



Bioprospecting of Hyoscyamine Alkaloid and Other Secondary Metabolites Production by Some Fungal Endophytes Isolated from *Hyoscyamus muticus*



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HYOSCYAMUS *muticus* L. (Egyptian Henbane), a shrub thriving in the sandy regions of Egypt, shows important pharmacological properties which may be attributed to its own bioactive compounds or due to associated endophytes. In the present study, roots, stems and leaves of *H. muticus* growing in Egypt were evaluated for the presence of associated fungal endophytes, and secondary metabolites produced from them especially the characteristic plant tropane alkaloid; hyoscyamine. Using culture-dependent approach, sixteen fungal endophytes belonging to five different genera were identified morphologically according to their culture appearance and detailed microscopic structures. The isolated endophytic fungi were cultured using three different media (PDB, RG and SDB) and each filtered broth was sequentially extracted with ethyl acetate. Crude extracts collected from different fungal isolates were subjected to phytochemical analysis using thin layer chromatography (TLC) which showed a distinct fingerprinting profile of several fungal secondary metabolites. The total secondary metabolite, phenolic compound, flavonoid, anthraquinone glycoside, coumarins and alkaloid content was investigated. Different activities of the used media were reported on the changing secondary metabolic profile of each fungus. Furthermore, the ability of the tested fungal endophytes to produce hyoscyamine, characteristic to *H. muticus* was tested by TLC analysis. Some isolated endophyte species belonging to *Aspergillus* and *Penicillium* showed positive tests for hyoscyamine.

Keywords: Egyptian Henbane, Fungal endophytes, Fungal identification, TLC, Tropane alkaloids.

Introduction

Hyoscyamus muticus L. (Egyptian Henbane) plant (F: Solanaceae) is a shrub growing in the sandy soils of Egypt (Wallis, 2005). It is characterized by high economic importance due to its content of bioactive alkaloids such as tropane alkaloids (hyoscyamine and hyoscyne) which exhibit mydriatic, anticholinergic, antispasmodic, sedative and hypnotic medical benefits (Batanouny et al., 1999; Griffin & Lin, 2000; Nassar et al., 2016).

Endophytes have been defined as microorganisms that live and survive asymptotically inside plant tissues for all or part of their life without causing any apparent damage

or diseases (Petrini et al., 1993). Many studies suggested that endophytes play a remarkable role in host protection against predators and pathogens (Azevedo et al., 2000). In this case, the endophytic fungi establish a mutualistic symbiosis relationship within the living tissue of the host plant where they can synthesize useful bioactive compounds used as a defense mechanism by the plant against pathogenic microorganisms (Schulz et al., 2002; Strobel, 2003; Corrado & Rodrigues, 2004; Owen & Hundley, 2004; Gimenez et al., 2007). However, endophytes may produce bioactive compounds like those of the host plant including flavonoids, coumarins, alkaloids, lignans, terpenoids, quinones phenylpropanoids, glycosides, saponins

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and xanthenes (Singh et al., 2021; Sehim & Dawwam, 2022). Also, the secondary metabolites derived from fungal endophytes exhibited therapeutic properties such as antioxidant agents, cholesterol inhibitors, anticancer, antimicrobials and antidiabetics (Strobel, 2003; Abdel-Aziz et al., 2018; Hamed et al., 2018; Eskander et al., 2020). Moreover, in nature there are several endophytic fungi harbored by *H. muticus* plants and displayed potential biocontrol agents against some phytopathogenic fungi and can produce many important secondary metabolites including alkaloids side by side with those of the plant itself (Abdel-Motaal et al., 2010; El-Zayat et al., 2008).

Accordingly, this study was undertaken to isolate and identify the endophytic fungi which inhabit different parts of *H. muticus* plant. Also, the effect of different growth media on the diversity of the fungal content of bioactive secondary metabolites evaluated such as hyoscyamine was investigated.

Materials and Methods

Collection of plant samples

The medicinal shrub, *H. muticus* used in the present study was cultivated in the medicinal plant farm, Faculty of Pharmacy, Tanta University, Egypt. Fresh, intact, and healthy leaves, stems and roots of the plant were collected in June and August 2021, January and May 2022 and used to isolate different harboring endophytic fungi.

Isolation of endophytic fungi

Media preparation

The used media were prepared according to the methods mentioned in Moubasher (1993).

Potato dextrose agar medium (PDA): This medium consists of potato tubers (200g), glucose (20g), agar (20g) and dist. H₂O (1L). Tubers of potatoes were peeled, cut to small pieces, and boiled for about one hour in one liter of distilled water. The potato pieces were mashed and squeezed through a fine sieve and thereafter glucose and agar were added. The mixture was completed to one liter by distilled water.

Sabouraud dextrose agar medium: Peptone (10g), glucose (40g), and agar (20g) were dissolved in 1L dist. H₂O.

Rice grains (RG): This growth medium was prepared as previously reported (Elkhouly et al., 2021). In detail, 100g clean washed rice grains were added to 100mL distilled water and 0.5g

NaCl in 250mL flasks and sterilized in autoclave.

First isolation process of endophytic fungi from the plant parts was carried out on June 13th, August 16th, 2021 and January 1st, 2022 where the work steps were directly done at the same time of collection from the farm according to a modified method (Tapfuma et al., 2019). Firstly, about 10g of each plant part (root, stem and leaf) was separately washed with running tap water for five minutes, followed by sterilized distilled water. Then soaked in 5% tween 80 with vigorous shaking for 5min. This was followed by washing the samples several times with sterile distilled water to completely remove tween 80. After that, samples were sterilized in 70% ethanol for 1min, washed with sterile distilled water five times followed by 1% sodium hypochlorite for 10min and finally washed several times with sterile distilled water. Control plates were prepared by cultivation of about 5 water drops from the final washing step on PDA plates to confirm the efficiency of surface sterilization process of plant parts. Then, the surface sterilized plant parts were mashed separately in 5mL sterile phosphate buffered saline (PBS) (Oxoid, Basingstoke, Hampshire, UK) using sterile mortar and pestle. Different dilutions (10⁻¹, 10⁻² and 10⁻³) were prepared and consequently cultivated on PDA plates supplemented with 250mg/L chloramphenicol antibiotic to prevent bacterial growth.

The second method of fungal isolation from fresh plant parts was performed in May 2022 according to a modified method (Elkhouly et al., 2021) The steps resemble those of the previous method except in cutting the plant parts to small segments (1cm³) after surface sterilization instead of mashing in saline. The plant segments were cultivated separately using PDA plates containing chloramphenicol. Then all the cultivated plates were incubated at 28°C for 7 days till fungal growth. Thereafter, each fungal species was sub-cultured and purified using new PDA plates. Pure cultures from all the isolated endophytic fungal species were clearly examined and identified morphologically and microscopically according to the identification keys cited in the literature (Ellis, 1971; Moubasher, 1993; Samson et al., 2011; Short et al., 2013). The identification process relied on the colony appearance (color, texture and bottom reverse) and microscopic examination of hyphae, conidia, conidiophores. using binocular biological light microscope (Model: XSZ-107BN) at different magnification powers (40, 100, 400

and 1000 ×).

Investigation of fungal secondary metabolites

Used reagents

The following color reagents were used for detection of different secondary metabolites in the fungal residues prepared according to reported methods (Stahl, 1962; Pascual et al., 2002). They include Dragendorf's, aluminum chloride (AlCl_3), ferric chloride (FeCl_3), potassium hydroxide (KOH), anisaldehyde sulfuric acid reagent and sulfuric acid reagents.

Preparation of fungal residues

This experiment was designated to detect the ability of the isolated endophytic fungi to produce hyoscyamine as well as some other types of fungal secondary metabolites such as phenolic compounds, flavonoids, coumarin and anthraquinone glycosides. Furthermore, the effect of different growth media on the production of these secondary metabolites by the tested fungi was also explained. For this purpose, sabouraud dextrose broth (SDB) and potato dextrose broth (PDB) media were used for cultivation of fungal species (Verma et al., 2011; Mane et al., 2018) in 500mL flasks. The inoculated flasks were incubated statically in dark at $28^\circ\text{C}\pm 2$ for 21 days for PDB and 10 days for SDB. After the incubation period, each culture flask was alkalized by adding 3-5 drops NH_3 , then the fungal mycelia were removed by filtration on cheese cloth. The separated fungal filtrates were extracted twice with equal volume of ethyl acetate, shaking vigorously for 10 min. The upper layer of ethyl acetate containing the fungal secondary metabolites was separated and dried in rotary vacuum evaporator to obtain a residue.

The RG growth medium was inoculated by the tested endophytes and incubated statically in dark at 30°C for 15 days. Then the grown fungal cultures were alkalized by 3-5 drops NH_3 , extracted twice with equal volume of ethyl acetate, shaking vigorously for 5min. After that, the rice grains and fungal mycelia were discarded by filtration and the filtrate was dried in rotary vacuum evaporator to afford a fungal residue.

Examination of different secondary metabolites by TLC

Different secondary metabolites including alkaloids especially hyoscyamine were detected in the previously prepared fungal residues using thin layer chromatography technique (TLC). For this purpose, 10mg from each residue was dissolved in 3mL mixture of chloroform: methanol (1:1

v/v). Separation process of different secondary metabolites was carried out using analytical TLC silica plates. From each fungal sample 300 μL , were spotted using capillary tube on a line about 1.5cm above the bottom edge of the silica plates. The TLC were developed using (CH_2Cl_2 : methanol: distilled water) 9:1:0.1 v/v or CH_2Cl_2 : methanol: Ammonia (8:2: few drops v/v) as mobile phase. The spots were visualized by Dragendorf's reagent. Other types of secondary metabolites such as glycosides and steroids were detected using other spray reagents. Different colored spots mainly violet and yellow using anisaldehyde sulfuric reagent then heating the TLC plates at $100\text{-}105^\circ\text{C}$ for about 1min till appearance of clear intense spots. Also, many colored spots were recognized with sulfuric acid reagent as general spraying detector of several types of secondary metabolites. FeCl_3 reagent was used for investigating phenolic compounds, AlCl_3 for flavonoids and KOH for anthraquinone glycoside and coumarins. Furthermore, ultraviolet light (UV) at short wavelength ($\lambda= 254\text{nm}$) and long wavelength ($\lambda= 365\text{nm}$) was used in examining the plates after development.

Results and Discussion

Morphological identification of endophytic fungi

Various fungal endophytes were isolated from different parts of *H. muticus* plant such as roots, stems and leaves. Initially, there was no microbial growth observed in the control PDA plates inoculated with water of the final surface-sterilization step of plant parts. This indicated that the obtained fungal isolates are endophytes hosted by different plant parts and proving that the surface-sterilization method was effective and successful. Thereafter, total of 16 endophytic fungal isolates were isolated in August 2021, January and May 2022 where the largest number of endophytic fungi was recorded from leaves (7 isolates) followed by the roots (5 isolates) and the smallest number was from stems (4 isolates). Table 1 and Fig. 1 show the isolated and identified fungal endophytes based on their cultural appearance features and the characteristic microscopic structures of each species according to different standard fungal manuals (Moubasher, 1993; Samson et al., 2011; Short et al., 2013; Walther et al., 2019). The identified fungal isolates were belonging to 5 fungal genera: 9 isolates of *Aspergillus*, 2 of *Alternaria*, 1 of *Fusarium*, 1 of *Lichtheimia* and 3 of *Penicillium*. There are three isolates belonging to *Lichtheimia* and *Penicillium*

were identified to genus name only and require molecular identification to recognize the species name. In addition, certain species namely *A. carbonarius* and *A. flavus* var. *columnaris* were the most abundant recorded species at all the time periods of isolation. Also, some similar isolates were obtained from different parts (Table 1). But obvious differences were found in the identified fungal isolates during different months of isolation which revealed that the time of isolation effectively influence the type of fungal endophytes. Consequently, the actual number of the identified endophytes was 12 different fungal species which were further selected for the investigation of different secondary metabolites.

Figure 1 shows detailed descriptions of macroscopic and microscopic features of the isolated fungal endophytes. *Aspergillus carbonarius* as a member of black Aspergilli is distinguished by producing copious amount of black colored spores. This species is characterized by long conidiophore which reached up to 5 mm, ended with spherical biserial vesicle. The conidia are globose, brown and conspicuously echinulate. On the other hand, culture of *Aspergillus flavus* var. *columnaris* is characterized by yellow green color and distinct columnar conidial heads which

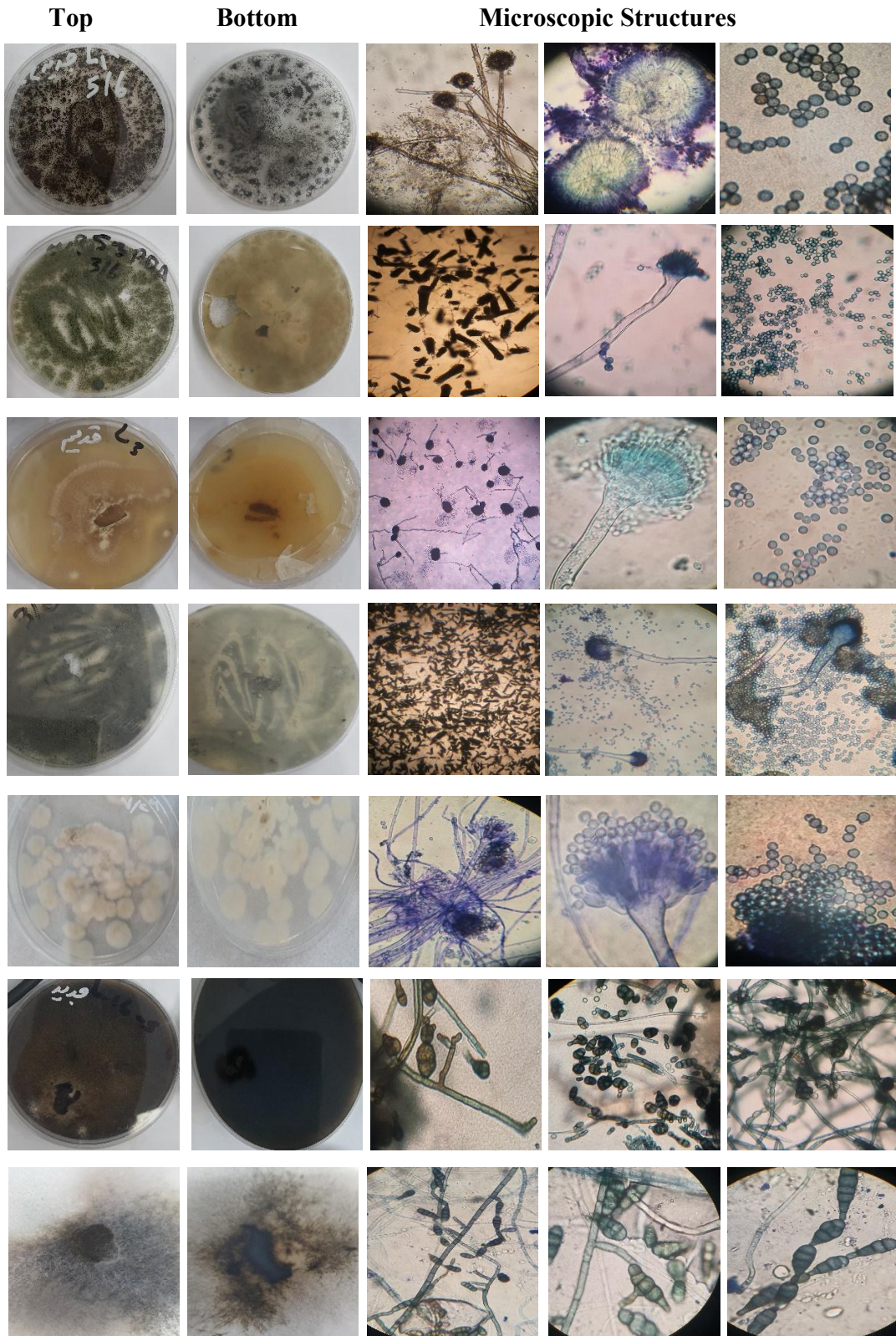
clearly appeared by low power microscopic lens. Vesicle is subglobose and fertile only on its upper half. Chains of smooth globose conidia were produced from conidiogenous cells which are born directly on the vesicle (Fig. 1). Culture of *Aspergillus terreus* var. *terreus* is buff tends to greyed orange color. Its smooth and colorless conidiophores ended with biserial dome shaped vesicles carrying compactly columnar chains of globose, subglobose and smooth walled conidia. While color of fast-growing colonies of *Aspergillus fumigatus* is dark smoky green. Tips of short conidiophores gradually enlarged to flask-shaped uniserial vesicles as shown in Fig. 1. Conidia are globose or subglobose with smooth walls. Colonies of *Aspergillus flavipes* are with pale buff color. Microscopically it is diagnosed by smooth conidiophores ended with ovate to elongate biserial and fertile at the upper part vesicles. Conidial heads radiate when young becoming columnar by age. Although Aspergilli were the most abundant species, but some other fungal species were also described. *Penicillium oxalicum* was characterized by greyed green colonies, smooth walled conidiophores, biverticillate asymmetrical penicilli and smooth elliptical conidia.

TABLE 1. The isolated endophytic fungi from *H. muticus*

	Month of isolation	Fungal isolate	Plant Part	Identification
1	August 2021	L ₁	Leaf	<i>Aspergillus carbonarius</i>
2	“	L ₂	Leaf	<i>Aspergillus flavus</i> var. <i>columnaris</i>
3	“	L ₃	Leaf	<i>Aspergillus terreus</i> var. <i>terreus</i>
4	“	L ₄	Leaf	<i>Penicillium oxalicum</i>
5	“	R ₂	Root	<i>Aspergillus flavus</i> var. <i>columnaris</i>
6	“	R ₃	Root	<i>Aspergillus terreus</i> var. <i>terreus</i>
7	“	R ₄	Root	<i>Lichtheimia</i> sp.
8	January and May 2022	L _{16s}	Leaf	<i>Alternaria chlamyospora</i>
9	“	L ₁ '	Leaf	<i>Aspergillus carbonarius</i>
10	“	L ₄ '	Leaf	<i>Aspergillus fumigatus</i>
11	“	R ₆	Root	<i>Fusarium keratoplasticum</i>
12	“	R _{16s}	Root	<i>Penicillium</i> sp.
13	“	S ₃	Stem	<i>Aspergillus flavus</i> var. <i>columnaris</i>
14	“	S ₅	Stem	<i>Aspergillus flavipes</i>
15	“	S ₆	Stem	<i>Alternaria alternata</i>
16		S1	Stem	<i>Penicillium</i> sp.

L: leaf R: root S: stem

Culture



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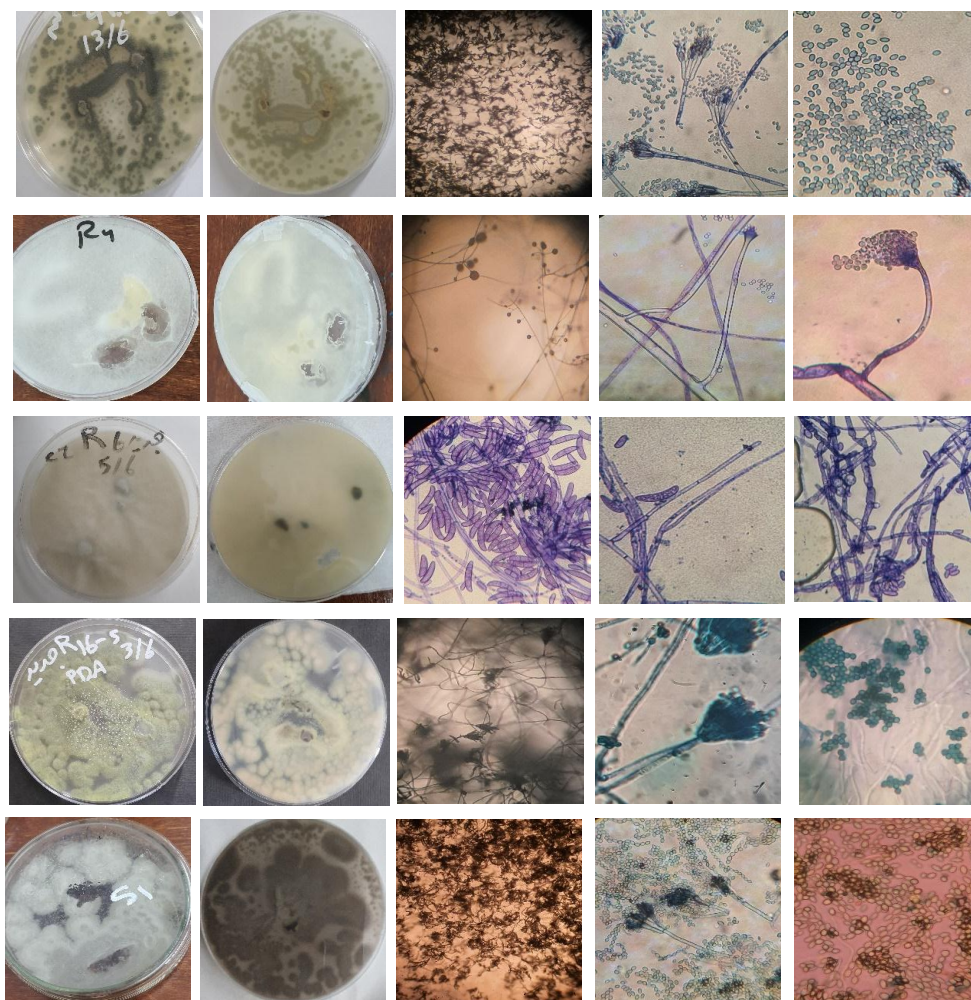


Fig. 1. The isolated fungal endophytes: species from top downwards; *Aspergillus carbonarius*, *Aspergillus flavus* var. *columnaris*, *Aspergillus terreus* var. *terreus*, *Aspergillus fumigatus*, *Aspergillus flavipes*, *Alternaria chlamydospora*, *Alternaria alternata*, *Penicillium oxalicum*, *Lichtheimia* sp., *Fusarium keratoplasticum*, *Penicillium* sp. and *Penicillium* sp.

Culture of the zygomycetous, *Lichtheimia* sp. are fast growing, floccose, white at first becoming pale grey. Sporangiophores are simple or sympodial branched, hyaline to faintly pigmented and arising solitarily or in groups. Sporangia are small and typically pyriform in shape with a characteristic conical-shaped columella and pronounced apophysis. Sporangiospores vary from globose, sub-globose to oblong-ellipsoidal (Fig. 1).

In our results, dematiaceous fungi are exemplified by *Alternaria alternata* and *Alternaria chlamydospora*. Colonies of the former species is greyish to olivaceous black color. Conidiophores are straight or curved, smooth and simple or branched. Conidia are varied in size, obclavate or long elliptical with beak which is equal one third

or one fourth of the conidial length. Cultures of *A. chlamydospora* are dark grey or dark olive to blackish colored. This species is characterized by its obclavate or obpyriform conidia.

Fusarium keratoplasticum is a member of *Fusarium solani* species complex. Its mature macroconidia are three septate and produced from sporodochia. While oval-shaped and curved cylindrical microconidia are formed on long phialides. Chlamydospores are formed terminally and intercalary (Fig. 1).

Numerous studies introduced fungal endophytes isolated from several medicinal plants and identified based on their morphological characteristics. In this study, the resulted endophytic

fungi were in accordance with those previously identified by El-Said et al. (2016) who reported that *Aspergillus*, *Alternaria* and *Penicillium* were found to be the most abundant genera isolated from *Datura innoxia* and *H. muticus*. Relevant results were obtained by other study indicated that the genus *Aspergillus* is the most abundant endophytic fungus isolated from *H. muticus* grown at extreme hot and aired weather of Egyptian southern desert at Aswan city which differed to that of Tanta city (El-Zayat et al., 2008). Moreover, Elkhoully et al. (2021) isolated *Aspergillus tubenginses* from *H. muticus* plant and found that its ethyl acetate extract showed antimicrobial, antibiofilm, antioxidant and anticancer activities. Also, fungal endophytic species, *Alternaria alternata*, *Alternaria* sp., *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium* sp. were reported from leaves and bark of *Tabebuia argentea* tree (Govindappa, 2014). While other fungal genera which were completely different from our identified genera were found associated with *Vernonia amygdalina* plant parts. These genera are *Phomopsis* sp., *Phoma* sp., *Lasiodiplodia* sp., *Colletotrichum* sp., and *Phyllosticta* sp. (Praptiwi et al., 2020).

Detection of various fungal secondary metabolites Hyoscyamine alkaloid

Production of several types of alkaloids by the tested endophytic fungi has been established by appearance of numerous reddish spots observed with dragendorff's reagent on TLC plates (Table 2). Certain tested fungal endophytes exhibited reddish orange spots with the same Rf of the authentic hyoscyamine. Our results were consistent with other studies revealed that TLC is the method of choice for routine alkaloid analysis before further sophisticated instrumental analysis is performed. It is considered simple and rapid method for the quantitative evaluation of hyoscyamine where discrete spots were quickly obtained by spraying with modified dragendorff's reagent (Chu et al., 1969; Christen et al., 2013; Kokotkiewicz et al., 2017). Presence of hyoscyamine in the endophytic fungi differs according to type of used growth media (SDB, PDB and RG) which affected alkaloids content especially hyoscyamine in each fungus. Investigation of TLC plates under UV short (254nm) and long (365nm) wavelength revealed invisible spots which are neither of the authentic hyoscyamine nor the tested fungal samples showed clear spots (Fig. 2). This was confirmed by Christen et al. (2013) who discussed that, few alkaloids contain an aromatic ring which makes them detectable by UV light, tropane alkaloids

including hyoscyamine are usually monitored around 205nm. Other species previously grown on SDB (L₁, L₂, L₄, R₂ and S₁) were characterized as good producers of hyoscyamine where reddish spots with the same Rf recognized as authentic hyoscyamine (Table 2 & Fig. 3). Fungal growth on RG medium led to absence of alkaloids in all the tested samples while positive results were obtained by L₁ and L₁' isolates grown on PDB (Fig. 4). It is noteworthy that, continuous subculture of the tested fungal endophytes led to disappearance of hyoscyamine when tested by TLC. Previous researchers reported production of tropane alkaloids such as hyoscyamine and scopolamine by endophytic fungi isolated from different medicinal plants like *Datura metel* (Naik et al., 2018).

Total secondary metabolites profile

Production of different secondary metabolites by each tested fungal residue samples were expressed by appearance of various colored spots on the TLC plates using anisaldehyde sulfuric and sulfuric acid reagents as well as detected by UV irradiation. The total secondary metabolite profile (SMP) in each fungal sample differed according to the type of growth medium (PDB, RG or SDB) of the tested endophytic fungi.

TLC plates were exposed to short and long UV after development step. Many dark spots with different Rf values were detected under short λ UV while fluorescent spots were obtained using long λ UV (Table 2 & Fig. 5). Obviously, fungal growth using different media led to variations in the obtained SMP spots which mean that the growth medium significantly affects the formation of certain fungal secondary metabolites. Also, various colored spots (violet, greenish blue, blue, yellow and brown) were obtained by spraying the plates with anisaldehyde sulfuric and sulfuric acid reagents separately (Figs. 6 and 7). The obtained results revealed that, the fungal samples previously grown on RG growth medium was richer with several types of secondary metabolites followed by these media PDB and SDB. This means that RG medium may encourage the biosynthesis of different types of SMP more than PDB and SDB. The present results established numerous secondary metabolites from cultures of tested endophytic fungi. This is consistent with many previous findings which illustrated that endophytes are able to produce a multitude of secondary metabolites with diverse biological activities (Uzma et al., 2018; Hyde et al., 2019; Newman & Cragg, 2020).

TABLE 2. Different secondary metabolites of the endophytic fungi.

Fungi	Fungal isolate	Color and number of spots on the TLC																	
		SMP			Phenolic compounds			Flavonoids			Anthraquinone glycoside and coumarins			Hyoscyamine alkaloid					
		PDB	RG	SDB	PDB	RG	SDB	PDB	RG	SDB	PDB	RG	SDB	PDB	RG	SDB			
<i>Aspergillus carbonarius</i>	L ₁	1 DG 1 LG 4 FG	6 B 1 FV 3 FO	1 BshG	3 Bsh	5 FO	-	-	2 Y	-	-	-	-	1 Fs	-	1 BkR			
<i>Aspergillus flavus</i> var. <i>columnaris</i>	L ₂	-	1 FO 1 V	2 FV	1 DRO	1 RO	1	RO	-	-	-	-	-	-	-	1 RO			
<i>Aspergillus terreus</i> var. <i>terreus</i>	L ₃	1 LG 1 V 1 R	7 V 1 Gr 1 O	1 B 2 Bck 1 O	1 O	1 FBI	1 O	1 Y	2 Y	1 FR	-	2 R	-	-	-	-			
<i>Penicillium oxalicum</i>	L ₄	-	2 FO 2 DV 1 RV	1 Gr	3 BI	-	1	Bsh	-	-	-	-	-	-	-	1 RO			
<i>Aspergillus flavus</i> var. <i>columnaris</i>	R ₂	2 V	1 FO 1 V	1 DV 1 FV	1 DRO	1 RO	1	RO	-	-	-	-	-	-	-	1 RO			
<i>Aspergillus terreus</i> var. <i>terreus</i>	R ₃	1 LG 1 V 1 R 3 FV	1 FG 4 FV 2 DV 2 Gr 1 BI	2 Bck	2 BI	1 FBI	1 BI	1 Y	1 Y	-	-	2 R	-	-	-	-			
<i>Lichtheimia</i> sp.	R ₄	-	5 FV	-	-	-	-	-	-	-	-	-	-	-	-	-			
<i>Alternaria chlamydospora</i>	L _{16s}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
<i>Aspergillus carbonarius</i>	L _{1'}	1 DG 1 LG 4 FG	6 B 1 FV 3 FO	1 BshG	3 Bsh	5 FO	-	1 Y	-	-	-	-	1 Fs	-	-	-			

TABLE 2. Cont.

Fungi	Fungal isolate	Color and number of spots on the TLC																			
		SMP				Phenolic compounds				Flavonoids				Anthraquinone glycoside and coumarins				Hyoscyamine alkaloid			
		PDB	RG	SDB	1 Gr	PDB	RG	SDB	1 Gr	PDB	RG	SDB	1 Gr	PDB	RG	SDB	1 Gr	PDB	RG	SDB	1 Gr
<i>Aspergillus fumigatus</i>	L ₄ '	3 Bsh	3 V	4 FV	1 R	1 FGr	1 R	-	-	-	-	-	-	-	-	1	-	-	-	-	-
		2 FV	1 R	1 B	3 B	1 FO	2 B	1 Y	-	-	-	-	-	-	-	FR	-	-	-	-	-
		5 FV	1 FV	4 Gr	1 Gr	1 Gr	1 Gr	1 Gr	-	-	-	-	-	-	-	-	-	-	-	-	-
		2 Gr	BshO	1 BI	3 Gr	1 Y	1 Gr	1 Gr	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Penicillium</i> sp.	R _{16s}	1 FO	1 V	1 DV	1 RO	1 RO	1 RO	1 RO	-	-	-	-	-	-	-	-	-	-	-	-	-
	S ₃	-	1 V	1 FV	1 FV	1 FV	1 FV	1 FV	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Aspergillus flavus</i> var. <i>columnaris</i>	S ₃	-	1 V	1 DV	1 RO	1 RO	1 RO	1 RO	-	-	-	-	-	-	-	-	-	-	-	-	-
	S ₅	1 FGr	1 RV	1 B	1 RB	1 Gr	1 Gr	1 Gr	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Aspergillus flavipes</i>	S ₅	1 FGr	1 RV	1 B	1 RB	1 Gr	1 Gr	1 Gr	-	-	-	-	-	-	-	-	-	-	-	-	-
	S ₆	GshBl color	1 RV	1 Bsh	1 O	1 O	1 O	1 O	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Alternaria</i> sp.	S ₆	4 FG	1 RV	1 Bsh	1 O	1 O	1 O	1 O	-	-	-	-	-	-	-	-	-	-	-	-	-
	S ₁	-	6 V	1 FV	1 FV	1 FV	1 FV	1 FV	-	-	-	-	-	-	-	-	-	-	-	-	1 RO

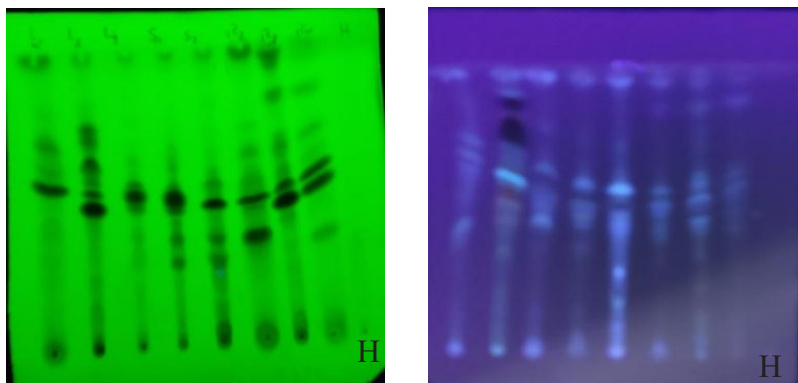


Fig. 2. Invisible hyoscyamine (H) explored by UV short λ (Left) and UV long λ (right)

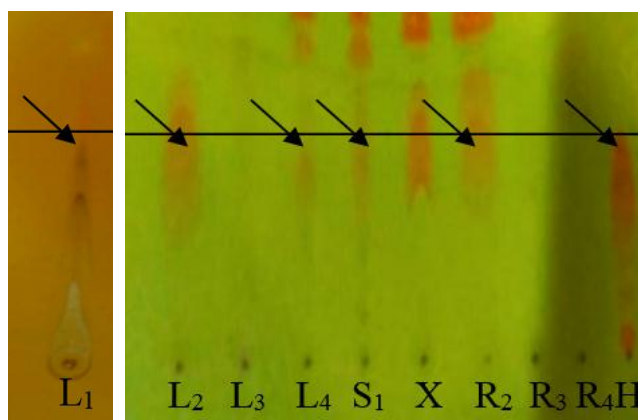


Fig. 3. TLC illustrate production of hyoscyamine (H) by some tested endophytic fungi (arrows) [X symbol donated neglected spot sample]

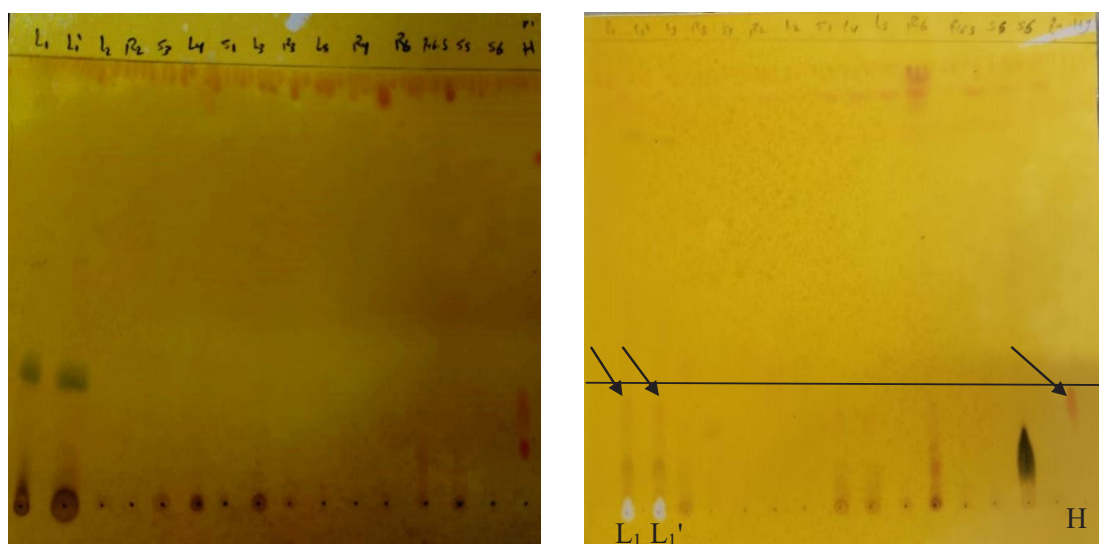
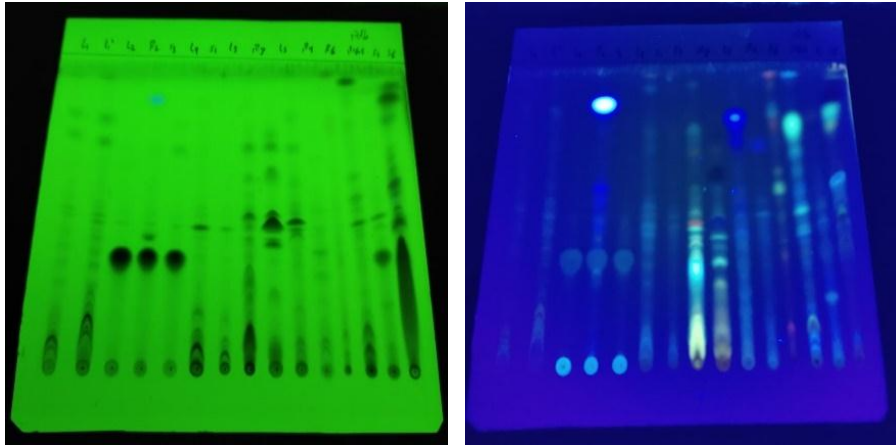
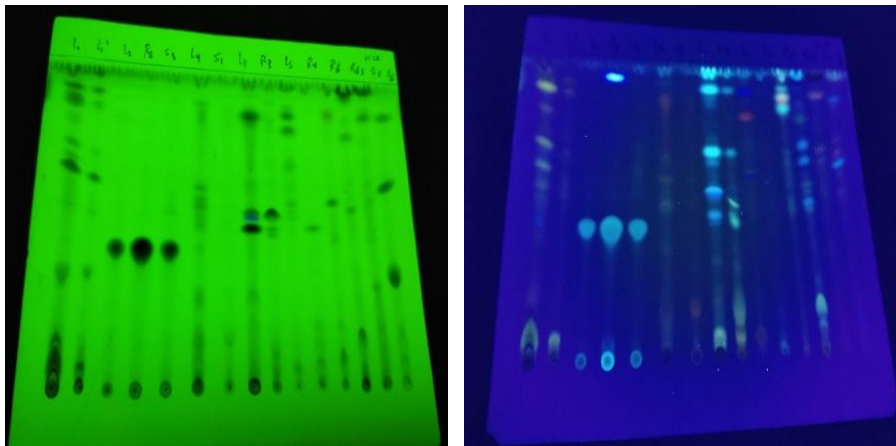


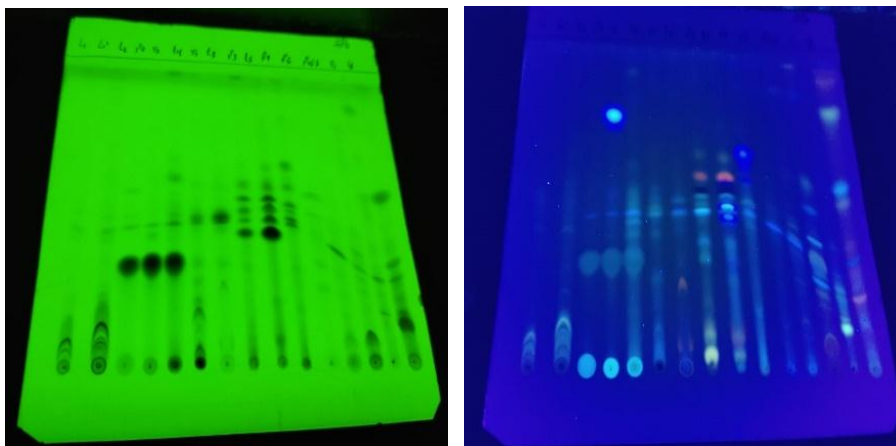
Fig. 4. TLC illustrate negative alkaloid production by fungal samples grown on RG medium (Left) and positive hyoscyamine (H) production by some tested samples (arrows) grown on PDB (right).



(PDB medium)



(RG)



(SDB)

Fig. 5. TLC plates showing different types of SMP using 254nm UV (left) and 365nm (right) [Fungal species on each plate from left to right: L₁, L₁', L₂, R₂, S₃, L₄, S₁, L₃, R₃, L₄', R₄, R₆, R₁₆, S₅ and S₆. Fungi were grown on different growth media: PDB, RG and SDB from top downwards]

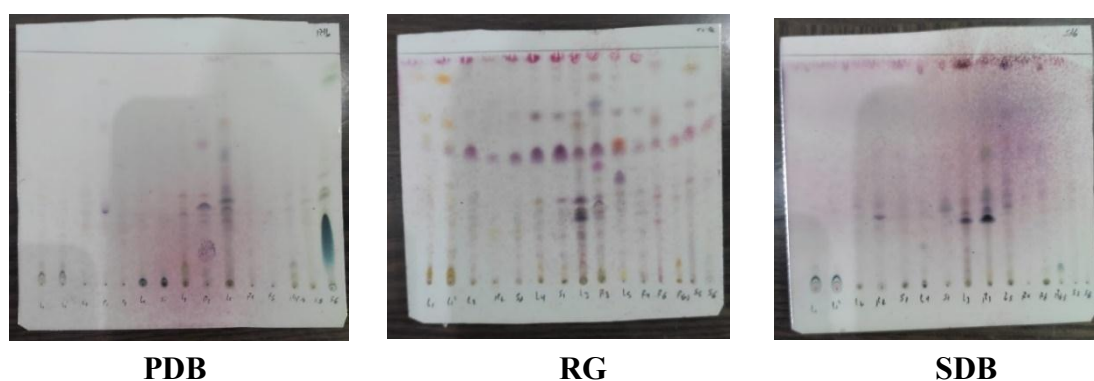


Fig. 6. Different colored spots of SMP on TLC plates sprayed with anisaldehyde sulfuric reagent [Fungal species on each plate from left to right: L_1 , L_1' , L_2 , R_2 , S_3 , L_4 , S_1 , L_3 , R_3 , L_4' , R_4 , R_6 , R_{16} , S_5 and S_6 . Fungi were grown on different growth media: PDB, RG and SDB]

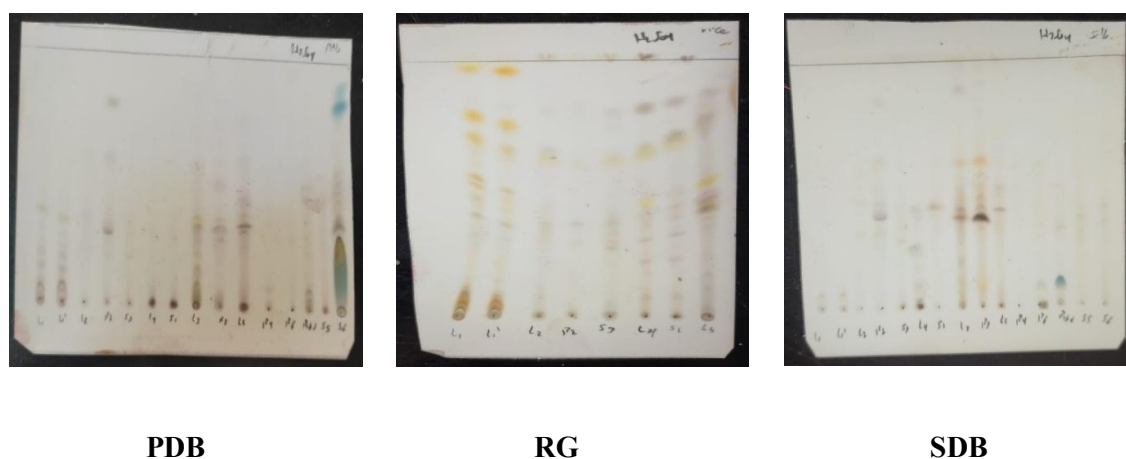


Fig. 7. Different colored spots of SMP on TLC plates sprayed with sulfuric acid reagent [Fungal species on each plate from left to right: L_1 , L_1' , L_2 , R_2 , S_3 , L_4 , S_1 , L_3 , R_3 , L_4' , R_4 , R_6 , R_{16} , S_5 and S_6 . Fungi were grown on different growth media: PDB, RG and SDB]

Phenolic compounds

Presence of phenolic compounds in the tested fungal samples was distinguished by formation of blue, green, orange and red colored spots after spraying the TLC plates by $FeCl_3$ reagent. Table 2 and Fig. 8 illustrate that L_2 , R_2 and S_3 fungal species were considered the most producers of phenolic compounds after their growth on all types of tested media where positive results appeared as reddish orange spots on the TLC plates. While only PDB and RG media encourage phenolic compounds formation by L_1 and L_1' endophytes in the form of blueish spots. Also, blue and green spots were observed by L_4 and S_6 fungi grown on PDB. Another reddish orange spot indicated other type of phenolic compounds was obtained by S_6 species grown on RG medium. Other faint blue spots by L_3 and R_3 were found using PDB and RG media. These findings indicated that PDB

and RG media enhance the formation of different types of phenolic compounds of some tested fungi especially those of the genus *Aspergillus*. These results indicated that the phenolic content of fungi differed according to the type of growth medium. Our results were in concordance with those obtained with previous study of Gautam et al. (2022) who found that the total phenolic content detected by qualitative phytochemical analysis was maximal in ethyl acetate crude extract of the endophytic fungus *Nigrospora sphaerica* fermented in PDB medium.

Flavonoids

The flavonoids were observed as bright yellow spots on TLC plates after spraying with $AlCl_3$ reagent and detection by UV λ 365nm. Fungal samples of L_1 , L_1' , L_3 and R_3 species grown on RG medium and L_3 and R_3 grown on SDB medium

illustrate the presence of flavonoids, while fungi grown on PDB medium indicate absence of flavonoid compounds (Table 2 and Fig. 9). Flavonoids production has been reported by some endophytic fungi isolated from different plants (Huang et al., 2007; Liu et al., 2007; Qiu et al., 2010). These studies revealed that the major bioactive constituents of the fungal cultures were found to contain phenolics and related derivatives such as flavonoids. In this study flavonoids have not been detected in fungal cultures grown on PDB medium. This disagreed the recent study of (Gautam et al., 2022) who reported a very heavy presence of flavonoids in methanol extract of PDB medium of *N. sphaerica*. But this disagreement in the results might be due to different tested fungal species.

Anthraquinone glycoside and coumarins

Faint red spots were obtained on TLC plates after spraying with KOH indicating the presence of

small concentrations of anthraquinone glycoside and coumarins secondary metabolites. Table 2 and Fig. 10 expressed that L_3 species grown on PDB, R_3 grown on SDB and L_4' grown on RG are potential producers of these compounds. Relevant results were previously reported (Yang et al., 2013) about the production of different types of anthraquinones from *Rumex nepalensis*, *Rumex hastatus*, and endophytic *Aspergillus fumigatus*. Additionally, the anthraquinone glycoside, 3-O-(α -D-ribofuranosyl) questinol was isolated from the endophytic fungal strain *Eurotium cristatum* EN-220, obtained from the marine alga *Sargassum thunbergia* (Du et al., 2014). Moreover, the medicinally important specific plant-derived coumarins are produced by several fungal endophytes belonging to certain genera such as *Penicillium*, *Botryodiplodia*, *Annulohyphoxylon*, *Fusarium*, *Septoria* and others (Singh et al., 2021).

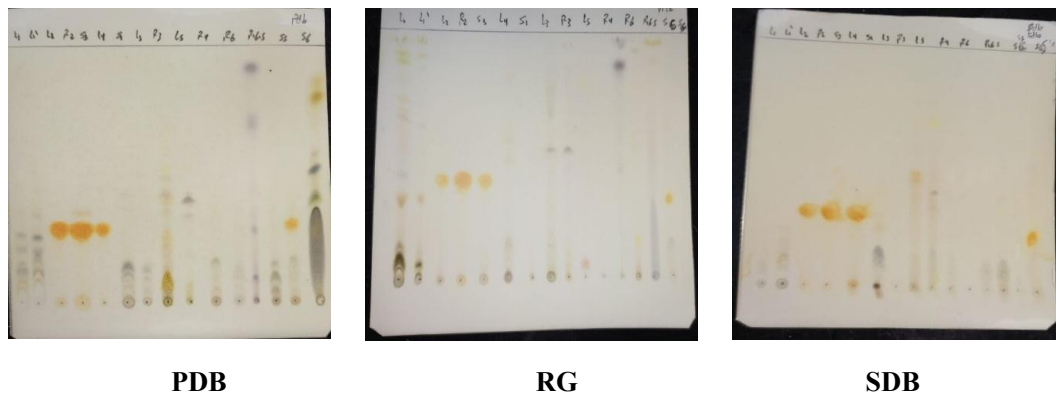


Fig. 8. Effect of different growth media on phenolic compounds formation. Reddish orange, blue and green spots indicating positive results [Fungal species on each plate from left to right: L_1 , L_1' , L_2 , R_2 , S_3 , L_4 , S_1 , L_3 , R_3 , L_4' , R_4 , R_6 , R_{16} , S_5 and S_6]

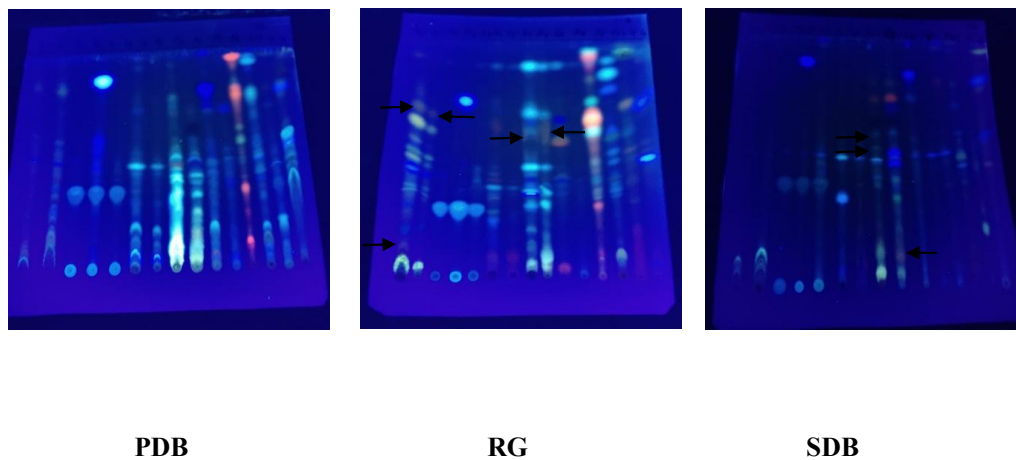
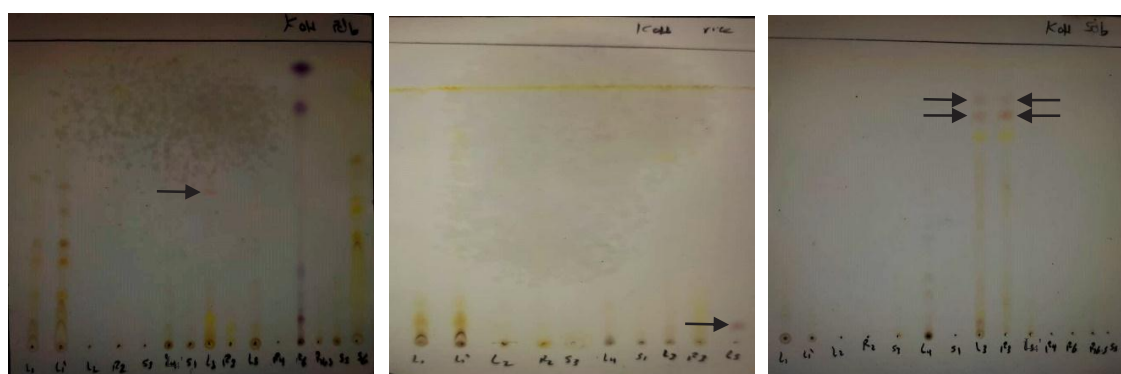


Fig. 9. Bright yellow spots (arrows) with UV 365nm indicating presence of flavonoids [Fungal species on each plate from left to right: L_1 , L_1' , L_2 , R_2 , S_3 , L_4 , S_1 , L_3 , R_3 , L_4' , R_4 , R_6 , R_{16} , S_5 and S_6]



PDB

RG

SDB

Fig. 10. Effect of different growth media on anthraquinone glycoside and coumarins formation. Faint red spots (arrows) indicating positive results [Fungal species on each plate from left to right: L₁, L₁', L₂, R₂, S₃, L₄, S₁, L₃, R₃, L₄', R₄, R₆, R₁₆, S₅ and S₆]

Conclusions

Sixteen entophytic fungi were isolated from roots, stems and leaves of the medicinal plant *H. muticus* and identified morphologically. The isolated fungi belonged to genera, *Alternaria*, *Aspergillus*, *Fusarium*, *Lichtheimia* and *Penicillium* where *Aspergillus* isolates were the most abundant. The phytochemical analysis of crude extracts of the isolated endophytic fungi using TLC showed presence of different types of secondary metabolites such as phenolic compound, flavonoid, anthraquinone glycoside, coumarins and alkaloids. The secondary metabolic profile of each tested fungus changed according to the type of used growth medium (PDB, RG and SDB). In addition, the tested species belonging to *Aspergillus* and *Penicillium* fungi were found to be good producers of the hyoscyamine alkaloid, characteristic to *H. muticus* plant.

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and TLC analysis, writing the manuscript and corresponding author for publication. Amal A. El-Somany carried out collection of plant samples, participated in isolation of endophytes, performed practical work of secondary metabolites extraction and TLC examination. Nagwa A. Shoeib and Ghada I. El-Shahat contributed in suggestion of the research idea and follow up the practical work. Abdel-Rahim S. Ibrahim contributed in putting the research idea, design of biochemical experiments, follow up the practical work and reviewing the typed manuscript.

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التقيب الحيوى عن قلويد الهوسيامين وبعض المواد الثانوية الاخرى المنتجة بواسطة بعض الفطريات الداخلية المعزولة من نبات الهوسياميس موتيكس

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يعد نبات الهوسياميس موتيكس (السكران المصرى) شجيرة تزدهر في المناطق الرملية في مصر، تُظهر خصائص دوائية مهمة يمكن أن تُعزى إلى مركباتها النشطة بيولوجيًا أو بسبب الميكروبات الداخلية المرتبطة بها. في هذه الدراسة، تم عزل مجموعة من الفطريات الداخلية المرتبطة بجذور وسيقان وأوراق النبات وتُعرفها تعريفًا مورفولوجيًا دقيقًا، والكشف عن المواد الثانوية المنتجة منها خاصة قلويد التروبان النباتي المميز؛ هوسيامين. تم عزل ستة عشر نوعًا من الفطريات الداخلية ينتمون إلى خمسة أجناس مختلفة شكليًا وتم تعريفها طبقًا للوصف المميز للمستعمرات والفحص المجهرى المفصل. تم استزراع الفطريات الداخلية المعزولة باستخدام ثلاث بيئات زراعة معملية مختلفة وهم: بيئة البطاطس، بيئة حبوب الأرزو بيئة السبرود. تم استخلاص المواد الفعالة من كل رشيق فطرى باستخدام أسيتات الإيثيل ثم خضعت المستخلصات الخام التي تم جمعها من العزلات الفطرية المختلفة للتحليل الكيميائي باستخدام تقنية (TLC) والتي أوضحت وجود العديد من المواد الثانوية المختلفة مثل الفينولات، الفلافونويدات، الأنثراكينون جليكوسيد، الكومارين القلويدات. علاوة على ذلك، تم اختبار قدرة الفطريات الداخلية المختبرة على إنتاج الهوسيامين حيث أظهرت بعض الأنواع التي تنتمي إلى *Aspergillus* و *Penicillium* نتائج إيجابية للهوسيامين.