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RESEARCH ARTICLES

α-Glucosidase inhibitor and phytotoxic metabolite produced by an endophytic fungus *Diaporthe melonis* isolated from *Acalypha indica* L

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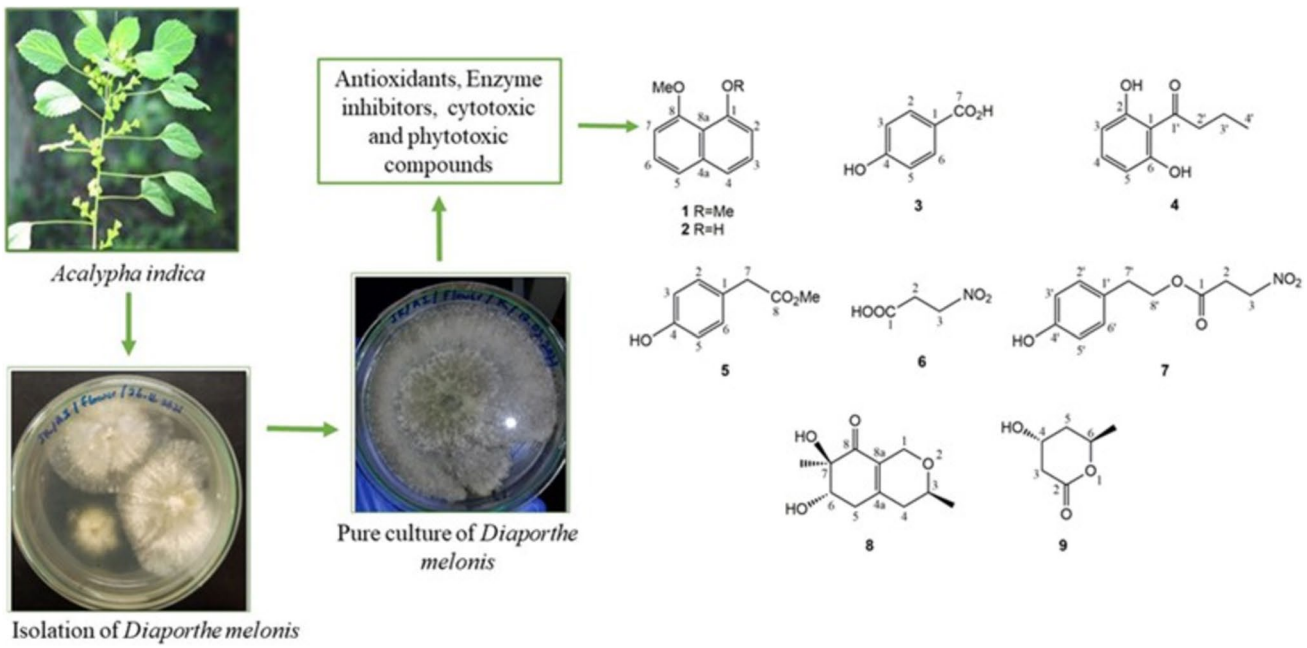
Abstract

Nine compounds from the fermentation of *Diaporthe melonis*, an endophytic fungus in the inflorescence of *Acalypha indica* L. (Euphorbiaceae), were isolated and identified. They are 1,8-dimethoxynaphthalene (**1**), 8-methoxynaphthalen-1-ol (**2**), 4-hydroxybenzoic acid (**3**), 1-(2,6-dihydroxyphenyl)butanone (**4**), methyl (4-hydroxyphenyl)acetate (**5**), 3-nitropropanoic acid (**6**), phomonitroester (**7**), felinone A (**8**) and (4*R*,6*R*)-4-hydroxy-6-methyltetrahydro-2*H*-pyran-2-one (**9**). The EtOAc crude extract resulted in strong cytotoxicity, radicle elongation inhibition and a strong potential to inhibit α-glucosidase enzyme. This is the first report of the strong α-glucosidase and strong phytotoxicity of compound **4**.

Graphical abstract



Keywords *Acalypha indica* · *Diaporthe melonis* · Enzyme inhibition · Phytotoxicity · Naphthalene derivatives

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Introduction

Endophytic fungi serve as a plentiful and reliable reservoir of unique and bioactive compounds, yet remain relatively understudied category of microorganisms (Kumar et al. 2014). We have previously reported several bioactive compounds from endophytic fungi from Sri Lankan plants (Bandara, et al., 2015; Piyasena et al. 2015; Siriwardena et al., 2015; Thanabalasingam et al. 2015; Quader et al., 2016; Munasinghe et al. 2017; Padmathilake et al., 2017; Quader et al., 2017a; Quader et al., 2017b; Rathnayake et al. 2018a, b; Rathnayake et al. 2018a, b; Kehelpannala et al. 2018; Sritharan et al., 2019; Dissanayake et al. 2020; Munasinghe et al. 2021; Thanbalasingam et al., 2024). Continuing our studies of chemical prospecting of endophytic fungi in Sri Lankan flora, the secondary metabolites in an endophytic fungus of *Acalypha indica* L. were studied. *A. indica* is a widely distributed medicinal herb of the family Euphorbiaceae. It is used to treat many diseases in the respiratory tract, urinary tract, nervous system and skin. The diversity of the endophytic community in *A. indica* and antimicrobial activities of their crude extracts have been previously reported (Kuradawad et al., 2014; Sowparthani et al., 2016). Here, we report the isolation of nine compounds from *Diaporthe melonis*, an endophytic fungus isolated from *A. indica*. Further, their antioxidant activity, cytotoxicity, phytotoxicity and potential to inhibit acetylcholinesterase (AChE), α-amylase and α-glucosidase activity.

Methods

General

The TLC was performed using pre-coated aluminum sheets with silica gel $60F_{254}$ (Merck 1.05554). For column chromatography, silica gel (Merck 1.07734) and Sephadex LH20 (Fluka, 84,952) were used. The ^{1}H NMR were recorded on a JEOL JNM-ECP500 or JEOL JNM-AL300 spectrometer in $CDCl_3$ or CD_3OD solution. UV absorbance values were taken using the multimode reader (BioTek Synergy HTX). AChE enzyme (from *Electrophorus electricus*), α-amylase and α-glucosidase (from *Saccharomyces cerevisiae*) were purchased from Sigma Aldrich. All the other chemicals used were analytical grade unless otherwise specified.

Collection and identification of plant material

Plant material: *Acalypha indica* is a native plant found in home gardens. This is a common plant, identified by Prof. Nimal Adikaram, Professor of Botany, National Institute of Fundamental Studies (NIFS), Sri Lanka and deposited at the Natural Product Laboratory of NIFS (Specimen No. NIFS-JK/AI/01).

Isolation and identification of the endophytic fungus

Plant leaves of *Acalypha indica* were washed and triple sterilized with 90% ethanol, 2.5% sodium hypochlorite, 90% ethanol and distilled water. Tiny pieces (≥ 5 mM^2) were placed on potato dextrose agar medium and incubated at room temperature (32 °C) for 7 days. The emerged fungus was carefully sub-cultured to obtain its pure cultures. The external characteristics and the spores from the pure cultures were microscopically observed. Molecular level identification was carried out by amplifying the Internal Transcribed Spacers (ITS) of rDNA gene using universal eukaryotic primers ITS 1F and ITS 4. The resulting sequence was compared for similarities by performing nucleotide BLAST. The amplified gene sequence had 99.29% similarity with *Diaporthe melonis* of the Diaporthaceae family (GenBank accession number: MW450847.1). The pure culture of the fungus (NIFS-JK/AI/K) and the photographic evidence were deposited at the Natural Products Laboratory of the National Institute of Fundamental Studies (NIFS).

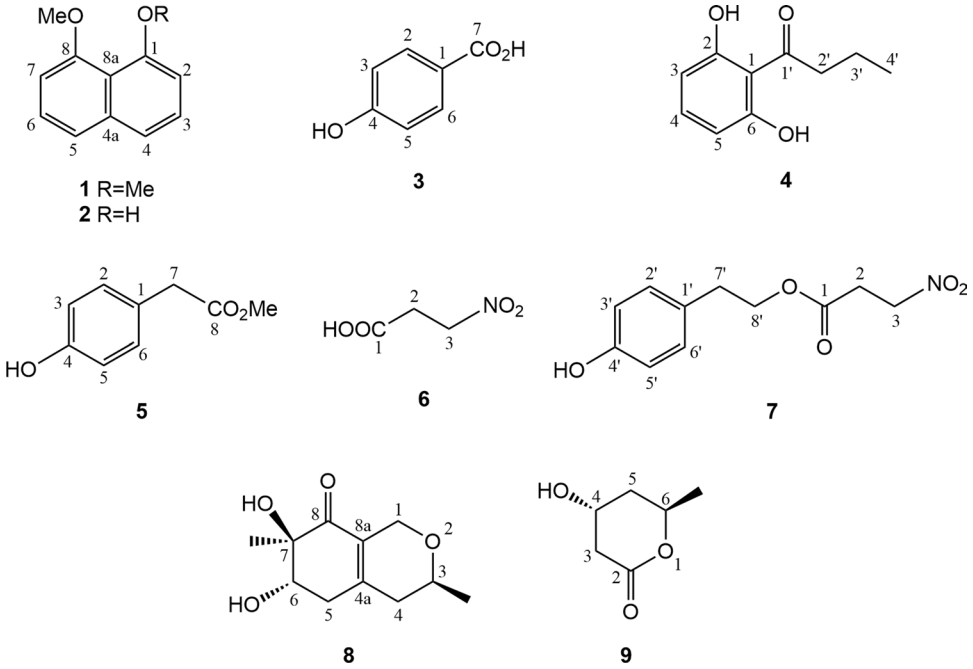
Fermentation and extraction of fungus

D. melonis was large-scale grown in 1 L conical flasks, each containing 400 mL of potato dextrose broth medium. They were incubated for 30 days at room temperature. The medium was filtered. The filtrate was partitioned with EtOAc and concentrated. The residual mycelium was crushed into pieces and extracted to EtOAc by sonication. Both EtOAc extracts were combined (10.83 g) after observing the similarities in their Thin Layer Chromatography (TLC).

Separation and identification of compounds

The resulting crude extract was subjected to silica gel column chromatography (*n*-hexane–EtOAc-MeOH) followed by Sephadex LH20 (100% MeOH and 30% $CHCl_3$/MeOH) and further purified using PTLC. This resulted compounds **1**–**9** were resulted during this process. Crude extract (concentration series of 2000–31.25 mg L^{-1}) and pure compounds (100–3.12 mg L^{-1}) were tested for their bioactivities (Fig. 1).

Fig. 1 Chemical structures of compounds **1**–**9**



Antioxidant assay

DPPH radicle scavenging assay

The 0.3 mM 2,2′-diphenyl-1-picrylhydrazyl (DPPH) solution was prepared by dissolving 1.2 mg in 10 mL of MeOH. For the tests, 150 µL of the sample and 60 µL of DPPH were added into a 96-well plate (Cellstar, 12.5 × 8.5 × 1 cm). For the negative control, 150 µL of MeOH and 60 µL of DPPH were added. The well plate was incubated in the dark for 30 min at room temperature. The absorbance (A) was measured at 517 nm. Ascorbic acid was used as the positive control. The percentage of Radical Scavenging Activity (RSA) was noted, and the IC_{50} value was calculated using the log dose scavenging activity curve (Aliyu et al. 2010).

$$\text{Percentage RSA}(\%) = \frac{\delta\text{A control} - \delta\text{A sample}}{\delta\text{A control}} \times 100$$

where; $\delta A_{control} = \text{Absorbance}_{control} - \text{Absorbance}_{control\ blank}$
δAsample = Absorbance sample − Absorbancesample blank

Ferric reducing antioxidant power (FRAP) assay

For the tests, 50 µL of sample followed by 200 µL of FRAP reagent, was added to the wells of a 96-well plate. For the test blanks, 50 µL of sample and 200 µL of distilled water were added. The wells of the control were filled with 50 µL distilled water and 200 µL of FRAP reagent. Then it was incubated for 4 min at 37 °C. The absorbance was measured at 593 nm. The antioxidant potential of the samples was determined against the standard curve of Fe_2SO_4. The final value was expressed as the concentration of antioxidants having a ferric-reducing ability equivalent to that of 1 mM Fe_2SO_4 (Langley-Evans et al., 2000).

Brine shrimp lethality assay

A teaspoon of Brine Shrimp's eggs (*Artemia salina*) was added to artificial sea water and kept at room temperature for 48 h. During that time, the eggs hatched and developed into nauplii (1st larval stage). Next, 2 mL from each concentration of the sample, followed by ten nauplii, were added to 24-semi microplates (Biologix, dimensions 12.5 × 8.5 × 2 cm). After 24 h, the number of dead larvae was counted. The negative and positive controls were seawater and $K_2Cr_2O_7$, respectively. The percentage lethality was calculated, and the LC_{50} value was determined from the graph between concentration and percentage lethality (Krishnaraju et al 2005).

$$\text{Percentage lethality}\ (\%) = \frac{\text{Number of died nauplii after 24 h}}{\text{Number of nauplii added}} \times 100$$

Phytotoxicity assay

Phytotoxicity was tested on the germination of *Lactuca sativa* (lettuce) seeds. Sterilized Whatmann No.01 filter paper discs (diameter 3.4 cm) were laid on the bottom of the wells of a 6-well plate (Biologix, 12.5 × 8.5 × 2 cm). 400 µL from each dilution was evenly distributed on the filter papers. Ten viable seeds were placed on it. The plates were sealed and incubated

in the dark at room temperature for five days. On the 5th day, the length of the hypocotyl and the radicle of each seed was measured. Negative control was distilled water and, abscisic acid was used as the positive control. The percentage inhibition of radicle elongation was calculated using the following equation. Similarly, the percentage inhibition of hypocotyl elongation was also calculated and the IC_{50} value was calculated (Baratelli et al., 2012).

$$\text{Inhibition of radicle elongation (\%)} = \frac{\text{Control radicle length} - \text{Treated radicle length}}{\text{Control radicle length}} \times 100$$

Acetylcholinesterase inhibitory assay

The AChE inhibitory bioassay was performed according to the published method with minor modifications (Ellmann et al., 1961). The samples were dissolved in 1% DMSO or distilled water. 100 µL of phosphate buffer (pH 8), 25 µL of sample and 50 µL of enzyme were added to the wells of 96-well plate. After 10 min, 50 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution and 25 µL of substrate solution (acetylthiocholine iodide) was added and incubated for 10 min at room temperature. The same procedure was followed for the controls and blanks. For the test-blanks, 50 µL of buffer was added instead of the enzyme. 25 µL of solvent was added to the controls instead of the sample. Absorbance was measured at 412 nm. Donepezil was used as the positive control. The percentage inhibition of AChE was calculated. The IC_{50} value of the sample was calculated by using the log dose-inhibitory curve.

$$\text{Enzyme inhibitory activity}(\%) = \frac{\delta\text{A control} - \delta\text{A sample}}{\delta\text{A control}} \times 100$$

α-Amylase inhibitory assay

The samples were dissolved in either phosphate buffer (pH 6.9) or distilled water. 100 µL of sample and 100 µL of enzyme solution were mixed in an Eppendorf tube. After 30 min at room temperature, 100 µL of starch solution was added. After 10 min, 100 µL of 96 mM 3,5-dinitrosalicylic acid (DNSA) was added, vortexed, and the tubes were placed inside the water bath at 85 °C for 15 min. Finally, the reaction mixtures were diluted with 900 µL of distilled water. The same procedure was followed for the controls and the blanks. For the test blanks, 100 µL of buffer was added instead of the enzyme. 100 µL of the solvent to control wells instead of the sample was added. 250 µL from each reaction mixture was added to a 96-well plate. The absorbance was measured at 540 nm. Acarbose was used as the positive control. The IC_{50} value of the sample was calculated by using the log dose-inhibitory curve. The percentage inhibition of α-amylase was determined (Nickavar et al. 2008).

α-Glucosidase inhibitory assay

The samples were dissolved in either phosphate buffer (pH 6.9) or distilled water. For the test wells of a 96-well plate, 100 µL of buffer, followed by 25 µL of sample and 25 µL enzyme, were added. The plate was incubated for 10 min at 37 °C. 50 µL of 4-nitrophenyl α-D-glucopyranoside (*p*NPG) was added and incubated for 30 min at 37 °C. The blanks and the controls were carried out in the same procedure. For the test blanks, 25 µL of buffer was added instead of the enzyme. 25 µL of the solvent to the control wells instead of the sample was added. The absorbance was measured at 410 nm. Acarbose was used as the positive control. The IC_{50} value of the sample was calculated by using the log dose-inhibitory curve. The percentage of α-glucosidase inhibitory activity was calculated (Sathya et al. 2020).

Statistical analysis

All measurements in this study were obtained by analysis in triplicate (n = 3). The results were presented as mean ± standard deviation (SD). Data were analyzed statistically by one-way ANOVA using the minitab 17 software package. When the F values were significant, mean differences were compared using Dunnett's test at the 5% level of probability.

Results and discussion

This study is the first report of isolating *D. melonis* from *A. indica*. The chromatographic separation of the combined crude extract furnished nine known compounds: 1,8-dimethoxynaphthalene (**1**) (Yang et al. 2008), 8-methoxynaphthalen-1-ol (**2**) (Ballantine et al. 2009; Wen et al. 2013), 4-hydroxybenzoic acid (**3**) (Yoshioka et al. 2004), 1-(2,6-dihydroxyphenyl)butanone (**4**) (Dai et al., 2006), methyl (4-hydroxyphenyl)acetate (**5**) (Fleming, et al., 2011), 3-nitropropionic acid (**6**) (Flores et al., 2013), phomonitroester (**7**) (Rukachaisirikul et al., 2008), felinone A (**8**) (Du et al. 2014) and (4*R*,6*R*)-4-hydroxy-6-methyltetrahydro-2*H*-pyran-2-one (**9**) (Sann et al. 2005). Their structures were elucidated by spectroscopic methods and confirmed by comparison with the literature. Due to the availability of metabolites, compounds, **1**–**4** were

Table 1 Results obtained for the bioassays of *D. melonis* crude extract and isolated compounds **1**–**4**

Bioassays	Sample					
	Crude extract	Comp. **1**	Compo. **2**	Compo. **3**	Comp. **4**	(+) control
DPPH radical scavenging assay IC_{50} value (mg L^{-1})	297.38 ± 2.10	ND	15.55[a] ± 0.53	ND	> 100	1.86[a] ± 0.43
FRAP assay (μmol $FeSO_4$ per mg of the sample)	0.58 ± 0.02	0.16 ± 0.01	9.68 ± 0.06	ND	0.85 ± 0.13	12.07[a] ± 0.30
Brine shrimp lethality assay LC_{50} value (mg L^{-1})	65.37 ± 0.65	> 100	54.67 ± 1.30	> 100	ND	34.40[a] ± 0.30
Hypocotylelongation inhibition IC_{50} value (mg L^{-1})	166.68 ± 3.16	> 100	39.54 ± 6.04	64.03 ± 9.62	> 100	1.46[a] ± 0.26
Radicle elongation inhibition IC_{50} value (mg L^{-1})	12.17[a] ± 4.73	4.41[a] ± 2.22	5.07[a] ± 0.86	11.47 ± 3.20	7.00[a] ± 3.25	1.85[a] ± 0.17
AChE inhibitory assay IC_{50} value (mg L^{-1})	763.53 ± 47.07	22.22 ± 1.33	19.41 ± 2.31	26.51 ± 3.61	ND	2.39[a] ± 0.78
α-amylase inhibitory assay IC_{50} value (mg L^{-1})	271.06 ± 1.10	ND	> 100	ND	ND	1.30[a] ± 0.57
α-glucosidase inhibitory assay IC_{50} value (mg L^{-1})	172.88 ± 81.37	59.39 ± 0.41	ND	> 100	30.97 ± 0.18	816.36[a] ± 22.08

The values in the table are given as mean ± SD of triplicates. The means of inhibition% that are labeled with the lowercase superscription letter 'a' are not significantly different from the positive control at 95% confident (α = 0.05). ND: Not detected (inhibition% less than 1% at all tested concentrations were considered as ND)

subjected to bioactivity determination and their results are presented in Table 1. The IC_{50} values, which had no significant difference ($p > 0.05$) with the positive controls, are mentioned as strong inhibitions. Moderate inhibitions were defined for crude extract when the IC_{50} was lower than 500 mg L^{-1} and pure compounds when the IC_{50} was lower than 100 mg L^{-1}. In vivo reaction of oxygen-centered radicals with biomolecules causes serious damage to the cellular structures and functions, which leads to many chronic diseases. *D. melonis* crude extract resulted in moderate antioxidant potential in the DPPH radical scavenging assay and the FRAP assay. However, strong antioxidant activity was observed in compound **2**, which had no significant difference ($p > 0.05$) with the positive control ascorbic acid in the DPPH assay and a high FRAP value as well. Rukachaisirikul et al. (2007) has reported similar results (Rukachaisirikul et al. 2007). Compound **2** neutralizes itself (after transferring the proton) by forming intermolecular hydrogen bonds and being resonance stabilized (Foti et al. 2002). Even though 4-hydroxybenzoic acid (**3**) is a phenolic compound, it had no activity. The carboxylic group in benzoic acids has a negative influence on the hydrogen-donating ability of hydroxybenzoic acids when the hydroxyl group is at ortho and para positions (Rice-Evans et al. 1996). The antioxidant potential of compounds **1**–**4** was mostly determined by the DPPH radical scavenging assay in the existing literature. Hence, their FRAP values are reported here for the first time. Further, compound **2** could be the major active compound in the crude extract of *D. melonis*. Identification of naturally occurring cytotoxic compounds has become one of the major research projects in the twenty-first century. This simple screening can be considered as an initial step in discovering highly effective cytotoxic compounds. The crude extract showed a moderate activity in the brine shrimp lethality assay, which had no significant difference ($p > 0.05$) with the positive control, $K_2Cr_2O_7$. Among the isolated compounds, only compound **2** showed the moderate percentage of lethality. Researchers suggested that methylation of the hydroxyl group might be the reason, and the phenolic hydroxyl moiety of naphthalene plays a crucial role in the activity (Li et al. 2008; Matsushita et al. 2011). Secondary metabolites from phytopathogenic fungi are found to be more phytotoxic and their activity is relatively easier to detect than screening higher plants (Xu et al. 2021). Henceforth, kingdom fungi were largely investigated for phytotoxic compounds. *D. melonis* crude extract resulted in a strong inhibition in radicle elongation of lettuce seeds, which had no significant difference ($p > 0.05$) with that of the positive control abscisic acid. But the inhibition of hypocotyl elongation resulted in a significant difference ($p < 0.05$) with that of abscisic acid. This is the first report on the phototoxic activity of *D. melonis*, although some *Diaporthe* species were reported to show phytotoxic activities. For example, crude extract of *D. schini* has the potential to inhibit the shoot growth of several grass species (Brunn et al., 2022) and crude extract of *D. kongii* has shown phytotoxicity on tomato leaves (Hilario et al., 2022). The two naphthalene derivatives **1** and **2** in this study exhibited almost the same potency in radicle elongation

inhibition. Naphthalene and its derivatives are known for being strong plant growth inhibitors (Henner et al., 1999; Dubrovskaya., 2016). 4-Hydroxybenzoic acid (**3**) is known to show phytotoxic effects to several plants including lettuce germination and is reported as one of the metabolites produced by *D. gulyae* (Cimmino, et al. 2011). This is the first report of the phytotoxicity of compound **4**. Inhibition of AChE is considered one of the best approaches to treat Alzheimer's disease (AD), because it permits the accumulation of acetylcholine in the synapse, which strengthen the neuro transmission. The natural AChE inhibitors have been explored from endophytic fungi as well. *D. melonis* crude extract inhibited moderately AChE enzyme. Compounds **1**–**3** showed high inhibitory potentials but with a significant difference ($p < 0.05$) with donepezil (positive control). The AChE inhibition observed in the crude extract of *D. melonis* could be due to compounds **1**–**3**. The AChE inhibition potential of naphthalene derivatives has been reported in extensive studies using mice brain and the inhibition potentials of compound **1** and **2** have been confirmed. AChE inhibition potential of naphthalene derivatives, including compounds **1** and **2**, has been reported in extensive studies using mice brain (Anwar et al., 2020). Settu & Arunachalam (2023) have reported that crude extract of *Phomopsis* sp. is a potent of antidiabetic source (Settu et al., 2023). This study also reported its ability to inhibit α-amylase and α-glucosidase enzymes which helps in lowering postprandial hyperglycemia. *D. melonis* crude extract inhibited α-amylase enzyme moderately but compounds **1**–**4** resulted no inhibition as shown in Table 1. In the α-glucosidase inhibitory assay compounds **1** and **4** have resulted a strong α-glucosidase inhibition potential which were significantly lower ($p > 0.05$) than the positive control acarbose. Several studies have reported the α-glucosidase inhibition potential of naphthalene derivatives (Ieyama et al. 2011; Jung et al. 2017). Some have mentioned that presence of naphthalene moiety in several antidiabetic compounds have improved their antidiabetic effect (Makar et al. 2019). These discoveries can be used as evidence for accepting the high α-glucosidase inhibitory potential of 1,8-dimethoxynaphthalene (**1**). However, there is a scarcity of literature data to discuss the results obtained for 1-(2,6-dihydroxyphenyl)-butanone (**4**). The potent α-glucosidase inhibitory activity found in the crude extract of *D. melonis* can be attributed to compounds **1** and **4**.

Compounds **1**–**5** have been previously isolated from several endophytes as secondary metabolites. 3-Nitropropionic acid (**6**), also reported from endophytic fungi (Flores et al., 2013; Chomcheon et al. 2005), is a mitochondrial toxin and a few papers have been reported in relation with Huchington's disease (Ayala et al. 2007; Upadhayay et al. 2023). Phomonitroester (**7**) is 4-hydroxyphenethyl alcohol ester of compound **6** and has been isolated from several fungi including *Phomopsis* sp. PSU-D15 (Rukachaisirikul et al., 2008). Felinone A (**8**) was isolated from a marine-derived entomopathogenic fungus, *Beauveria felina* and its absolute stereochemistry was proposed as 3*R*,6*R*,7*S* based on a CD study (Du et al. 2014). Later, its absolute stereochemistry was revised to 3*S*,6*S*,7*R*-enantiomer by the chemical synthesis of the optically active the 3*R*,6*R*,7*S*-compound (Abe et al. 2017). Compound **9** (4*R*,6*R*-isomer) has been isolated from *Daldinia* sp. and chemically synthesized by several groups (Buchanan, et al. 1996).

Conclusion

A. indica exhibits a rich diversity of endophytic fungi, yet their bioactive compounds have been inadequately explored. As an attempt to fill this gap, *D. melonis* from *A. indica* was isolated for the first time. *D. melonis* was only one endophytic fungus obtained in this study. Based on the screening of crude extract, *D. melonis* resulted strong cytotoxicity in brine shrimp lethality assay and phytotoxicity against the radicle elongation inhibition in lettuce germination. *D. melonis* crude extract also had strong potential to inhibit α-glucosidase which can be attributed to the activity of compound **1** and **4**. Further, compound **2** resulted a significant antioxidant activity. This study is the first to document enzyme inhibitory potentials of these compounds as well as first report about the phytotoxicity of compound **4**. Consequently, further studies are recommended to harness the potential of these bioactive compounds for the well-being of living organisms and to encourage chemical prospecting of endophytic fungi.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42535-025-01313-y.

Declarations

Conflict of interest The authors declare no conflict of interest. Data sharing is not applicable to this article as no data sets were generated or analyzed during this study.

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