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Antifungal activity of prenylated flavonoids isolated from *Tephrosia apollinea* L. against four phytopathogenic fungi

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ABSTRACT

Four prenylated flavonoids, isoglabratephrin, (+)-glabratephrin, tephroapollin-F and lanceolatin-A were isolated from *Tephrosia apollinea* L. growing in Egypt. The structures of compounds have been elucidated using physical and spectroscopic methods including (UV, IR, ^{1}H NMR, ^{13}C NMR, DEPT, 2D ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY). The isolated flavonoids showed considerable antifungal activity against four phytopathogenic fungi, namely *Alternaria alternata*, *Helminthosporium* sp., *Colletotrichum acutatum* and *Pestalotiopsis* sp. in a dose-dependent manner using the agar well-diffusion bioassay. They differ significantly in their activity with tephroapollin-F was the most effective. In a test using a concentration of 4 mg/ml of tephroapollin-F, strong fungicidal activities (32.8–58.3%) were produced against the test fungi, where *C. acutatum*, *Helminthosporium* sp. and *Pestalotiopsis* sp. showed greater susceptibility, while *A. alternata* was the least susceptible. Using the same concentration, the two flavonoids isoglabratephrin and (+)-glabratephrin showed moderate activities with % inhibition of fungal growth were ranged between (16.1–37.8) against *A. alternata*, *Helminthosporium* sp. and *Pestalotiopsis* sp., while showed a strong antifungal activity against *C. acutatum* (% growth inhibition were 46.4 and 42.9, respectively). In all treatments, the flavonoid lanceolatin-A exhibited weak to moderate activities. Using lower concentrations of the test flavonoids (2 and 1 mg/ml), weak to moderate antifungal activities were observed against all of the test fungal strains. In all cases and regardless of the flavonoid tested, *C. acutatum* was the most susceptible, while *A. alternata* was the least. The study recommends the use of the test compounds as rational fungicides of natural origin.

1. Introduction

During their life cycle, plants are frequently subjected to attack by a broad range of pathogens which, if uncontrolled, can result in reduced productivity and high mortality rates. It is estimated that there are about 250,000 species of higher plants, but six times as many (1.5 million) species of fungi. Therefore, plants are continuously exposed to the biotic stress exerted by organisms with which plants have symbiotic or pathogenic interaction. Indeed, in interactions between plants and microbial pathogens, resistance is the rule and disease is the exception (Lattanzio et al., 2006). Fungal diseases of plants have always been one of the major constraints in crop production causing alterations during developmental stages, severe losses and quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life (Agrios, 2004). Generally, phytopathogenic fungi are controlled by synthetic fungicides and soil fumigants, however, the use of these chemicals is increasingly restricted due to decreasing efficacy because of developed resistance toward these poisons. The well-documented hazardous effects of these chemicals or their degradation products on agroecosystems and the environment as well as human health impacts are also of major concern (Harris et al., 2001; Pretty, 2008). For the development of new types of fungicides and effective control of fungicide resistant pathogens, novel target sites of fungicidal action have been extensively studied (Köller, 1992). An appropriate response to attack by pathogenic organisms, including fungi can lead to produce a broad range of secondary metabolites in plants that are toxic to pathogens. These metabolites occur constitutively in healthy plants as a part of their normal program of growth and development (preformed antimicrobial compounds or phytoanticipins represent in-built chemical barriers to infection by

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potential pathogens). Alternatively, they may be synthesized in response to pathogen attack as part of the plant defense response, phytoalexins (Osbourn, 1996). The multicomponent defense response induced after the pathogen attack are now well-documented to be induced in plants through secondary metabolites, including peptides, proteins, phenolics, saponins, cyanogenic glycosides, and glucosinolates (Osbourn, 1996, 1999; Brunet et al., 2009). Plant phenolics are among secondary metabolites involved in the plant defense mechanisms against fungal pathogens. The fungicidal activity of phenolic compounds and their derivatives on several pathogenic fungi have been extensively reported (Jayasinghe et al., 2004; Li et al., 2005; Galeotti et al., 2008; Kanwal et al., 2010; Cotoras et al., 2011). The genus *Tephrosia* (Leguminosae; subf. Papilionoideae; tribe Tephrosieae) includes about 400 species (Shaw and Willis, 1973). Extracts of some *Tephrosia* species such as *T. purpurea* and *T. apollinea* have revealed the presence of rotenoids, isoflavones, flavanones, flavanols and flavones (Abou-Douh et al., 2005). These phytochemicals have been reported to possess several biological activities including insecticidal, antibacterial, antifungal, antiviral and estrogenic activities (Pelter et al., 1981; Sánchez et al., 2000; Khalafallah et al., 2010; Hegazy et al., 2011). Previous phytochemical investigations on *T. apollinea* growing in Egypt gave prenylated flavonoids as the main constituents (Abou-Douh et al., 2005; Abd El-Razek et al., 2007; Khalafallah et al., 2009). In the present study, four prenylated flavonoids were isolated from *T. apollinea* and tested for their antifungal activities against certain phytopathogenic fungi.

2. Materials and methods

2.1. Fungal species

Four fungal species namely, *Alternaria alternata*, *Colletotrichum acutatum*, *Helminthosporium* sp. and *Pestalotiopsis* sp. were included in the present study. Detailed information about the test fungi are described in Table 1.

2.2. Collection and preparation of the test plant

T. apollinea was collected from Aswan Island, Aswan, South Egypt. A voucher specimen has been deposited in the Herbarium of the Department of Botany, Faculty of Science, South Valley University, Aswan, Egypt (voucher no. T ap-5). The aerial parts were air-dried for 10 days in the shade at environmental temperature (32–35 °C day time) and powdered mechanically by using an electric blender (Braun Multiquick Immersion Hand Blender, B White Mixer MR 5550 CA, Germany). Powdered samples were maintained in tightly closed dry bags for subsequent extraction.

Table 1
Fungal species tested in the present study.

Fungi	Host	Location	Symptoms	Pathogenic capability
Alternaria alternata	Apple (*Malus domestica*)	Saudi Arabia	Fruit black spot	Pathogenic
Colletotrichum acutatum	Date Palm (*Phoenix dactylifera*)	Saudi Arabia	Offshoots decline	Pathogenic (Ammar and El-Naggar, 2011)
Helminthosporium spp.	Date Palm (*Phoenix dactylifera*)	Saudi Arabia	Leaf spot (rachides)	Pathogenic (Ammar and El-Naggar, 2011)
Pestalotiopsis spp.	Apricot (*Prunus armeniaca*)	Egypt	Root cankers	Pathogenic

2.3. Isolation of phytochemicals from T. apollinea

Prenylated flavonoids were isolated from *T. apollinea* according the method adopted by Abd El-Razek et al. (2007). The air-dried powdered aerial parts of *T. apollinea* (900 g) were extracted with CH_2Cl_2–MeOH (1:1) at room temperature. The extract was concentrated in vacuum to give a residue (40 g), which was chromatographed by using flash column chromatography on a silica gel (kieselgel 60 Merck; 230–400 mesh) eluted with *n*-hexane (2 L) followed by a gradient of *n*-hexane–CH_2Cl_2 up to CH_2Cl_2 and CH_2Cl_2–MeOH up to 15% MeOH (2 L each of the solvent mixture). The *n*-hexane–CH_2Cl_2 fraction (1:3) was chromatographed on a Sephadex LH-20 column (Pharmacia Co., Tokyo, Japan) eluted with *n*-hexane–CH_2Cl_2–MeOH (7:4:0.25) with increasing polarity to give compounds 3 and 4. The CH_2Cl_2 fraction (100%) was chromatographed on a Sephadex LH-20 column and eluted with *n*-hexane–CH_2Cl_2–MeOH (7:4:0.5), afforded compounds 1 and 2.

2.4. Identification of compounds

The isolated phytochemicals were developed on TLC precoated plates using (silica gel 60, F_{254}, 0.25 mm, Merck Co.), and spots were detected under UV light and colored by spraying with 10% H_2SO_4 solution followed by heating. The R_f values of the isolated compounds were compared relative to the authentic standards. The structures of the isolated compounds were established by (^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, NOESY and HR-MS).

2.5. In vitro antifungal activity

An appropriate amount of each of the isolated flavonoids was dissolved in DEMSO to obtain a stock solution of 4 mg/ml. Serial dilutions of the stock solution of the test flavonoids were made to final concentrations of 4, 3, 2 and 1 mg/ml. The isolated compounds were screened for antifungal activity using the agar well diffusion bioassay (Perez et al., 1990). A 5 mm mycelial disc of 5-day-old fungal culture grown individually on Potato dextrose agar (PDA) medium was placed one cm at the periphery of the PDA culture plates. In agar well diffusion methods, 40 μl of each concentration of the test flavonoids were applied to an appropriate well made on the opposite side, one cm on other side of the same culture plates using a cork borer (Imtiaj and Lee, 2008). Control set was made using the solvent used without any of the tested materials and served as a negative control. The fungicide standard, Carbendazim was dissolved in sterilized water and tested by the same manner and used as a positive control. Plates were incubated at 25 °C and the experiment was carried out in four replicates. The antifungal activity was recorded until the growth of fungi was reached at the opposite side of the plate in control treatments using the following equation: Growth inhibition (%) = $[(A - B)/A] \times 100$, where A represents the length of mycelia in the negative control plate and B = the length of mycelia in the treated plate (Park et al., 2008).

2.6. Minimal inhibitory concentrations (MICs) of the tested extracts

The minimal inhibitory concentration (MIC) of each of the tested flavonoids was determined according to a standard procedure (CLSI, 2002) using the broth two-fold macro dilution method. Serial dilutions of each of the tested compounds together with the antifungal standard over the range 0.07–2.25 mg/ml were prepared in fungal broth media using tomato dextrose agar (PDA) and incubated at 25 °C for 48 h. The lowest concentration of each compound that inhibits the growth of each of tested phytopathogenic fungi was recorded.