



# Endophytic fungi from the medicinal herb *Euphorbia geniculata* as a potential source for bioactive metabolites

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## Abstract

Many researchers proved that plant endophytes manage successful issues to synthesize active chemicals within plant cells. These bioactive compounds might support a range of plant defense mechanism against many pathogenic microorganisms. In this study, a total of 22 isolates representing 21 fungal species belonging to 15 fungal genera in addition to one variety were isolated and identified for the first time from *Euphorbia geniculata* plants. The genus *Aspergillus* was the most common fungus isolated from the studied plant. The fungus *Isaria feline* was recorded in both leaves and stem, while *Aspergillus flavus*, *A. ochraceus*, *A. terreus* var. *terreus*, *Emercilla nidulans* var. *acristata*, *Macrophomina phaseolina* colonized both stem and root. The isolated fungi showed antagonistic activities against six strains of plant pathogenic fungi viz., *Eupenicillium brefeldianum*, *Penicillium echinulatum*, *Alternaria phragmospora*, *Fusarium oxysporum*, *Fusarium verticilloid*, and *Alternaria alternata* in dual culture assay. The highest antagonistic activity fungal species (*Aspergillus flavus*, *A. fumigatus*, and *Fusarium lateritium*) and the lowest (*Cladosporium herbarum*, *F. culomrum*, and *Sporotrichum thermophile*) showed twining in their secondary metabolites especially terpens and alkaloids with that of their host *E. geniculata*. Three concentrations of (0.5, 1.0, and 2.0 mg/ml) of these secondary metabolites extracted by ethyl acetate and *n*-butanol from the above six endophytic fungal species were tested against three pathogenic fungi isolated from infected tomato plant (*E. brefeldianum*-EBT-1, *P. echinulatum*-PET-2, and *A. phragmospora*-APT-3), whereas these pathogens showed promising sensitivity to these fungal secondary metabolites. In conclusion, this is the first report on the isolation of endophytic fungi from *E. geniculata* and evaluation of their antifungal activity.

**Keywords** *Euphorbia geniculata* · Endophytes · Secondary metabolites · Antifungal activity

## Introduction

*Euphorbia geniculata* (syn. *E. heterophylla*) commonly known as spurge weed belongs to the family Euphorbiaceae which represented by trees, shrubs, and herbs. This plant was characterized by the presence of white milky latex which is more or less toxic (Kumar et al. 2010). It was locally abundant, erected about 3 ft. high and annual weed (Fred-Jaiyesimi and Abo 2010). In East Africa, *E. geniculata* is used for the treatment of gonorrhoea, as a purgative, a lactogenic agent and as a cure for migraine and warts (Dokosi 1998;

Yesilada et al. 1999; Falodun and Agbakwuru 2004; Falodun et al. 2003; James and Friday 2010). The latex of the plant is used as fish poison, insecticide, and poisons (Rodriguez et al. 1976). Moreover, the antibacterial and anti-inflammatory activities of *E. geniculata* have been reported (Falodun et al. 2003, 2006; Okoli et al. 2009).

Endophytic fungi are commonly found in almost all plants. Most endophytic fungi belong to the Ascomycetes and fungi Imperfecti. Under normal circumstances, they can survive in plants for all or part of their life without causing any damage or diseases (Petrini et al. 1993; Ali et al. 2018). Some endophytic fungi were isolated from *Euphorbia* species such as *Chaetomium strumarium* from *E. hirta* (Anitha and Mythili 2017), *Fusarium sp.* from *E. pekinensis*, and *E. milii* (Dai et al. 2008; Rao et al. 2018), while the endophytic fungal population in *E. geniculata* was not studied yet.

Many studies revealed that endophytes play an important role in host protection against pathogens (Azevedo et al.

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2000; Firáková et al. 2007; Giménez et al. 2007). In this case, the endophytic fungi establish mutualistic symbiosis. Many are capable of synthesizing antimicrobial secondary metabolites, which support plants defense against pathogenic microorganisms, and enhance plant growth (Carrol 1988; Schulz et al. 2002; Strobel 2003; Corrado and Rodrigues 2004; Owen and Hundley 2004; Strobel et al. 2004; Giménez et al. 2007). It is believed that screening for antimicrobial compounds from endophytes is a convenient way to overcome the increasing threat of drug-resistant microbes of human and plant pathogen (Tan and Zou 2001; Yu et al. 2010).

In this study, we focus on the isolation of endophytic fungi from the medicinal herb *E. geniculata*. Then, to investigate the antagonism between these endophytic fungi and some selected plant pathogens, the antifungal activity of secondary metabolites produced by selected endophytes against some tomato pathogens. Finally, comparing the spiking secondary metabolites of the host plant (*E. geniculata*) and inhabiting endophyte.

## Materials and methods

### Plant materials

*Euphorbia geniculata* plant was collected from their growing habitat in the campus of Aswan University, Aswan governorate, Egypt. Fourteen healthy medicinal plants were selected from three different sites in Aswan university area. The climatic environment in this region ranged between moderately cold dry in winter to very hot summer (Abdelrahman et al. 2016; Ali et al. 2018).

### Isolation and identification of endophytic fungi from *Euphorbia geniculata*

*Euphorbia geniculata* plants were collected and surface sterilized in 70% (v/v) ethanol for 1 min and then in 5% (v/v) sodium hypochlorite solution for 5 min. Sterilized plants were subsequently washed twice with sterilized distilled water (Rossman et al. 1998). The separated plant parts (roots, stems, and leaves) were longitudinally cut into 0.5–1.0 cm segments, and the resultant specimens were directly placed on a sterilized Petri dish containing Potato Dextrose Agar (PDA) medium. Three replicated plates were incubated at 28 °C and other three replicas at 45 °C for 2–3 weeks. The growing fungi were identified on the basis of their morphological characteristics according to Raper and Thom (1949), Raper and Fennell (1965), Ellis (1971, 1976), Booth (1977), Christensen and Raber (1978); Pit (1979, 1985), and Moubasher (1993).

### Pathogenic fungi

Six plant pathogenic fungi, *Eupenicillium brefeldianum*-EBT-1, *Penicillium echinulatum*-PET-2 and *Alternaria phragmospora*-APT-3 isolated from the infected tomato leaves and fruits, in addition to, *Alternaria alternata*-AAP-1 from infected pepper fruits were obtained from mycology lab of faculty of science, Aswan University. *Fusarium oxysporum*-FOP-1 from the rotting roots of palm, *Fusarium verticilloid*-FVZ-1 from *Zea maize* seeds obtained from Agriculture Research Center in Aswan Governorate.

### Dual-culture experiment

In vitro screening for antagonistic activity of all isolated endophytic fungi against six pathogenic fungal strains was evaluated by dual culture assay (Jinantana and Sariah 1998; Albert et al. 2011). Control plates were established by co-inoculating pathogens with PDA agar plug. All tests were conducted in three replicates at  $28 \pm 2$  °C. The diameter of pathogen colony was measured regularly. The inhibition percentage (IP %) of pathogen was recorded after incubation for 5 days following the formula:

$$\% \text{ Inhibition} = \frac{D_c - D_s}{D_c} \times 100,$$

where  $D_c$  is the average increase in mycelial growth in control, and  $D_s$  is the Average increase in mycelial growth in treatment (Singh and Tripathi 1999).

### Secondary metabolites extractions from selected endophytes

The most active isolates (*A. flavus*, *A. fumigatus*, and *F. lateritium*) and the lowest (*F. culomrum*, *C. herbarum*, and *S. thermophile*) were incubated with potato dextrose broth (PDB) in 500 ml flask under shaking condition (200 rpm) for 7 days. Ethyl acetate (EtOAc) was added to the fungal culture and incubated overnight, and then, the extract was separated and dried, *n*-butanol (*n*-BuOH) was added to the remaining media and kept overnight, and then, extraction was separated and dried (Abdel-Motaal et al. 2010a).

### Control of some tomato pathogenic fungi experiment

Fungal ethyl acetate and *n*-butanol extracts which mentioned above were incorporated into the potato dextrose agar at different concentrations (0.5 mg/ml, 1.0 mg/ml, and 2.0 mg/ml), mixed well and kept at 4 °C overnight. The mycelial disc (0.7 cm in diameter), was deposited in the center of

the plate (6.0 cm in diameter) according to Poisoned food method which used to evaluate the antifungal effect against pathogens (Balouiri et al. 2016). After further incubation for 7 days at 28 °C for the fungal strains tested, the diameters of fungal growth for control and treated plates were measured, and the inhibition percentage was calculated (Singh and Tripathi 1999).

The minimum inhibitory concentration (MIC) values of each extract for fungal growth compared to the control sample were determined according to Li and Rinaldi (1999) and Abdel-Motaal et al. (2010b).

### ***Euphorbia geniculata* plant extraction**

The plant materials were collected, washed, and dried. The dried plant materials were powdered by grinding and extracted with EtOAc and n-BuOH similar to fungal extraction process (Abbasi et al. 2013).

### **Screening of secondary metabolites produced by *E. geniculata* and some associated endophytes**

Detection of active secondary metabolites constituents was carried out for *E. geniculata* and the mentioned above fungal extracts, such as alkaloids, flavonoids, terpenoids, steroids, phenols, tannins, and saponins according to Visweswari et al. (2013).

### **Statistical analysis**

Data obtained were subjected to a one-way analysis of variance (ANOVA). Significant differences between the control and treatments ( $P \leq 0.05$ ) obtained by student's *t* test using SPSS software (SPSS20.0, SPSS Inc., USA). Values shown in the figures are the means  $\pm$  standard errors (SEs) of four independent replicates. Correlation analysis was carried out using Corrplot in R program (R- 3.4.3, <https://www.r-project.org/>).

## **Results and discussion**

### **Endophytic fungi associated with *E. geniculata***

From 14 individual plants corresponding to *E. geniculata* (roots, stems, and leaves), a total of 21 fungal species belonging to 15 fungal genera in addition to one variety were isolated. According to our knowledge, this is the first report describing the isolation of endophytic fungi associated with *E. geniculata* plants. Peláez et al. (1998); Schulthess and Faeth (1998) reported that many fungi were known to grow endophytically in many plants such as *Festuca arizonica*, *Phragmites australis*, and *Scirpus maritimus*. *Aspergillus*

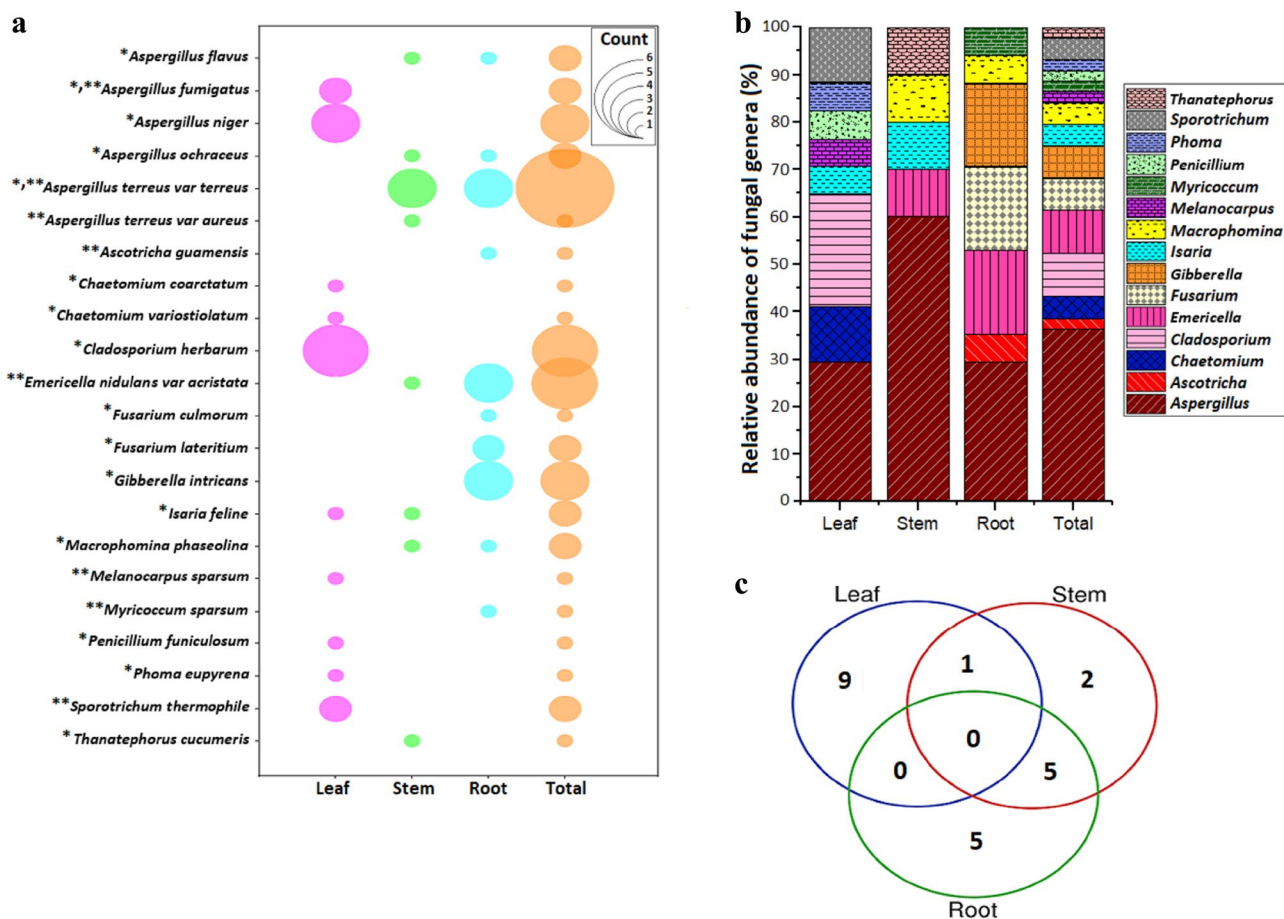
was the most predominant fungal genus in all plant parts of *E. geniculata* (36%), followed by *Cladosporium* and *Emmericella* (9% for each). Anith and Mythili (2017) isolated *Chaetomium strumarium* as endophytic fungi from *E. hirta*, also from *Euphorbia pekinensis*, Dai et al. (2008) isolated *Fusarium* sp. as endophytic fungi. Many endophytes restricted to a small tissue area or in specific plants parts such as roots, leaves, or twigs (Stone et al. 2004). In this study, the relative abundance of *Aspergillus* in the different parts of the studied plant was (28%, 60%, and 28% of the leaves, stem, and roots, respectively) represented by five species and two varieties of *A. terreus*. Distribution of fungal species was variable in different parts of the studied plant, whereas *Cladosporium herbarum* was the most common fungal species in leaves (4 colonies), *A. terreus* var. *terreus* in the stem (3 colonies), and *A. terreus* var. *terreus*, *E. nidulans* var. *acristata*, and *Gibberella intricans* were common in the root (3 colonies for each) (Fig. 1a, b). It was interesting to recognize that only one fungal species (*Isaria feline*) present in both stem and leaves, while *A. flavus*, *A. ochraceus*, *A. terreus* var. *terreus*, *E. nidulans* var. *acristata*, and *Macrophomina phaseolina* were in mutual occurrence in both stem and root (Fig. 1c).

Some isolated endophytic fungal species were thermo-tolerant (*A. fumigatus* and *A. terreus* var. *terreus*) which are able to grow at 28 °C and 45 °C. Others were thermophilic fungi (*A. terreus* var. *aureus*, *Ascotricha guamensis*, *E. nidulans* var. *acristata*, *Myricoccum sparsum*, *Melanocarpus sparsum*, and *Sporotrichum thermophile*) growing at 45 °C only but the remaining fungi were mesophilic (grow at 28 °C only) (Fig. 1a).

Similar endophytic fungal species were isolated from variable plants, whereas *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. ochraceous*, *A. terreus*, and *C. herbarum* isolated from *Hyoscyamus muticus* (Abdel-Motaal et al. 2010a), *Emericella nidulans* from *Ipomea batatas* plant (Hipol 2012), *Fusarium lateritium* from Yew plant (Strobel et al. 1996), *Penicillium funiculosum* from soybean plants (Khan and Lee 2013), and *Phoma eupyrena* isolated from leaves of *Avicennia schaueriana* (Costa et al. 2012).

### **Antagonistic activity of the isolated endophytic fungi against selected pathogenic fungi**

The dual culture test revealed that all endophytic fungi isolated from *E. geniculata* could inhibit most of the studied pathogenic fungi. The serious pathogen APT-3 which could damage the tomato crop by causing leaf spot was controlled by the thermophilic endophytic fungal species, *A. terreus* var. *aureus* and the mesophilic fungal species *Chaetomium variostiolatum* and *A. flavus* with inhibition percentage 41.67%, 42.46%, and 41.32%, respectively. The other tomato pathogen, EBT-1 which spoil tomato fruit was inhibited by



**Fig. 1** Diversity profile of endophytic fungi associated with *Euphorbia geniculata*. **a** Total count of fungal species, asterisks indicate isolation temperatures (\*temp=28 °C; \*\*temp=45 °C); **b** relative

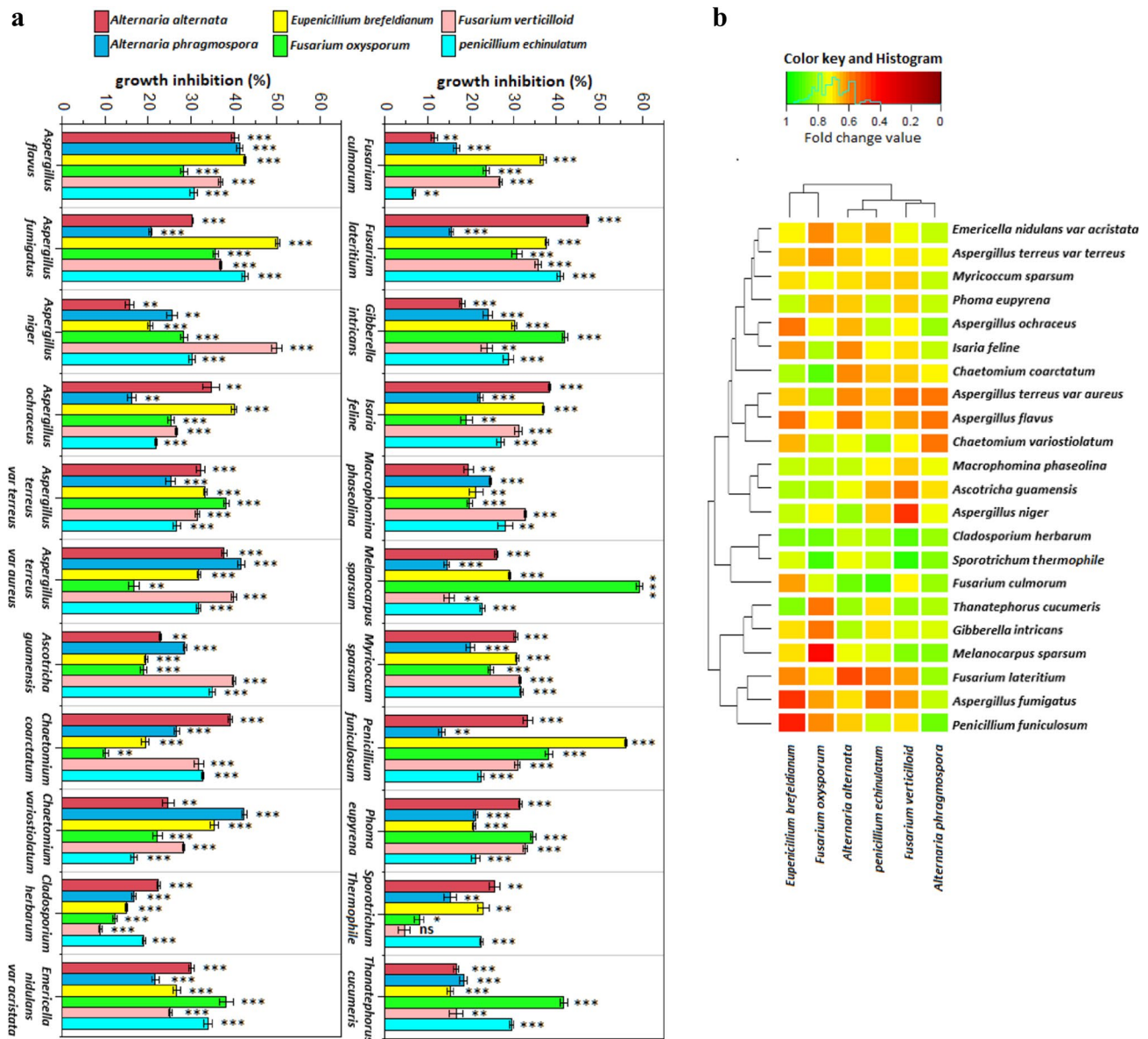
abundance of fungal genera; **c** venn diagram of the total number and mutual occurrence of fungal species of the root, stem, and leaf

the mesophilic fungal species *P. funiculosum*, *A. flavus*, *A. ochraceus*, and thermo-tolerant fungal species *A. fumigatus* with inhibition percentage 56.11%, 42.50%, 40.09, and %50.20%, respectively. PET-2 (fruit tomato pathogen) growth was controlled by the thermo-tolerant fungus, *A. fumigatus* and the mesophilic fungus *Fusarium lateritium* with inhibition percentage of 42.63% and 40.88%, respectively. The palm rotting root pathogen (FOP-1) was inhibited by the mesophilic fungal species *Gibberella intricans*, *Thanatephorus cucumeris*, and the thermophilic fungal species *Melanocarpus sparsum* with percentage (41.93%, 41.67%, and 59.37%, respectively). The *zea maize* pathogen FVZ-1 was inhibited by *A. niger* with inhibition percentage 50.0%. *Alternaria phragmospora* was reported as pathogen that causes damage to the seedlings of radish (Emden 1970) and *A. alternata* caused rotting of tomato fruits (Abdel-Mallek et al. 1995). Berbee (2001) proved that *F. oxysporum*, *Eupencillium sp.*, and *A. alternata* were plant and mammal pathogens. *Penicillium echinulatum* was recorded as pathogen which causes blue mold symptoms to grape fruits (Kim

et al. 2007). Liu et al. (2001) reported that 21 endophytes isolated from *Artimisia annua* had strong antifungal activity against six phytopathogenic fungi causing wheat take-all, sharp eye spot, and common rot. Rhizospheric fungi such as *A. flavus*, *A. niger*, and *A. terreus* inhibit the growth of *F. oxysporum* (Alwathnani and Perveen 2012). Faba bean (*Vicia faba* L.) leaf spot disease caused by *A. alternata* has been limited by *Trichoderma harzianum* (Kayim et al. 2018). In this study, AAP-1 (pepper pathogen) was controlled by the mesophilic fungal species *A. flavus* and *F. lateritium* (40.17% and 47.29%, respectively). In vitro *F. verticilloid* was inhibited by *Chaetomium zea* (Gao et al. 2010).

Figure 2b shows that the tested pathogenic fungi reaction to the endophytic fungi was variable. Whereas the pathogenic EBT-1 and FOP-1 in a clade, while AAP-1 and PET-2 in another clade and the last clade included FVZ-1 and APT-3. Each clade showed similar sensitivity and resistance to the endophytic fungi. Moreover, the endophytic fungi, *A. flavus*, *A. fumigatus*, and *F. lateritium* virulence, was clear as the most active effect against almost all pathogenic fungi.





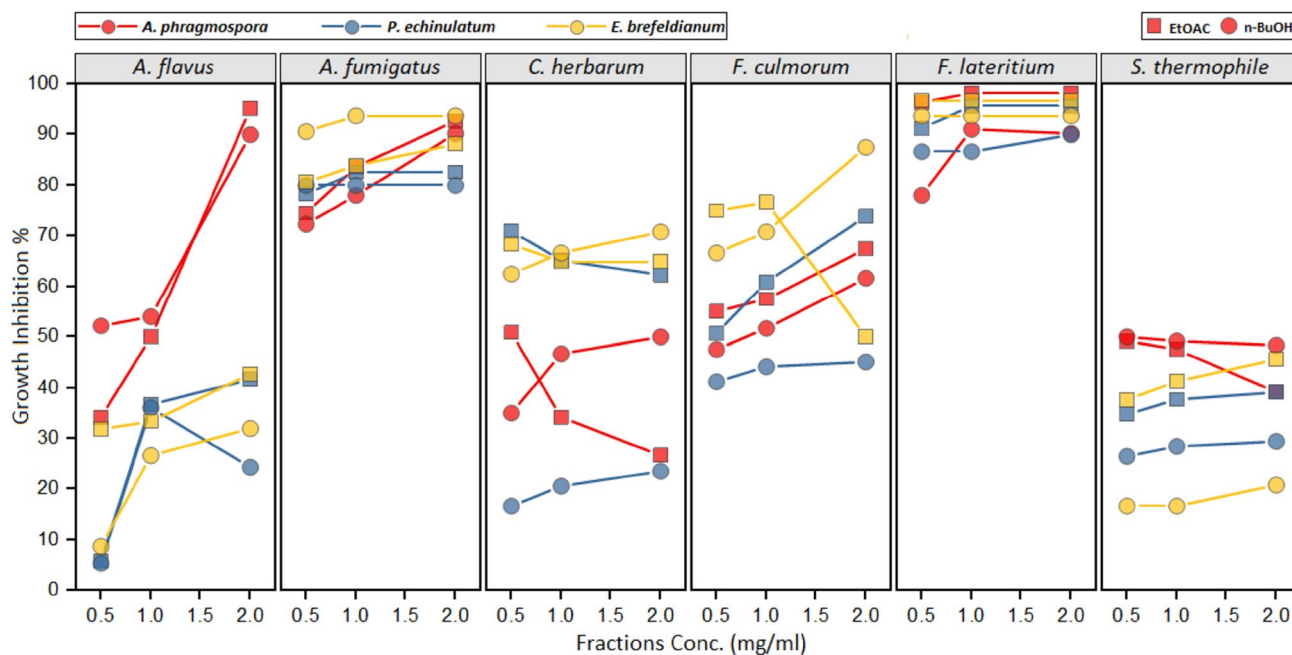
**Fig. 2** Antagonistic activity of Endophytic fungi isolated from *Euphorbia geniculata* against different plant pathogens, **a** pathogen growth inhibition percentage, asterisks indicate significance of inhibi-

tion (\*low; \*\*moderate; \*\*\*high); ns (not significance), **b** heatmap illustrate the sensitivity of selected plant pathogenic fungi to the endophytic fungi

### Antifungal activity of selected endophytic fungal extracts against three tomato's pathogenic fungi

The percentage of growth inhibition of the selected phytopathogenic fungi (EBT-1, PET-2, and APT-3) by different concentrations (0.5, 1.0, and 2.0 mg/ml) broth of selected endophyte culture (*A. flavus*, *A. fumigatus*, *C. herbarum*, *F. lateritium*, *F. culomrum*, and *S. thermophile*) was determined by measuring the diameter of inhibition zones and minimum inhibitory concentration (MIC mg/ml) (Balouiri et al. 2016), and the data were shown in (Fig. 3) not paralleled to the antagonistic action mentioned above, the

antifungal activities of selected endophyte cultures were significantly different. The three concentrations of the selected endophytes cultures were active against the three tested plant pathogens with variable inhibitory effects. *Fusarium lateritium*, an endophyte producing the most active substance, the EtOAc fractions of which exhibited abroad antifungal spectrum at a concentration of 0.5 mg/ml (MIC<sub>90</sub>) followed by n-BuOH fraction (MIC<sub>78,93</sub>) against all tomato pathogens. In contrast, n-BuOH fraction of *A. fumigatus* culture was more effective than EtOAc fraction especially against EBT-1 (MIC<sub>72-90</sub>, MIC<sub>74-80</sub>), respectively. However, the pathogen APT-3 had marked sensitivity towards both



**Fig. 3** Antifungal activity (inhibition percentage) of selected fungal endophytes secondary metabolites extracted by ethylacetate (EtOAc) and *n*-butanol (*n*-BuOH) with the concentrations of (0.5, 1.0, and 2.0 mg/ml) against three tomato pathogenic fungi

*n*-BuOH and EtOAc of *A. flavus* with the three concentrations which inhibited at 0.5 mg/ml with MIC<sub>50</sub> *n*-BuOH, MIC<sub>90</sub> at 1.0 mg/ml EtOAc and MIC<sub>95</sub> at 2.0 mg/ml EtOAc. Furthermore, the MICs of EtOAc fraction of *C. herbarum* and *F. culmorum* against all tested pathogen was determined to be 50–75, while *n*-BuOH fraction possessed moderate activity 40–66 at 0.5 mg/ml for each of them. *Sporotrichum thermophile* had the lowest inhibition activity in comparison with endophyte cultures tested on phytopathogenic fungi, the *n*-BuOH fraction of which only detected with MIC<sub>50</sub> at concentration 0.5 mg/ml against APT-3. Both EtOAc and *n*-BuOH fractions of *A. flavus*, *A. fumigatus*, and *F. lateritium* showed broader antifungal spectrum and stronger toxicity mycelium growth of APT-3.

In the microscopic examination, swollen in hyphae and formation of chlamydo spores were recorded in APT-3 which treated with both extracts (EtOAc and *n*-BuOH) of *A. flavus*, *A. fumigatus*, and *F. lateritium* compared to the control. Sporulation also affected compared to control, whereas asexual spores were disappeared in treated colonies. In addition, melanin pigmentation of hypha under microscope was reduced in 2.0 mg/ml of both extracts in *A. flavus*, *A. fumigatus*, and *F. lateritium*. Pathogen melanins may act as a supportive weapon in host penetration and suppression of host's responses (Mérillon and Ramawat 2017). Thus, reducing melanin formation makes the pathogen hypha very weak and easy to control. The pigmentation of the spores was reduced in PET-2 colonies which treated with 1.0 mg/ml *n*-BuOH fraction of *A. flavus*. The antifungal activity of the

chemicals biosynthesized by the plant endophytes has been reported by several groups (Rojas et al. 1992). The present results indicate that the metabolites (EtOAc and *n*-BuOH fractions) of endophytic fungi from *E. geniculata* have extensive inhibition to the growth of phytopathogenic fungi but with variable inhibitory effect. This could be related to variation in quality and quantity of active compounds in their extracts and could be attributed to structural nature of the pathogen and endophytic fungi constituents (Yao and Moellering 1995).

### Twining between the secondary metabolites of *E. geniculata* plant and some selected endophytic fungi inhabiting it as a model

Natural products are naturally derived compounds present as metabolites or byproducts from microorganisms, plants, or animals. Microorganisms are one of the most important sources of these compounds. Recently, there is a special focus on endophytic fungi residing medicinal plants, whereas they are able to synthesize similar secondary metabolites of great potential for the discovery of new bioactive compounds (Kusari et al. 2008). Endophytes are synergistic to their host in producing secondary metabolites to avoid other microorganism and insects attack (Strobel and Daisy 2003). Some endophytic fungi would produce different bioactive compounds, such as alkaloids, terpenes, and flavonoids to increase the resistance to biotic and abiotic stresses for their host plants (Firáková et al. 2007; Rodriguez et al.

2009). Steroids, terpenoids, flavonoids, etc. were detected in many endophytes (Zhang et al. 2006). Flavonoids are able to inhibit spore germination of plant pathogens; therefore, they have been proposed for the use against fungal pathogens (Cushnie and Lamb 2005).

In the current study, we have tried to confirm this relationship between the host plant (*E. geniculata*) and selected endophytic fungi (*A. flavus*, *A. fumigatus*, *C. herbarum*, *F. lateritium*, *F. culomrum*, and *S. thermophile*) and their co-operation to produce similar secondary metabolites. The highest antagonistic activity fungi were (*A. flavus*, *A. fumigatus*, and *F. lateritium*) and that the lowest activity (*C. herbarum*, *F. culomrum*, and *S. thermophile*) against most studied pathogenic fungi in this work (Fig. 2).

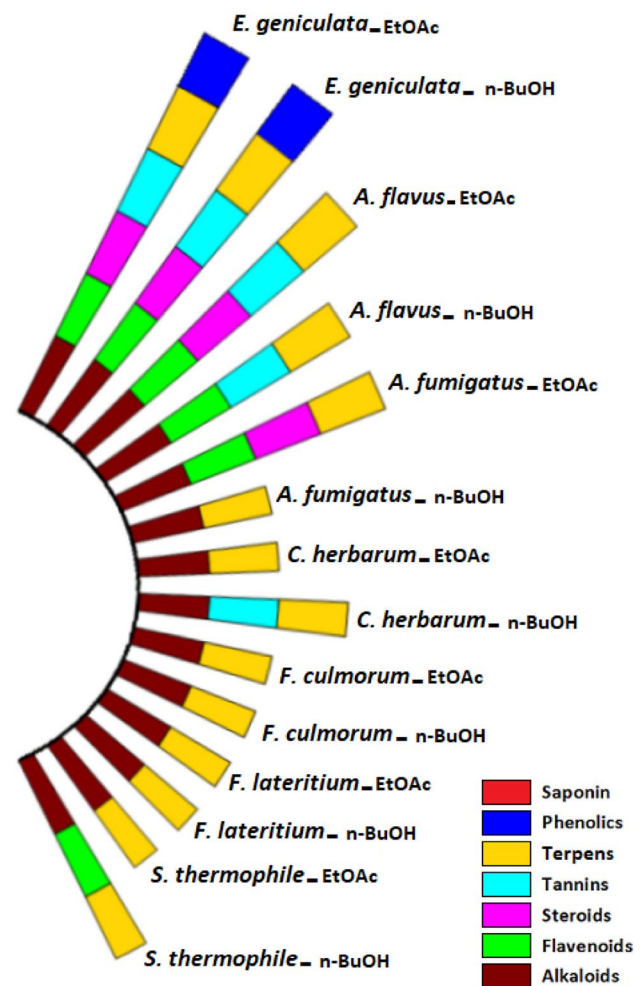
Interestingly, the secondary metabolites, alkaloids, and terpenes were detected in both EtOAc and n-BuOH extracts of *E. geniculata* herb and all the selected endophytes, while saponin was absent in both host plant and its associated tested fungi (Fig. 4). The most twinning fungus was *A. flavus*, whereas besides alkaloids and terpenes, other chemical classes were detected such as flavenoids and tannins in both fractions and steroids in EtOAc fraction. In addition, the EtOAc fraction of *A. fumigatus* contains two more active chemical classes (flavenoids and steroids). While the n-BuOH fraction contains tannins and flavenoids in *C. herbarum* and *S. thermophile*, respectively (Fig. 4). These results may open a complete new dimension for the production of natural antimicrobial compounds in an extremely effective manner, stating that the relationship between endophytic fungi and their host medicinal plants is completely understood. A result that warrants more investigation in the future in our lab is that phenolic compounds were detected only in plant extract and how all selected fungi respond to these compounds.

## Conclusion

This study reported for the first time the endophytic fungal community associated with the medicinal herb *Euphorbia geniculata* and screening their antifungal activity. Endophytic fungi associated with *E. geniculata* are a promising safe tool to control urgent plant disease. Twinning of secondary metabolites compounds between plant and its associated endophytes confirm the positive role of endophytes in supporting their host plant.

Further detailed studies of these endophytes after colonizing the tomato plant and their roles in protection against pathogens and improving plant morphology and physiology are currently being undertaken in our laboratory.

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**Fig. 4** Twinning between some secondary metabolism extracted by ethylacetate (EtOAc) and *n*-butanol (*n*-BuOH) from both *E. geniculata* plant and the selected endophytic fungi residing it

providing facilities in the unit laboratories to accomplish this work. We are very thankful to the researcher Mr. Rasheed Zidan for his help in the statistical analysis.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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