Endophytic fungi isolated from all the plant parts were each inoculated in a 500 ml conical flask containing 250 ml of Potato Dextrose broth according to Al-mahi Idris *et al.,* (2013) and incubated for 7 days at 25±2°C. The broth was then filtered and mixed with 250 ml ethyl acetate in a 500 ml conical, shaken to mix and transferred to a separating funnel, and left to stand for 15 minutes. After the solutions were separated, the lower one having PDB was discarded and the upper one was collected and evaporated in a rotor evaporator at 45°C to dryness. The extracts were reconstituted in DMSO for secondary metabolite determination.

Screening endophytic fungal extracts for secondary metabolites

Test for Steroids

2ml of chloroform and 2ml of concentrated H2SO4 was added together with 5ml aqueous fungal crude extract and then heated. The formation of a brown ring indicated the presence of steroids (Setyawati et al. 2019).

Test for Terpenoids

Two milliliters of chloroform was mixed with 5 ml aqueous fungal extract and heated in water bath and then boiled with 3 ml of concentrated H_2SO_4. Formation of grey colour indicated presence of terpenoids (Bhandary et al. 2024).

Test for Saponins

Five milliliters of test solution was mixed with 5ml of water, shaken vigorously, and observed for the formation of foam, which was stable for 15 minutes for a positive result (Gul et al. 2017).

Test for Alkaloids

Presence of alkaloids was determined according to Sheel et al. (2014). Five milliliters of fungal extract was added to Dragendorff's reagent. Appearance of orange red precipitate indicated a positive result.

Test for Flavonoids

An alkaline reagent test was carried out where 2 ml of 2.0% NaOH was mixed with 5 ml aqueous fungal crude extract. A concentrated yellow colour was produced, which became colourless when 2 drops of diluted H2SO4 acid were added. Colourless appearance indicated the presence of flavonoids. (Gul et al. 2017) while persistence of the yellow colour indicated absence of flavonoids.

Test for Tannins

Presence of tannins in the fungal extract was determined by Ferric Chloride Test method as described by Sheel et al. (2014). 50mg of the extract was boiled with 5 ml of 45% solution of ethanol for 5 minutes, cooled and filtered. 1ml of filtrate was diluted with distilled water in a ration of 1:1 and two drops of ferric chloride added. A transient greenish-to-black colour indicated the presence of Tannins.

Data analysis

Data on percentage inhibition of the endophytes was subjected to one way analysis of variance (ANOVA) and means separated by least significant difference at $p \leq 0.05$.

RESULTS

There was no significant difference ($p > 0.05$) in the antagonistic potential of fungal isolates against *Cercosporae zeae maydis* and *Fusarium verticilloides* regardless of the plant species and plant part. Endophytic fungal isolates from Microglossa pyrifolia exhibited varied growth inhibition percentages against the two pathogens (Plate 1 & 2). The highest mean inhibition percentage against *Cercosporae zeae maydis* and *Fusarium verticillioides* was from DENS4 from stem at 67 % and 62 % respectively. The lowest growth inhibition against *Cercosporae zeae maydis* and *Fusarium verticillioides* was produced by isolate DENL2 (40%) and DENR1 (48%) from the leaf and root (Table 1).

Fungal endophytes isolated from stems and leaves of *Steganotaenia aralicea* inhibited the growth of *Cercosporae zeae* maydis and *Fusarium verticilloides* with no significant difference Table 2. Isolate DSTS4 from the stem produced the largest inhibition percentage (76%) against *Cercosporae zeae* maydis followed by isolate DSTS2 (69%) also from the stem. Highest inhibition percentage against *Fusarium verticilloides* was produced by DSTL2 (64.67%) from the leaf followed by DSTS2 (63.3%) from the stem. *Carissa edulis* had only two isolates which also inhibited the growth of the two pathogens without no significant difference. Isolate DSRR2 was more active against *C. zeae maydis* and *F. verticilloides* compared to isolate DSRL3.

Screening of Secondary metabolites of the fungal endophytes extracts

All tested endophytic isolate extracts exhibited positive results for tannins and alkaloids (Plate 3) but negative for saponins regardless of the plant species or plant part from which they were extracted (Plate 2 and Table 3). Terpenoids and flavonoids were detected in extracts from six fungal isolates: DENL1, DENS4, DENR2, DSTL1, DSTS5 and DSRL2. Among these, DENL1, DENS4 and DENR2 were isolated from the leaves, stems and root of *Microglossa pyrifolia*, DSRL2 from *Carissa edulis* leaves while DSTL1 and DSTS5 were from leaf and stem of *S. araliacea* (Table 4). Isolate DENS3 and DSRR2, tested positive for

terpenoids but negative for flavonoids and were isolated from the stem of M. pyrifolia and root of C. edulis. Conversely, DENR1 extract did not exhibit the presence of terpenoids and flavonoids. Ten isolates tested positive for flavonoids were terpenoids (DENL2, DENS1, DENS2, DENR3, DSTR1, DSTS1, DSTS2, DSTS3, DSTS4, and DSRL3).

Table 1: Inhibition % of fungal isolates from *Microglossa pyrifolia* against *Cercosporae zeae maydis* and *Fusarium verticilloides*

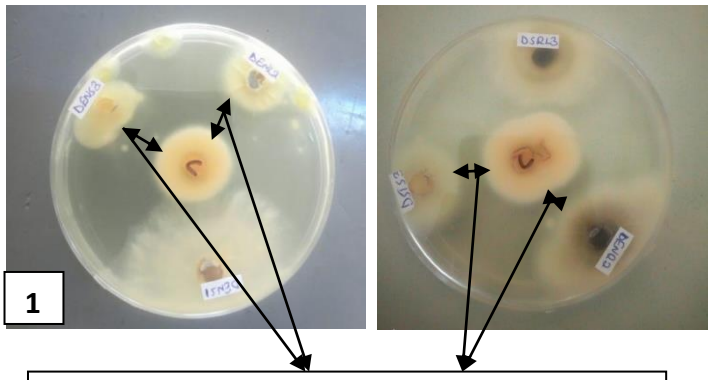
Pathogen	Fungal isolates from *Microglossa pyrifolia*							
	Leaf	Root			Stem			P value
	DENL2	DENR1	DENR2	DENR3	DENS1	DENS3	DENS4	
C. zeae maydis	40[a]	54[a]	40.67[a]	49.67[a]	51[a]	40.33[a]	67[a]	0.75
F. verticilloides	61.67[a]	48[a]	61.33[a]	62[a]	60[a]	49.67[a]	62[a]	0.89

Inhibition % followed by same super script letter is not significantly different

Table 2: Inhibition % of fungal isolates from *Steganotaenia aralicea* and *Carissa edulis* against *Cercosporae zeae maydis* and *Fusarium verticilloides*

Pathogen	Fungal isolates from *Steganotaenia aralicea*							Fungal isolates from *Carissa edulis*		
	Leaf		Stem				P	Leaf	Root	P
	DSTL1	DSTL2	DSTS2	DSTS3	DSTS4	DSTS5	value	DSRL3	DSRR2	value
C. zeae maydis	50[a]	63.33[a]	69[a]	41.67[a]	76[a]	46.67[a]	0.32	51.67[a]	59.33[a]	0.68
F. verticilloides	53.33[a]	64.67[a]	63.33[a]	55.67[a]	59.33[a]	52.33[a]	0.94	59.33[a]	73[a]	0.26

Inhibition % followed by same super script letter is not significantly different



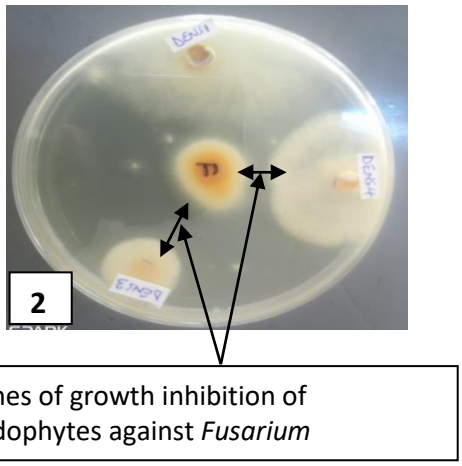


Plate 1 and 2: Zones of inhibition against *C. zeae maydis* and *F. verticilloides*

DISCUSSION

This study findings shows that fungal endophytes isolated from leaves, stems and roots of *Steganotaenia araliacea, Carissa edulis* and *Microglossa pyrifolia* possess a heightened ability to inhibit the growth of *Cercosporae zea maydis* and *Fusarium verticilloides* which are important fungal pathogens of maize. The inhibition of growth could be attributed to their efficient utilization of nutrients in the media, enabling faster synthesis and secretion of antifungal compounds. The selection of endophytes by each plant is influenced by various environmental factors, both abiotic and biotic which also determine the synthesis of potent antifungal chemicals (Hamzah et al., 2018). The prevalence of host medicinal plants like *Steganotaenia araliacea* in environments with low air humidity, such as riverine

and forest edges or open woodlands, affects the diversity of soil microorganisms recruited as endophytes (Opande, 2022) which could have influenced the composition of secondary metabolites synthesised that contains antifungal activities.

Table 4: Secondary metabolites from endophytic extracts

ISOLATE	SAPONNINS	TANNINS	FLAVONOIDS	TERPENOIDS	ALKALOIDS
DSTL1	-	+	+	+	+
DENS3	-	+	-	+	+
DSTS4	-	+	+	-	+
DSTR1	-	+	+	-	+
DSTS2	-	+	+	-	+
DENR3	-	+	+	-	+
DSTS3	-	+	+	-	+
DENS2	-	+	+	-	+
DENL2	-	+	+	-	+
DSRR2	-	+	-	+	+
DENL1	-	+	+	+	+
DENR1	-	+	-	-	+
DENS1	-	+	+	-	+
DSRL3	-	+	+	-	+
DSTS1	-	+	+	-	+
DSRL2	-	+	+	+	+
DENR2	-	+	+	+	+
DSTS5	-	+	+	+	+
DSTL2	-	+	+	+	+
DENS4	-	+	+	+	+

Key: DSR- Carissa edulis DEN- Microglossa pyrifolia DST- Steganotaenea aralicea L- Leaf, S- Stem and R-Root

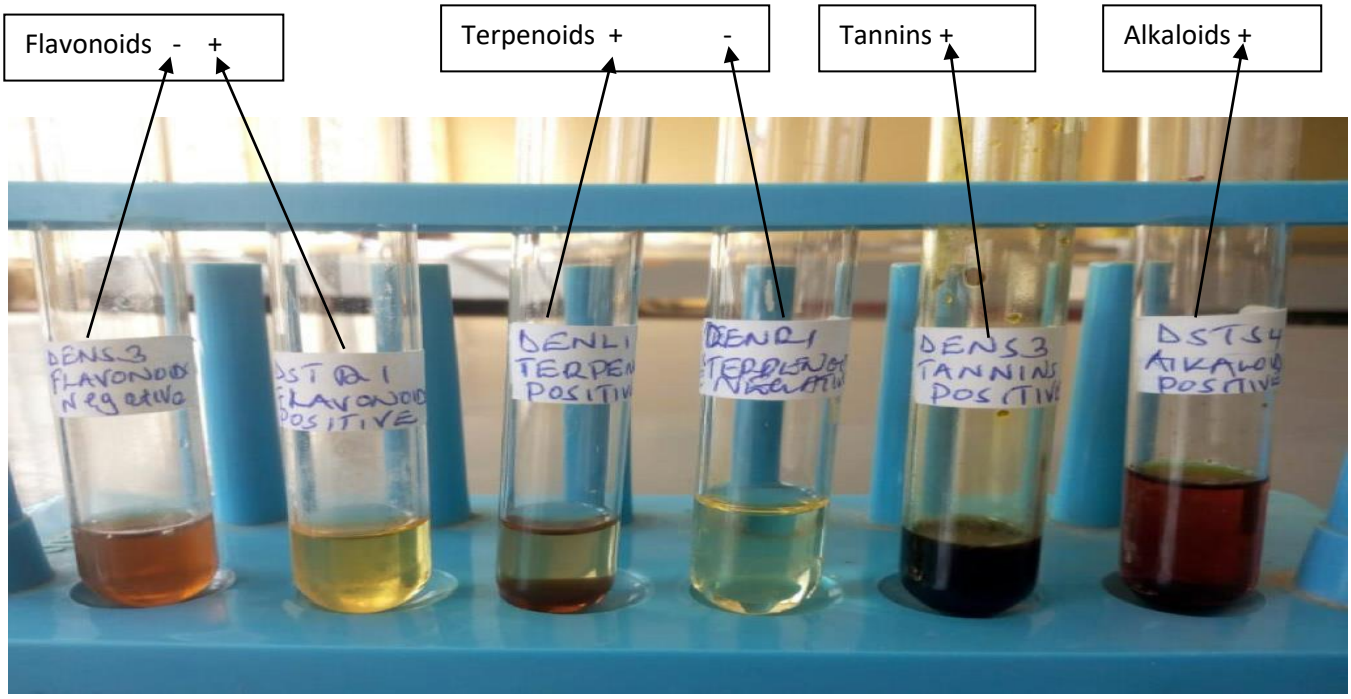


Plate 3: Tubes showing secondary metabolites from endophytic extracts

The inhibitory effects of endophytic fungi against *Cercosporae zeae maydis* and *Fusarium verticilloides* are attributed to their ability to synthesize extracellular enzymes and antifungal compounds in the growth media. These results are in agreement with Grabka et al. (2022) who investigated their role in pests and pathogens management. These compounds, such as volatile oil, n-butanol, and ethyl acetate, inhibit pathogen growth by various mechanisms, including hydrolyzing pathogen proteins and presence of biocontrol genes in some endophytic fungi (Adeleke et al., 2022), which could have been the same mechanisms used in this case.

Notably, endophytic fungi isolated from stems exhibited the highest growth inhibition potential against *Cercosporae zeae maydis*, possibly due to the storage of different secondary metabolites with antifungal properties in the stem. Stems, being prone to attack by feeding insects that may introduce pathogens, harbour more endophytes capable of synthesizing protective chemicals against fungal pathogens (Zaynab et al. 2018). In contrast, leaves contained fewer endophytic fungal isolates with growth-inhibiting abilities. This could be attributed to the fact that endophytes in leaves often prioritize synthesizing chemicals to deter herbivory rather than microbial attacks. The bioprospecting of fungal endophytes associated with medicinal plants holds significant promise in agriculture, primarily due to the presence of secondary metabolites in their crude fungal extracts (Raghav *et al.* 2022; Toppo *et al.* 2023).

The secondary metabolites screening from fungal endophytic extracts revealed presence of Tannins, alkaloids, flavonoids, terpenoids and absence of saponins in the endophytic fungi. The presence of most secondary metabolites could be attributed to the medicinal values of the host plants which are mimicked by the endophytic fungi. Similarly the presence of these secondary metabolites could be because host plants are prone to attack by insects, microbial pathogens and other animals of which they have evolved mechanisms of protecting the host plant against such. These results are in agreement with the report by Milugo et al. (2013) that presence of alkaloids and terpenoids in the absence of saponins confer strongest pathogen inhibition. These results were contrary to those reported by Dawa et al. (2021) in *Carissa edulis* fungal extracts contain saponins. Difference in the secondary metabolites detected in *C. edulis* could be attributed to difference in solvents used in extraction as he used hexane while in this study, ethyl acetate was used. Also, the difference could be because these plants occupied different ecological zones.

Our findings corroborate these previous studies (Chakraborty et al. 2021; Wang et al. 2023; Dawa et al. 2021), highlighting the consistent presence of alkaloids, flavonoids, phenols, terpenoids, and sterols in various fungal extracts.

Some of the fungal extracts tested positive for all the secondary metabolites and negative for saponins. This could be probably because the endophytic fungi and their host medicinal plants could be having a wide range of microbial pathogens, therefore the need to confer protection to the plants. These could also be attributed to other ecological functions of the metabolites such as symbiotic interactions with their host plants and endophytes, competition by other microbes and defense against predation. These results are in agreement with reports from Clemensen et al. (2020) that secondary metabolites have ecological implications that enhance agricultural sustainability. According to reports by Zaynab et al. (2018), secondary metabolites prominently function in protecting plants against microbes and insect attacks and therefore applicable in controlling fungal pathogens and insects. Similar reports have been recorded by Demain and Fang (2000) that secondary metabolites function as competitive weapons against pathogens and agents of symbiotic interactions between microbes, plants, and other organisms.

The presence of alkaloids and the absence of saponins could be attributed to the use of these plants for medicinal purposes which have been exhibited in endophytes to inhibit the growth of *Cercosporae zeae maydis* in this study. These results are in agreement with reports by Milugo et al. (2013) that alkaloid-saponin interaction significantly reduced antagonistic activities as recorded in the antagonistic activity in extracts from *Rauvolfia caffra* but the presence of alkaloids in the absence of saponin had higher inhibition activity. Presence of alkaloids in the extracts indicates that these fungi synthesize bio compounds with diverse activities such as pharmacological characteristics which include antimicrobial and anti-inflammatory effects, which suggests their applications in agriculture, medicine and other industrial uses (Behar et al. 2024).

The presence of flavonoids in some endophytic extracts could also have contributed to their antifungal properties. Flavonoids can crosslink microbial enzymes, and inhibit secretion of cellulases and other microbial enzymes which may act as a physical barrier during pathogen attack (Das et al. 2024). Detection of terpenoids in the extracts could be attributed to pathogenic attacks on host plants which activated the recruitment of endophytes with the ability to synthesize terpenoids by the host medicinal plant. Several terpenoids have their roles in plant defense against biotic and abiotic stresses or they are treated as signal molecules to attract the insects of pollination (Singh and Sharma, 2015).

CONCLUSION

The present study establishes a significant platform for exploring the potential of endophytic fungi derived from *Carissa edulis, Microglossa pyrifolia* and *Steganotaenia araliacea* plants. These endophytes exhibit promising traits such as the production of bioactive secondary metabolites, facilitation of plant growth, and the secretion of extracellular enzymes. The secondary metabolites screening from fungal endophytic extracts revealed presence of Tannins, alkaloids, flavonoids, terpenoids and the absence of saponins. The presence of most secondary metabolites was attributed to their antagonistic activities against plant pathogens since most secondary metabolites are known to possess antagonistic characteristics. Leveraging these fungal endophytes offers a sustainable and environmentally friendly approach to supporting crop development, particularly crucial in the context of contemporary challenges posed by climate change.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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