

***In vitro*- exploration of fungal endophytes of Egyptian *Cynara scolymus* L. (artichoke) and investigation of some their bioactive potentials**

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Abstract

Plant-associated microorganisms, especially endophytic fungi, represent an untapped resource for the discovery of biologically active natural products. The objectives of this study were to isolate, identify endophytic fungi, and produce their bioactive metabolites from the leaves of two varieties of Egyptian artichoke namely: French Hyrious and Egyptian Baladi. In addition, assess of their total antioxidant capacity (TAC), total phenolic content (TPC) and total flavonoid content (TFC). The results of this novel study show a total of 35 endophytic fungal species belonging to 14 genera were isolated from both artichoke leaves with gross total counts of colonizing endophytic fungi ranged from 71 to 123 cfu which is matching 78.89% to 136.67% of colonization frequency. All taxa recovered were assigned to Ascomycetes. In addition, there is high species richness and diversity indices of endophytic filamentous fungi in the leaves Baladi Artichoke as compared to its French rival. *Alternaria alternata* were found to be the most frequently isolated dominant species. The TAC, TPC and TFC of the fungal cultures ranged from 163 to 681 mgAAE/gDW, 10.38 to 40.30 mgGAE/gDW, and 13.92 to 173.55 mgQE/gDW, respectively. Furthermore, LC-ESI-MS/MS confirmed the presence of 1,3-dicaffeoylquinic acid and 1,5-dicaffeoylquinic acid in the methanolic extract of *A. alternata*. Hence, this novel study suggested that the metabolites produced by endophytic fungi associated with Egyptian artichoke could be explored as an economic and potential natural resources with diverse pharmaceutical and biological activities.

Keywords: Artichoke, fungal endophytes, antioxidant capacity, total flavonoids, total phenolics contents.

1. Introduction

As an alternative to drug therapy, it is desirable to use foods, food components, and/or medicinal plants for the

prevention and treatment of different diseases and thereby avoid the adverse effects of organically synthesized drugs as well as the high-cost drug therapy (Roghani-Dehkordi and Kamkhah, 2009). Medicinal plants are the richest bio-resource of drugs from traditional systems and modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. World Health Organization (WHO) encourages, advises and promotes herbal remedies in national health care programmers due to the low cost of these drugs (Amita Pandey, 2014). Recently, medicinal plants have received the attention of the pharmaceutical and scientific societies. The biomolecules of pharmaceutical and nutraceutical interest are mainly represented by flavonoids and phenolic acids, particularly caffeic acid and its derivatives mono- and bi-caffeoylquinic acids (Saez *et al.*, 2013). Recent studies have reported that bioactive phenolic constituents from plants and endophytic microbe have a wide variety of biological activities, such as antioxidants, hepatoprotective, anti-inflammatory, antifibrosis, and antiviral activities, and anticarcinogenic effects against different cancer cell line (Chutulo and Chalannavar, 2018; Gebhardt and Fausel, 1997; Liang *et al.*, 2012; Llorach *et al.*, 2002; Sihem DABBOU *et al.*, 2017; Simona Spînu *et al.*, 2017; Wang *et al.*, 2014). Also, Plant-derived bioactive compounds have achieved an extensive interest due to their versatile applications and beneficial effects; for instance, antioxidant activity, digestive stimulation action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic anticarcinogenic potential (Jacociunas *et al.*, 2013; Wojdylo *et al.*, 2007), on health promotion because the low side effects in comparison to the synthetic drugs (Farag *et al.*, 2013; Ncube and Afolayan, 2008). Th. Thus, many publications recorded its medicinal values as natural compounds.

Artichoke (*Cynara scolymus* L.) is an ancient herbaceous perennial plant species belonging to the Asteraceae family, genus *Cynara* (Farag *et al.*, 2013; Xia *et al.*, 2014), originating from the Mediterranean region, and it is now cultivated worldwide. According to FAO estimation, Italy was the world's largest artichoke producer in 2016 followed by Egypt, Spain Peru (FAO, 2016). The artichoke was used as food and medicine by ancient Egyptians, Greeks, and Romans. Three *Cynara scolymus* (artichoke) cultivars: American Green Globe, French Hyrious, and Egyptian Baladi have been cultivated in the Northern part of Egypt (Farag *et al.*, 2013). Moreover, the artichoke is one of the oldest medicinal plants with multiple health benefits (Xia *et al.*, 2014). Artichoke has many natural antioxidants and multiple pharmacological actions. The antioxidant properties of artichoke are thought to be related to its abundant phenolic composition (El Morsy and Kamel, 2014). Recent studies have reported that Artichoke has antitoxic activity (Heidarian and Rafieian-Kopaei, 2013), cholesterol-reducing effect (Kusku-Kiraz *et al.*, 2010) anticarcinogenic, antigenotoxic, hepatoprotective, bile expelling, diuretic, and anti-inflammatory, as well as anti-HIV, antifungal, and antibacterial (Abu-Reidah *et al.*, 2013). Additionally, its leaves are used in traditional medicine as an herbal medicament for the treatment of hepatitis, choleretic, diuretic, hyperlipidemia, obesity and dyspeptic disorders (Nassar *et al.*, 2013). Artichoke was ranked fourth out of more than 1000 food products in antioxidant content, and the first among several selected vegetable crops (Yoo *et al.*, 2012). Artichoke by-products (leaves, external bracts and stems) represent an enormous amount of discarded materials (representing 80–85% of the biomass) (Lattanzio *et al.*, 2009). Recently, the possibility to recover them has been proposed for economic, environmental concerns, adding value to agro-industrial by-products (Ruiz-Cano *et al.*, 2014).

Endophytic fungi residing internally in the healthy living plants tissues without causing any immediate harm to their host (Yadav *et al.*, 2014). All higher plants host one or more endophytic microbe on this green planet (Strobel and Daisy, 2003) from the arctic to the tropics. In recent years, endophytic fungi have been received great attention because they have the ability to benefit host plant growth. The research on endophytes showed that they are obviously a rich and reliable source of bioactive secondary metabolites and chemically novel compounds with huge medicinal, agricultural potential, and industry (Yi-Shuan Chen *et al.*, 2015). Endophytic fungi and their associated higher plants are shown to be a good source of novel antioxidants (Sandhu and Gupta, 2015). The endophytic fungi seem to yield bioactive compounds, originally isolated from their host plants, as well as bioactive metabolites that are clearly different from those of plants and feature unique structural characteristics, which may have potential use in agriculture and medicine (Silva-Hughes *et al.*, 2015; Stierle *et al.*, 1995; Wang *et al.*, 2013). In addition, it is well known as a microbial source of a valuable products and it is generally easier and

more economically to produce (Chen *et al.*, 2010). Metabolites isolated from the fungal endophytes are good sources of novel secondary metabolic products possess diverse and unique structural groups (Chutulo and Chalannavar, 2018; Schulz *et al.*, 2002; Sharma *et al.*, 2016). Numerous work has been focused on the bioactive potential of endophytes, such as antiviral, antibacterial, antifungal, anticancer, antiviral, insecticide, antidiabetic and immunosuppressive activities, but few is known about their antioxidant capacity (Yadav *et al.*, 2014). Shukla *et al.* was isolating endophytic fungi from *Ocimum sanctum* and showing their antioxidant activity. A strong antioxidant activity was found in the endophytic fungus of *Phyllosticta* sp. isolated from *Guazumato mentos* plant. Therefore, by fermentation an endophytic fungus isolated from Artichoke (*Cynara scolymus* L.) can produce bioactive compounds such as chlorogenic acid, caffeoylquinic acids, caffeic acid and its derivatives (apigenin and luteolin) just like its host plant. This will protect the plant from extinction. Thus, an immense opportunity to discover novel natural products from hitherto less investigated fungal endophytes that reside in hitherto assumed medicinal plants. Also, microbial natural products represent a huge and largely important resource of unique chemical structures and probably play an important role for adaptation as a response to habitat (Gunatilaka, 2006).

Based on this theory, the aim of this study is: (1) to isolate and identify each endophytic fungus from two artichoke cultivars (French Hyrious and Egyptian Baladi), (2) to investigate the total polyphenols and the total flavonoids content and (3) to evaluate the antioxidant activities using in vitro study of the Methanolic crude extracts of *C. scolymus* L. and its fungal endophytes. To the best of our knowledge, no previous work on fungal endophytes and its metabolites investigated from Artichoke plant, so this research considered a new record on this investigation. By implementation of our present work, it becomes easier, cheaper and time consuming to get the bioactive compounds (like flavonoids, phenolic compound, anti-microbial or anti-cancer compounds) of plants by their endophytic fungi via fermentation processes. Additionally, we open the way for all to discover novel endophytes isolated from new medicinal plants to get novel bioactive compound. Also, that is invitation for preserving medicinal plants by decrease plant uprooting and use their endophytes to improve environmental science research.

2. Materials and methods

2.1. Chemicals and reagents

Aluminum chloride, sodium acetate, sulfuric acid, Folin-Ciocalteu (FC) reagent, gallic acid (GAE), ascorbic acid (AA), quercetin (QE), luteolin-7-O-rutinoside, 1,3-dicaffeoylquinic acid (1,3-diCQA), 1,5-dicaffeoylquinic acid (1,5-diCQA), chlorogenic acid, caffeic acid, ethanol, methanol and formic acid of analytical or HPLC grade were purchased from Sigma Aldrich. Ammonium molybdate was purchased from Fluka Co. (Bushes, Switzerland). Assay of all standards, solvents, and

reagents were $\geq 99\%$. Water was purified by using a Milli-Q system.

2.2. Plant material

Two Artichoke varieties, *Cynara scolymus* L, cultivars namely; Egyptian Baladi and French Hyrious, were collected from Markaz Abu Al Matamir, Beheira governorate, Egypt, during February-April 2015. The collected plants have been confirmed by plant taxonomists at Botany and Microbiology Department, Faculty of Science, Assiut University, 71516-Assiut, Egypt.

2.3. Evaluation for presence of fungal endophytes

2.3.1. Surface sterilization

To ascertain the possible presence of the fungus in the internal plant tissues, one hundred separate healthy artichoke plants were collected from the field, but we choose 10 samples randomly for investigation. From each sample, three leaves were randomly selected and individually washed thoroughly in running tap water to remove dust and debris then surface-disinfected by washing with 70% ethanol for 2 min, submersing in 5-6% sodium hypochlorite for 3 min then 70% ethanol for 30 sec. respectively, followed by two rinses in sterile autoclaved distilled water and finally were dried between sterilized filter paper (Arnold *et al.*, 2001). Foliar leaves were cut using a sterile scalpel into 1 cm² in diameter with and without midrib. Then, three leaf segments were inserted in sterilized petri dishes (9 cm diameter) individually containing sterile media, replicated three times. All Petri plates were incubated at 28 ± 2 °C in darkness for 10-15 days.

2.3.2. Used cultivated media

Three different types of media were used in this investigation namely; potato dextrose agar (PDA), Glucose- Czapek's (CZ) and Cellulose-Czapek's (C) agar media (Smith and Onions, 1994). To count and collect all filamentous fungal endophytes which can grow on this media and was expected in studied leaves.

2.3.3. Efficacy of surface sterilization

To confirm the sterilization, the final rinse water was plated to determine whether the sterilization process was successful in eliminating epiphytic microorganisms. Also, another sterilized leaf segments (10 gm) were placed in a conical flask containing 200 mL sterile distilled water, the suspension was shaken by a mechanical shaker at 150 rpm for 10 minutes. One mL of this dilution (1/200) was transferred aseptically into five replicates of sterilized Petri dishes and 12-15 mL of PDA agar medium, cooled to just above the solidifying temperature, were added to each dish. The dishes were rotated by hand for good dispersion. The absence of growth of any fungi on the all Petri dishes confirmed that the surface sterilization procedure was effective (Schulz *et al.*, 1993). All Petri plates were incubated at 28 ± 2 °C in darkness for 10-15 days.

2.3.4. Phenotypic identification of fungal endophytes

Periodically the colonies were examined, counted and each colony that emerged from segments was transferred to antibiotic-free Potato Dextrose Agar medium (PDA) to aid macroscopic and microscopic identification using morphological criteria of fruiting body and spores. Standard taxonomic manuals were used to identify the fungal genera. Any fungal growth was sub-cultured on individual plates containing PDA for preservation at Multidisciplinary Research Center (MRCE), Assiut University for further work. The percentage of fungal endophytes present in the tissues sampled was assessed.

2.3.5. Calculation of colonization frequency

Colonization frequency (CF), %, of an endophyte species was calculated as reported (Pandey *et al.*, 2003; Suryanarayanan, 2003).

$$CF \% = N_{col} / N_t \times 100$$

Where: N_{col} is the number of segments colonized by a single endophyte, and N_t is the total number of segments examined.

2.4. Preparation of fungal extracts

Fungal extracts were prepared according to the reported method with slight modifications (Sutjaritvorakul, 2011). Briefly, 1 cm² disc of 7-day-old colony of each tested fungal isolated was inoculated into 250 mL Erlenmeyer conical flask containing 150 mL potato dextrose broth (PDB) media. Cultures of colonies were incubated at 120 rpm and 28 ± 2 °C for 10 days. Next, the growing mycelia were extracted using methanol: PDB (2: 1) and homogenized at 16000 rpm. Then, the methanol extracts were filtered through filter paper No. 1 and the filtrates were evaporated under reduced pressure at 40 °C till dryness. Finally, the obtained residues were stored at -20 °C for further investigations.

2.5. Total phenolics content

The total phenolics content (TPC) of all extracts was performed using the Folin-Ciocalteu (FC) reagent with slight modifications of the reported method in the literature (Ainsworth and Gillespie, 2007). Initially, 300 μ L of methanolic extracts, or gallic acid (GA) standard in methanol were mixed well with 0.6 mL of 10% (v/v) FC reagent, then vortexed thoroughly for 10 sec, and incubated at room temperature for 3 min. After the addition of 2.4 mL of 700 mM Na₂CO₃ to the mixture, followed by vortexing, covered and incubating for 2 h at room temperature, the absorbance was measured at 765 nm using a Cary 60, Agilent technologies, UV/VIS spectrophotometer. The TPC was expressed as mggallic acid equivalent (GAE) per g of plant dry weight (mgGAE/gDW). A calibration curve was constructed by using series of calibration standards of GA in methanol (1, 15.62, 31.5, 62.5, 125, 250 and 500 mg/L). All tests were carried out in triplicate and the results were averaged.

Table 1. Total count (TC), percentage of total count (%TC), percentage of colonization frequency (%CF), number of cases of isolation (NCI; out of 10 samples), and occurrence remark (OR*), of endophytic fungi isolated from Egyptian Baladi Artichoke fresh leaves per 90 segments on the three types of media (PDA, CZ, & cellulose)

Types of media	PDA					CZ					C				
Endophytic fungi	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR
Alternaria	58	55.24	64.44	10	H	36	37.89	40	10	H	52	42.28	57.78	10	H
<i>A. alternata</i> (Fr.) Keiss	51	48.57	56.67	10	H	30	31.58	33.33	10	H	48	39.02	53.33	10	H
<i>A. atra</i> (Preuss) Woudenb. & Crous						1	1.05	1.11	1	L					
<i>A. cheiranthi</i> (Lib.) P.C. Bolle	2	1.90	2.22	2	L										
<i>A. chlamydospora</i> Mouch	2	1.90	2.22	1	L	2	2.11	2.22	1	L	1	0.81	1.11	1	L
<i>A. graminicola</i> E.G. Simmons sp	1	0.95	1.11	1	L										
<i>A. citri</i> Ellis & N. Pierce	1	0.95	1.11	1	L	2	2.11	2.22	2	L					
<i>A. mali</i> Roberts											1	0.81	1.11	1	L
<i>A. tenuissima</i> (Kunze) Wiltshire	1	0.95	1.11	1	L						2	1.63	2.22	2	L
<i>A. phragmospora</i> Emden						1	1.05	1.11	1	L					
Aspergillus	11	10.48	12.22	6	H	14	14.74	15.56	8	H	19	15.45	21.11	4	M
<i>A. flavus</i> Link						4	4.21	4.44	3	L					
<i>A. niger</i> Tiegh	10	9.52	11.11	5	M	10	10.53	11.11	5	M	9	7.32	10.00	4	M
<i>A. terreus</i> Thom	1	0.95	1.11	1	L						10	8.13	11.11	3	L
<i>Chaetomium globosum</i> Kunze	1	0.95	1.11	1	L	1	1.05	1.11	1	L	4	3.25	4.44	2	L
Cladosporium						4	4.21	4.44	4	M	4	3.25	4.44	1	L
<i>C. cladosporioides</i> (Fresen.) G.A. de Vries						2	2.11	2.22	2	L	4	3.25	4.44	1	L
<i>C. herbarum</i> (Pers.) Link						1	1.05	1.11	1	L					
<i>C. sphaerospermum</i> Penz						1	1.05	1.11	1	L					
<i>Curvularia australiensis</i> (M.B. Ellis)	1	0.95	1.11	1	L										
<i>Epicoccum nigrum</i> Link											4	3.25	4.44	4	M
<i>Fusarium fujikuroi</i> Nirenberg						4	4.21	4.44	3	L					
<i>Nigrospora oryzae</i> (Berk. & Broome) Petch	15	14.29	16.67	4	M	20	21.05	22.22	9	H	19	15.45	21.11	5	M
Penicillium	5	4.76	5.56	3	L	4	4.21	4.44	3	L	3	2.44	3.33	2	L
<i>P. citrinum</i> Thom	3	2.86	3.33	2	L	1	1.05	1.11	1	L					
<i>p. glabrum</i> (Wehmer) Westling						3	3.16	3.33	2	L	3	2.44	3.33	2	L

[Continued]

Types of media		PDA					CZ				C				
Endophytic fungi	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR
<i>P. fasciculatum</i> <i>Sommerf.</i>	2	1.90	2.22	1	L										
<i>Stachybotrys</i> <i>chartarum</i> (Ehrenb.) S. <i>Hughes</i>	1	0.95	1.11	1	L	2	2.11	2.22	1	L	1	0.81	1.11	1	L
<i>Stemphylium</i>	6	5.71	6.67	4	M	10	10.53	11.11	7	H	15	12.20	16.67	6	H
<i>S. botryosum</i> <i>Wallr</i>	5	4.76	5.56	3	L	5	5.26	5.56	3	M	3	2.44	3.33	1	L
<i>S. vesicarium</i> (Wallr.) E.G. <i>Simmons</i>											5	4.07	5.56	2	L
<i>Stemphylium</i> sp.	1	0.95	1.11	1	L	5	5.26	5.56	4	M	7	5.69	7.78	3	L
<i>Talaromyces</i>	2	1.90	2.22	2	L						2	1.63	2.22	2	L
<i>T. duclauxii</i> (Delacr.) <i>Samson, N.</i> <i>Yilmaz, Frisvad &</i> <i>Seifert</i>	1	0.95	1.11	1	L						1	0.81	1.11	1	L
<i>T. funiculosus</i> (Thom) <i>Samson,</i> <i>N. Yilmaz,</i> <i>Frisvad & Seifert</i>	1	0.95	1.11	1	L										
<i>T.</i> <i>purpureogenus</i> <i>Stoll</i>											1	0.81	1.11	1	L
<i>Trichoderma</i> Sp.	2	1.90	2.22	2	L										
<i>White Sterile</i> <i>mycelium</i>	3	2.86	3.33	3	L										
Total count	105	100	113.33	—	—	95	100	105.56	—	—	123	100	136.67	—	—
No. of genera	10+1	9	10												
No. of species	19+1	18	17												

* OR=occurrence remark: H=high occurrence, between 7-10 cases (out of 10); M=moderate occurrence, 4-6 cases; L=low occurrence, 1-3 cases; R=rare occurrence only one

2.6. Total flavonoids content

The total flavonoids content (TFC) was determined by the colorimetric method using quercetin standard as reported (Hossain and Rahman, 2011). Typically, 0.5 mL of methanolic extract, or standard in methanol was mixed with 100 µL of aluminum chloride (10%), 100 µL of sodium acetate (1%) and 4.3 mL distilled water. After an incubation period of the mixture at room temperature for 30 min, the absorbance was measured at 415 nm using a Cary 60, Agilent technologies, UV/VIS spectrophotometer. The TFC was calculated using a standard calibration curve of quercetin (12.5–400 mg/L). The TFC was expressed as quercetin equivalent (QE) per g of plant dry weight (mgQE/gDW). All determinations were performed in triplicate and the results were averaged.

2.7. Total antioxidant capacity

The total antioxidant capacity (TAC) of the extracts was evaluated by phosphomolybdenum method with minor modifications as recorded in the literature (Hossain and Rahman, 2011; Prieto *et al.*, 1999). Firstly, 100 µL of the extract was combined with equivolume of the reagent solution, 3 mL, which consists of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate

in glass tube. Next, the tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples cooled to room temperature, the absorbance of the developed color was measured at 695 nm against a blank using a Cary 60, Agilent technologies, UV/VIS spectrophotometer. A typical reagent blank was carried out by mixing 100 µL of methanol with 3 mL of the reagent solution and then follow the same procedure for the extract. The experiments were performed in triplicate. The TAC was expressed as mg ascorbic acid equivalent (AA.E) per gram dry weight (mgAA.E/gDW). The standard calibration curve was constructed by measuring the absorbance of different concentrations of ascorbic acid (1, 12.5, 25, 50, 100, 200 and 400 mg/L).

2.8. Liquid chromatography-electrospray ionization tandem mass spectrometry

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) is one of the top choices for analysis of plant polyphenols in complex mixtures due to its high sensitivity and efficiency (Desta *et al.*, 2017). Analyses of bioactive compounds isolated from the endophytic fungi of the artichoke were carried out with Triple Quadrupole LC-DAD-ESI-MS/MS, Agilent

6420 Series, system which consisted of a capillary HPLC equipped with a binary pump solvent delivery, thermostatic column compartment, diode array detector (DAD), and a QQQ 6420 mass detector coupled with a pneumatic nebulizer-assisted electrospray interface (ESI). A ZORBAX Eclipse XDB-C18 column (150 mm× 4.6 mm i.d., particle size 5 µm (Agilent Technologies) was used. The mobile phase consisted of acetonitrile (A) and water/formic acid (99/1, v/v) (B) with the gradient system. The flow was maintained at 0.8 mL/min; sample injection was 3 µL. Negative electrospray mode was used for ionization of molecules.

3. Results and discussion

3.1. Endophytes analysis

Endophytic organisms have received considerable attention since 1981 when Weber discovered their great abilities to protect their host against insect pests, pathogens and even domestic herbivores (Webber, 1981). Additionally, they are now considered as an outstanding

source of bioactive natural products especially those which colonize medicinal plants (Arnold *et al.*, 2000; Strobel and Daisy, 2003; Wang *et al.*, 2006; Xuan, 2011). Because the lack information about the endophytic fungal biodiversity in Egypt, the present work was initiated to explore the endophytic fungal population in two types of Artichoke which are widely used as medicinal plants and food. And, evaluation of the explored endophytic fungi abilities to produce flavonoids, phenolics and antioxidant compounds. List of recovered endophytic fungi, their total count, colonization frequency and occurrence in each studied plant from each studied media are presented in Tables 1 and 2. A total of 35 endophytic fungal species belonging to 14 genera were explored from total of 540 apparently healthy segments out of 60 plant samples from two Artichoke studied types. The gross total counts of colonizing endophytic fungi obtained from Baladi artichoke fresh leaves was 323 cfu on three different studied media with the majority of 123 cfu originating from cellulose medium while the French artichoke fresh leaves recorded 259 cfu.

Table 2. Total count (TC), percentage of total count (%TC), percentage of colonization frequency (%CF), number of cases of isolation (NCI; out of 10 samples), occurrence remark (OR*), of endophytic fungi isolated from French Hyrious Artichoke fresh leaves per 90 segments on the three types of media (PDA, CZ, & cellulose)

Types of media	PDA					CZ					C				
	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR
Alternaria	44	50.00	48.89	10	H	47	47.00	52.22	10	H	39	54.93	43.33	8	H
<i>A. alternata</i> (Fr.) Keissl	44	50.00	48.89	10	H	45	47.00	50.00	10	H	39	54.93	43.33	8	H
<i>A. radicina</i> Meier, Drechsler & E.D. Eddy						1	1.00	1.11	1	L					
<i>A. tenuissima</i> (Kunze)Wiltshire						1	1.00	1.11	1	L					
Aspergillus	5	5.68	5.56	4	M	5	5.00	5.56	4	M	4	5.63	4.44	3	L
<i>A. flavus</i> Link	1	1.14	1.11	1	L										
<i>A. niger</i> Tiegh	4	4.55	4.44	3	L	4	4.00	4.44	3	L	4	5.63	4.44	3	L
<i>A. terreus</i> Thom						1	1.00	1.11	1	L					
<i>Chaetomium</i> <i>globosum</i> Kunze	6	6.82	6.67	4	M	7	7.00	7.78	4	M	1	1.41	1.11	1	L
Cladosporium						2	2.00	2.22	2	L					
<i>C. cladosporioides</i> (Fresen.) G.A. de Vries						1	1.00	1.11	1	L					
<i>C. sphaerospermum</i> Penz						1	1.00	1.11	1	L					
<i>Epicoccum</i> <i>nigrum</i> Link											1	1.41	1.11	1	L
Fusarium	1	1.14	1.11	1	L						2	2.82	2.22	1	L
<i>F. napiforme</i> Marasas, P.E. Nelson & Rabie	1	1.14	1.11	1	L						1	1.41	1.11	1	L
<i>F. fujikuroi</i> Nirenberg											1	1.41	1.11	1	L
<i>Nigrospora</i> <i>panici</i> Zimm						12	12.00	13.33	6	M					

[Continued]

Types of media		PDA					CZ					C				
Endophytic fungi	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR	Tc	%Tc	%CF	NCI	OR	
<i>Penicillium</i>											2	2.82	2.22	2	L	
<i>P. fasciculatum</i> <i>Sommerf.</i>											1	1.41	1.11	1	L	
<i>p. glabrum</i> (Wehmer) <i>Westling</i>											1	1.41	1.11	1	L	
<i>Sarocladium</i> <i>strictum</i> (W. <i>Gams</i>) <i>Summerb</i>											1	1.41	1.11	1	L	
<i>Stachybotrys</i> <i>chartarum</i> (Ehrenb.) S. <i>Hughes</i>	1	1.14	1.11	1	L	1	1.00	1.11	1	L						
<i>Stemphylium</i>	26	29.55	23.33	9	H	23	23.00	25.56	8	H	21	25.35	20.00	6	M	
<i>S. botryosum</i> <i>Wallr.</i>	20	22.73	22.22	8	H	22	22.00	24.44	7	H	3	4.23	3.33	3	L	
<i>S. vesicarium</i> (Wallr.) E.G. <i>Simmons</i>	6	6.82	6.67	3	L	1	1.00	1.11	1	L	18	25.35	20.00	6	M	
<i>Talaromyces</i> <i>funiculosus</i> (Thom) Samson, <i>N. Yilmaz,</i> <i>Frisvad & Seifert</i>	4	4.55	4.44	4	M											
<i>Sterile mycelium</i>	1	1.14	1.11	1	L	3	3.00	3.33	1	L						
Total count	88	100.00	96.67	—	—	100	100.00	111.11	—	—	71	100.00	78.89	—	—	
No. of genera	7+1	7+1	7													
No. of species	9+1	12+1	11													

* OR=occurrence remark: H=high occurrence, between 7-10 cases (out of 10); M=moderate occurrence, 4-6 cases; L=low occurrence, 1-3 cases; R=rare occurrence only one

The total counts and colonization frequencies of endophytes in this study almost were within the range of many results obtained previously from other plants (Fröhlich *et al.*, 2000; Liu *et al.*, 2010; Orachapunlap *et al.*, 2009; Shankar Naik *et al.*, 2008). Our results referred that there is high species richness and diversity indices of endophytic filamentous fungi in the leaves of Baladi Artichoke as compared to its French rival. Total fungal counts and colonization frequencies of endophytic fungi from both tested plants were higher spectrum on PDA than other tested media. In this respect, Mohanta *et al.* (Mohanta *et al.*, 2008) found maximum number of endophytes on PDA when they investigated endophytes from three ethno-medicinal plants of Similipal Biosphere Reserve, India. Variation in the total counts and colonization frequencies between the two types of Artichoke on three different types of media that might be due to differences in medium constituents and site-specific factor. Variety of media composition help different fungal endophytes to grow; Such as *Alternaria graminicola* and *Curvularia australiensis* recovered one time on PDA medium only. Additionally, *Alternaria atra*, *A. radicina* and *A. phragmospora* recovered from Glucose-CZ medium only for one time while *Alternaria mali* and *Sarocladium strictum* recovered from Cellulose medium. Furthermore, higher spectrum of the number of the

collected endophytic species isolated from the Egyptian Baladi artichoke were observed compared to the French Hyrious artichoke in all tested media as shown in Figure 1.

All taxa recovered from Artichoke were belonging to Ascomycetes, a large number of genera and species of fungal Ascomycetes are able to live endophytically in plants (Petrini, 1986; Saar *et al.*, 2001). There is no clear correlation between these fungal group and host categories. *Alternaria alternata* was the most common species as fungal endophyte in leaves of Baladi and French Artichoke, it was recovered in high frequency of occurrence comprising range from 36 to 58 cfu (colony forming unit) as total count matching range from 40 to 64.44% of colonization frequency. Genus *Alternaria* was frequently isolated from leaves Artichoke, this genus demonstrated as endophytes of many plants previously (Fernandes *et al.*, 2015; Moharram *et al.*, 2016; Roy, 2001). In addition, fungal endophytes are reported to be host specific at the same time several species can also be isolated from different host (Suryanarayanan *et al.*, 2002).

These results strongly suggested that existence of some degree of host specificity among fungal endophytes. Such host specific endophytes (isolated from only one plant) have also been observed in the present study: *Alternaria atra* (1.05%), *A. cheiranthi* (1.90%), *A. chlamydospora*

(1.90%), *A. graminicola* (0.95%), *A. citri* (0.95%), *A. mali* (0.81%), *A. phragmospora* (1.05%), *Cladosporium herbarum* (1.05%), *Curvularia australiensis australiensis* (0.95%), *Penicillium citrinum* (1.05 and 2.86%) and *Talaromyces duclauxii* (0.95 and 0.81%) were represented as fungal endophytes in Baladi Artichoke leaves only while *A. radicina* (1%), *Nigrospora panici* (12%), *Stachybotrys chartarum* (1 and 1.54%). were represented as fungal endophytes in French Artichoke leaves.

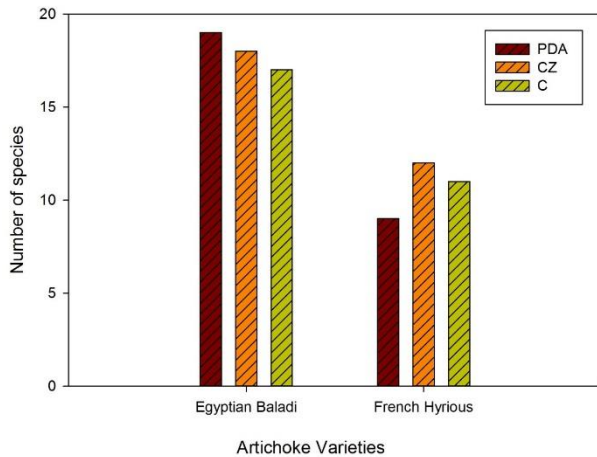


Figure 1. Distribution of endophytic fungi species isolated from the leaves of two varieties of Egyptian artichoke (French Hyrious and Egyptian Baladi) using three different types of tested media (PDA, CZ, C)

Table 3. Total phenolics (TPC), total flavonoids (TFC) content and total antioxidant (TAC) as mg standard equivalent per g dry weight (mg standard/gDW)

Fungal species	Egyptian Baladi Artichoke			French Hyrious Artichoke		
	TPC	TFC	TAC	TPC	TFC	TAC
<i>A. alternata</i>	32.41 ± 0.01	20.88 ± 0.14	310.09 ± 0.29	36.42 ± 0.04	25.63 ± 3.23	322.59 ± 0.19
<i>A. cheiranthi</i>	20.38 ± 0.03	25.83 ± 0.60	268.50 ± 35.11			
<i>A. chlamydospora</i>	12.88 ± 0.01	34.01 ± 0.11	207.20 ± 0.20			
<i>A. graminicola</i>	29.72 ± 0.25	53.86 ± 0.14	357.03 ± 0.27			
<i>A. radicina</i>				24.91 ± 0.03	27.29 ± 0.75	278.67 ± 0.17
<i>A. flavus</i>	31.98 ± 0.03	73.90 ± 2.35	314.97 ± 0.45	22.36 ± 0.02	69.31 ± 0.38	294.14 ± 0.22
<i>A. niger</i>	21.06 ± 0.01	58.81 ± 0.15	193.55 ± 0.34	14.89 ± 0.02	43.38 ± 0.11	170.09 ± 0.15
<i>Epicocum nigrum</i>	10.38 ± 0.01	132.71 ± 0.31	162.96 ± 0.30	19.48 ± 0.02	173.55 ± 0.59	203.33 ± 0.20
<i>F. fujikuroi</i>	39.20 ± 0.06	132.03 ± 1.27	190.17 ± 0.14	33.19 ± 0.06	17.55 ± 0.43	244.06 ± 0.39
<i>P. glabrum</i>	18.73 ± 0.02	29.65 ± 0.05	364.21 ± 0.70	23.98 ± 0.13	30.18 ± 0.54	308.69 ± 0.49
<i>S. vesicarium</i>	17.33 ± 0.04	13.92 ± 0.06	306.55 ± 1.10	18.49 ± 0.00	13.43 ± 0.77	230.02 ± 0.19
<i>Talaromyces sp.</i>	40.30 ± 0.07	76.67 ± 1.25	680.92 ± 0.59	40.01 ± 0.10	48.95 ± 0.71	399.96 ± 0.06
<i>Sterile mycelium</i>	28.67 ± 0.10	29.65 ± 0.05	308.69 ± 0.49	26.08 ± 0.04	24.17 ± 0.26	206.94 ± 6.70

All values expressed as mean of triplicate measurements of TPC, TFC, and TAC ± standard deviation (S.D.)

The results revealed that TPCs of endophytic fungi were higher than those in its host plant. Also, a wide range of TPC in endophytic fungal extracts was observed, from 10.38 to 40.30 mg GAE/g DW, and 14.89 to 40.01 mg GAE/g DW for the Egyptian Baladi and French Hyrious, respectively. The highest concentration of total phenolics was observed in *Talaromyces sp.* isolated from both Egyptian Baladi, and French Hyrious Artichoke leaves' extract to be 40.30 mg GAE/g DW, and 40.01 mg GAE/g DW, respectively. On the other hand, the lowest TPC were found in case of *Epicocum nigrum* (10.38 mg GAE/g DW),

3.2. Analysis of phenolic compounds and antioxidant activity

3.2.1. Total phenolics content

Natural phenolics are a major group of compounds acting as primary antioxidants and free radical scavengers (Benedec *et al.*, 2012). Recently, they have attracted much interest due to their biological properties like anticancer, antimicrobial and antivirus activities (de Falco *et al.*, 2015; Petropoulos *et al.*, 2017; Simona Spînu *et al.*, 2017; Zhou *et al.*, 2016; Zhu *et al.*, 2004). The TPC depends on the solvent used in the extraction method. For instance, Liu *et al.* proved that higher TPC was obtained for different extracts of endophyte using highly polar solvents (Liu *et al.*, 2007). Thus, in our study, methanol was used as an extraction solvent due to it is the best solvent for the selectively of low and high molecular weight of phenolic compounds' extracts (Bimakr *et al.*, 2011; Liu *et al.*, 2007). In our study, the Egyptian Baladi Artichoke leaves of host plant contained 4.1 mg GAE/g DW, whereas French Hyrious Artichoke leaves had 4.8 mg GAE/g DW. Moreover, the TPC of the extract of endophytic fungi, belong to the Ascomycetes group isolated from the two leaves of *Cynara scolymus* L. artichoke; French Hyrious and Egyptian Baladi, was shown in Table 3.

and *A. niger* (14.89 mg GAE/g DW) isolated from the Egyptian Baladi and French Hyrious Artichoke leaves' respectively. Our results showed higher polyphenolic content in *A. alternata*, *Fusarium* and *Sterile mycelium*, *Penicillium species* and *Aspergillus species* compared to the reported studies (Eskandarighadikolai *et al.*, 2015; Fernandes *et al.*, 2009; Huang *et al.*, 2007; Nagda *et al.*, 2017; Yadav *et al.*, 2014). Whereas the TPC values of *Aspergillus sp.* were slightly lower than those observed in endophytic fungi isolated from *Eugenia jambolana* and *Aegle marmelos* (Patil *et al.*, 2015; Yadav *et al.*, 2014).

3.2.2. Total flavonoids content

Flavonoids (or bioflavonoids) are a class of plant and fungus secondary metabolites which are one of the most important polyphenols. The total flavonoid contents (TFC) of the methanolic extracts of endophytic fungi are summarized in Table 3. The TFC values of the leaves' extract were found to be 68.30 mg QE/g DW and 76.21 mgQE/gDW for the Egyptian Baladi and French Hyrious, respectively. Also, the endophytic fungi showed different amounts of flavonoids ranging from 13.92 to 173.99 mg QE/g DW. As indicated in Table 3, *Epicocum nigrum* had the highest amount of the TFC in both varieties of Artichoke plant. While, the results illustrated that *S. vesicarium*, in the Egyptian Baladi and French Hyrious, had the lowest values of the TFC. The TFC of *Penicillium species* and *Aspergillus species* that have been reported in the literature (Nagda *et al.*, 2017) have higher values compared to that isolated from Artichoke. The results suggested that the isolated endophytic fungi contain high antioxidants from the two different Artichoke plants as they have higher TFC compared to their hosts.

3.2.3. Total antioxidant capacity

The phosphomolybdate method has been used to evaluate the total antioxidant capacity (TAC) of endophytic fungi extracts. This method is based on the reduction of Mo(VI) to Mo(V) in the presence of the extracts, and it forms a green-colored phosphomolybdenum (V) complex, which registers a maximum absorbance at 695 nm (Prieto *et al.*, 1999). Table 3 lists the total antioxidant capacity (TAC) of the methanolic extracts of the endophytic fungi. The extracts from leaves of the Egyptian Baladi and French Hyrious showed antioxidant activity with the value of 109.8 and 84.8 mgAA.E/g DW, respectively. On the other hand, the extracts of the endophytic fungi contain high amount of TAC compared to the leaves of the plant. For instance, in the Egyptian Baladi, the TAC values varied widely and ranged from 163 to 681 mgAA.E/g DW, while in the French Hyrious ranged from 170 to 400 mgAA.E/g DW. Therefore, the endophytic extracts could demonstrate to be more effective in the reduction of Mo(VI) to Mo(V) comparing to that of the Artichoke leaves. It is clear from Table 3 that the samples which had low phenolics content, it had lower antioxidant activity and vice-versa. Thus, the results are in agreement with the literature that demonstrate the linear relation between the total phenolics and total antioxidants content (Sultana *et al.*, 2007; Yadav *et al.*, 2014). For example, the *Epicocum nigrum* isolated from the Egyptian Baladi artichoke has the lowest TPC (10.38 mg GAE/g DW) and hence had the lowest TAC value (162.96 mgAA.E/g DW). As well as, the TPC and TAC values of *A. niger* isolated from the French Hyrious artichoke were 14.89 mg GAE/g DW and 170.09 mg AA.E/g DW, respectively. So, it is widely accepted that antioxidant activity is strongly linked to the concentration of total phenolics and flavonoids. In general, our results indicate that the total antioxidant capacity of the different endophytic fungi were highly correlated to the total phenolics, as shown in Figure 2, which is in agreement

with the reported in the literature (Fernandes *et al.*, 2009; Nath *et al.*, 2012; Wang *et al.*, 2003; Yadav *et al.*, 2014). Also Figure 2 depicts a good correlation between the total phenolics content and total antioxidant capacity estimation with R^2 value of 0.764 thereby confirm the anticipated linear relation.

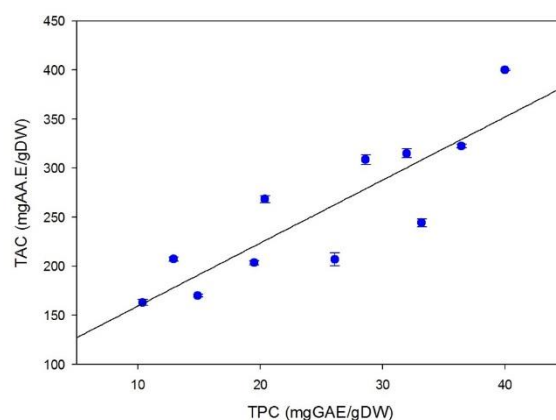


Figure 2. Correlation between TPC and TAC of the endophytic fungal extracts isolated from artichoke plant (French Hyrious and Egyptian Baladi)

3.3. Identification of bioactive compounds isolated from endophytic fungi using HPLC-ESI-MS/MS

Liquid chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) is an extremely important analytical technique which is used for the separation, identification, and quantitation of an analyte of interest even if present in a complex mixture of different sample constituents (Abu-Reidah *et al.*, 2015; Ramos *et al.*, 2014). In the present work, a qualitative analysis of the bioactive compounds of the endophytic fungi isolated from the two varieties of artichoke (French Hyrious and Egyptian Baladi) has been carried out using HPLC-DAD-ESI-MS/MS in a negative ionization mode. Figure 3A shows 5 peaks in the chromatogram of the phenolic compounds standard mixture at different retention time; 0.993, 1.347, 1.410, 1.704 and 1.997 min, which are corresponding to caffeic acid, chlorogenic acid, 1,3-dicaffeoylquinic acid (1,3-diCQA), 1,5-dicaffeoylquinic acid (1,5-diCQA), and Luteolin-7-O-rutoniside, respectively. Furthermore, fragmentation of the standard mixture was performed using LC-ESI-MS/MS to confirm the molecular and precursor ions of each compound. For instance, the precursor ions $[M-H]^-$ were found to be 179, 353, 515, 593 m/z which are corresponding to caffeic acid ($C_9H_8O_4$), chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acid ($C_{25}H_{23}O_{12}$), and Luteolin-7-O-rutoniside ($C_{27}H_{29}O_{15}$), respectively.

The fragmentation patterns of those compounds agree with the reported one (Abu-Reidah *et al.*, 2012; Abu-Reidah *et al.*, 2013; Andrei Fedosov *et al.*, 2016; Chen *et al.*, 2012; D'Antuono *et al.*, 2015; Farag *et al.*, 2013; Gebhardt, 1998; Gebhardt and Fausel, 1997; Llorach *et al.*, 2002; Moglia *et al.*, 2008; Sanchez-Rabaneda *et al.*, 2003; Schutz *et al.*, 2006; Sonnante *et al.*, 2010; Wu *et al.*, 2013).

Moreover, identification of those constituents of the endophytic fungi, *Alternaria alternata*, isolated from the two varieties of artichoke was performed using LC, and LC-MS/MS. Figure 3B and 3C confirmed the presence of 1,3-diCQA and 1,5-diCQA (cynarin) in the endophytic fungi, *Alternaria alternata*, by comparing its retention time with the corresponding reference standards. Therefore, this novel study confirms the production of bioactive compounds from the endophytic fungi isolated from Artichoke. The obtained observations from this investigation are in matching with the compounds isolated from the artichoke plant as reported in the literature (Abu-Reidah *et al.*, 2013; Farag *et al.*, 2013). Thus, those endophytic fungus can be considered as potential source of antioxidants, anticancer, etc for further applications as well as good source for valuable products to produce from an easier and economic process.

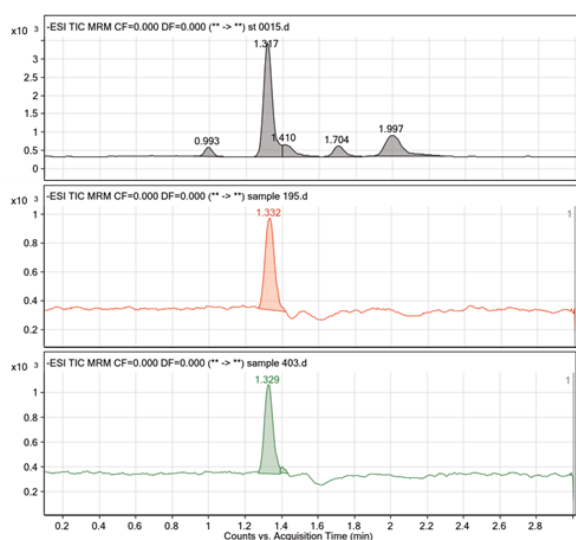


Figure 3. Total ion chromatograms (TIC) in negative mode of endophytic fungal methanolic extracts from two varieties of artichoke (French Hyrious and Egyptian Baladi)

4. Conclusions

This is the first study that reports the isolation and identification of the endophytic fungus from leaves of two varieties of Egyptian artichoke (French Hyrious and Egyptian Baladi) as well as the evaluation of their in vitro total antioxidant capacity (TAC), total phenolics content (TPC) and total flavonoids content (TFC). Significantly the endophytic fungi exhibited higher content of total phenolics, total flavonoids and antioxidant activity than their host plant leaves of Egyptian artichoke. Also, a total of 35 endophytic fungal species belonging to 14 genera were isolated from both artichoke leaves. It was found that *Alternaria* was the most frequently isolated genus and *A. alternata* was the most dominant species. All taxa recovered were assigned to Ascomycetes. The TAC, TPC and TFC of the fungal cultures ranged from 163 to 681 mgAA.E/g DW, 10.38 to 40.30 mgGAE/gDW, and 13.92 to 173.99 mgQE/gDW, respectively. Moreover, 1,3-dicaffeoylquinic acid and 1,5-dicaffeoylquinic acid were

identified in the methanolic extract of *A. alternata* using LC-ESI-MS/MS. In addition, this study demonstrated that the endophytic fungi have higher amount of bioactive compounds compared to that produced by its host plant. Therefore, the metabolites produced by the endophytic fungi isolated from the two varieties of Egyptian Artichoke may serve as an economic and potential source of natural antioxidants. Further investigations are needed to conform the identification of the isolated endophytic fungi.

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