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Phytochemical screening, Antioxidative and anti- α- amylase properties of endophytic fungal extracts isolated from *Ocimum basilicum*

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Abstract

Ocimum basilicum belongs to the family Lamiaceae which is known to have anticancer and many other bioactivities. *Phoma eupyrena*, *Emericella nidulans lata* and *Chaetomium olivaccium* were isolated from the differ organs of the Basil plant. Root, stem and leaf's endophytes extracts of this plant, were tested for phytochemical constituents, antioxidant and anti- α-amylase activities. Phytochemical screening of fungal endophyte extracts revealed the presence of alkaloids, steroids, terpenoids and saponins. Quantitative determination of total phenolics, total flavonoids and various in vitro antioxidant activities (DPPH, H2O2, α-Amylase, reducing power and antioxidant capacity) of methanolic extract was carried out using colorimetric methods. The results of this study displayed that antioxidant activities differ significantly in the endophyte's extract of the plant. The flavonoid content appeared in phoma extract only. The IC_{50} values for DPPH radical scavenging was in the order of PHE $>$ EME $>$ CHE, the values were 63.3% $>$ 56.2% $>$ 26.8% at 100µg/ml, respectively. The scavenging activity for hydrogen peroxide of various extracts was in the order of PHE $>$ CHE $>$ EME respectively . The highest inhibitory activity of α-amylase was in PHE followed by EME then CHE. Finally we concluded that Ocimum basilium has strong scavenging activity and phytochemical contents, and confirm this plant may be good as antitumor and also is very useful for the management of other diseases.

Keywords: *Ocimum basilicum*,Endophyte,Phytochemical screening, Antioxidant activity, DPPH

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1 Introduction

Endophytes are microorganisms that colonize the plant tissue without causing any adverse effect or producing symptoms on the plant.Several endophytes are reported having excellent antioxidant activities (Basu and Narayan, 2017). Antioxidant among excess of substances is considered an important compound and potential therapeutic agent against the oxidative damage. Oxidative damage to cell component plays vital role in many human diseases, viz., cancer, Alzheimer's disease, and kidney disease. Antioxidant compounds can be thiols, ascorbic acids (AAs), Phenolic acids and flavonoids are major bioactive components isolated from endophytic fungi (Das et al., 2017).

Many highly reactive molecules derived from the metabolism of oxygen are reactive oxygen species (ROS), Oxidative damage caused by rapid production of free radicals may lead to degenerative disorders such as cancer, diabetes, neural disorders and ageing. Antioxidants are molecules, which can scavenge free radicals andprevent cellular damage by reducing the oxidative stress and thereforehave a beneficial effect on human health. A number of antioxidants isknown to provide protection against several diseases (Singh et al., 2016). In human body there are various enzyme systems for free radical scavenging but micronutrients like vitamin E, beta-carotene and vitamin C are the major antioxidants. These must be provided in diet as body cannot produce these nutrients. Protection against free radicals can be enhanced by taking sufficient amounts of exogenous antioxidants. An antioxidant is a stable molecule which donates an electron to a rampaging free radical andterminates the chain reaction before vital molecules are damaged. Free radical scavenging property of antioxidants delays or inhibits cellular damage (Yadav et al., 2014). Oxidative stress phenomenon caused by releasing free radicals which proved their harmful effect to living system either by development of degenerative diseases or by rapid ageing (Mahapatra & Banerjee, 2012).

Ocimum (Basil) is the most important genus of the subfamilyNepetoideae under the family Lamiaceae. The word Ocimum is derived from the Greek word "ozo" meaning smell and is called as "king of herbs" due to its immense use in traditional system of medicine, perfumery and pharmaceutical industry (Chowdhury et al., 2016). The genus *Ocimum* (basil) belonging to the Lamiaceae family is the most popular group of herbs in the world. It comprises many wild and domesticated, annual and perennial species distributed in tropical and subtropical regions of Asia, Africa, Center America (Rewers & Jedrzejczyk, 2016). At present, this annual aromatic plant, native to Southeast Asia, is globally cultivated and has significant economic value. The plants are ornamental, the leaves are used in cooking, and its essential oil is oftenan ingredient used in the personal care, and household cleaning products industry. Basil essential oil has been recognized as an antioxidant and local anaesthetic, and some of the compounds found in it are antibacterial, fungistatic and insecticidal. There is also a long tradition of using basil as a medicinal plant in treating coughs, diarrhoea, worm infestations and kidney malfunctions. Recent studies suggest that basil oil displays great potential as a stress repressor, and it is also used as a component in drugs for leukaemia treatment (Filip et al., 2017). The most used species, for essential oil production and as a pot herb are *O. basilicum* L.,*O. americanum* L., and their putative hybrid *O. africanum* Lour., (Klandija et al., 2011).

One of the valuable contents in basil is oleoresin. *Basil oleoresin* contains the main component of volatile substances (essential oils) and non-volatile (resin and gum)(Tambun et al., 2017). *Ocimum*

basilicum L. is one of the most important species amongst the member of the *Ocimum* genus. Its aromatic leaves, flowering tops and essential oils are used in food industry (flavouring foods and beverages), in perfum- ery (e.g. hair dressings, perfumes, soaps, dental creams, mouth washes) and in traditional medicine. It has antibacterial, antifungal, insecticid- al, and hepatoprotective activity and contains antimicrobial substances, also. It has antioxidative- and antiulcerogenic effect by the literature references. The plant accumulates essential oil in high amount (monoterpenes, sesquiterpenes and phenylpropanoids), which can be found in the organs of the plant in different amount (Bernhardt et al., 2014). This study aims to check the antioxidant potentials and anti-α-amylase of the endophytic fungal strains isolated from the Ocimum Basilicium plant.

2 Materials and Methods

2.1. Plants materials

Fresh and healthy plants materials (root,stem and leaves) of Ocimum basilicum collected from Aswan university campus, Aswan,Egyptwere carefully chosen for sampling during 2014 and 2015 in Aswan city. The plant materials were randomly collected from the study area . The samples were brought to the laboratory in sterile bags to reduce the contamination chance.

Isolation of endophytic fungi

The collected plant samples were rinsed gently in running water to removed dust and debris. The whole process of endophytic fungi is carried out aseptic condition(Saithong et al. 2010). Sterile glassware and mechanical tools, such as scissor, forceps, scalpel, were used in all experiment. The roots, stem and leaves samples from the plant were surface sterilized firstly. The samples were washed in 70% ethanol for 1 min, dipped in 4% sodium hypochlorite (NaOCl) for 3 min and then with 70% ethanol for 30 seconds. Later the plant pieces were rinsed three times with sterile distilled water. The plant pieces were blotted on sterile blotting paper to ensure surface drying (Fisher et al. 1993). Then, all sterilized dry samples were cut approximately in 1 cm. the sterilized samples were plated on potato Dextrose Agar (PDA) medium supplemented with antibiotics, chloramphenicol (0.5 g per liter). The dishes were sealed with parafilm and incubated at 28°C. Most of the fungal growth was initiated within two weeks. Isolation from the master plates was done by the transfer of hyphal tips to a new fresh Potato Dextrose Agar (PDA) plates to obtain pure cultures for identification.

2.2. Extraction Preparation

Phoma eupyrena, *Emericella nidulans lata* and *Chaetomium olivaccium* were cultured in potato dextrose broth media and the culture media and the mycelia were separated by filtration. The mycelia were soaked in methanol. The methanolic extract of mycelia was collected after 2 days of soaking, Liquid - liquid extraction using ethyl acetate was carried out three times for solvent, mixed well with shaker for 30 minutes and kept for about 2 hour till the two clear immiscible layers formed. The upper layer of solvent containing the extracted compounds was separated using separating funnel. The organic extract was evaporated by rotary evaporator to obtain solid residues. Finally 10mg from the resulting crude extracts was dissolved in 1ml of 80% methanol and stored at 4° for antioxidant analysis. Different concentrations ranging from 25µg to 100µg were used during determination of some experiments (TAC, DPPH, α –Amylase, H2O2 scavenging).

2.3. Phytochemical screening

The crude methanolic extracts were tested for the presence of alkaloids, steroids, tannins, saponins and glycosides. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

2.3.1. Test for alkaloids

Few mg (about 15 mg) of each extract was separately stirred with 1% HCL (6ml) on a water bath for 5 min and filtered. These filtrates were divided in to three equal parts.

a. Dragendorff's test: To one portion of the filtrate, Dragendorff's reagent (Potassium bismuth iodide solution) (1 ml) was added, an orange red precipitate shows the presence of alkaloids.
b. Mayer's test: To one portion of filtrate, Mayer's reagent (Potassium mercuric iodide solution) (1ml) was added, formation of cream colored precipitate gives an indication of the presence of alkaloids.
c. Wagner' test: Potassium iodide (2g) and iodine (1.27g) were dissolved in distilled water (5ml) and the solution was diluted to 100ml with distilled water, few drops of this solution were added to the filtrate , a brown colored precipitate indicates the presence of alkaloids (Iqbal et al. 2015, Abdullahi et al.2013).

2.3.2. Tests for steroids and terpenoids

a. Salkowski test: The crude extract (about 100mg) was separately shaken with chloroform (2ml) followed by the addition of concentrated H2SO4 (2ml) along the side of the test tube, a reddish brown coloration of the interface indicates the presence of terpenoid (Ayoola et al. 2008).
b. Liebermann-Burchard test: Each extract (100 mg) was shaken with chloroform in a test tube, few drops of acetic anhydride was added to the test tube and boiled in a water bath and rapidly cooled in iced water. Concentrated H2SO4 (2 ml) was added alongside of the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids while formation of deep red color indicates the presence of triterpenoids. (Iqbal et al. 2015).

2.3.3. Test for flavonoids

A few drops of concentrated hydrochloric acid were added to a small amount of the extracts of the plant material. Immediate development of a red colour was taken as an indication of the presence of flavonoids.

2.3.4. Test for Phenolics

Red coloured solution with acetic acid indicates the presence of phenolic compounds.

2.3.5. Test for Saponins

Each extracts (0.5 g) was separately shaken with distilled water (10ml) in a test tube. The formation of frothing, which persists on warming in a water bath for 5 min, shows the presence of saponins (Benso $ Adeyemo, 2006).

2.4. DPPH radical scavenging activity

The free radical scavenging capacity of methanolic extract was determined by using DPPH assay according to the method described by (Abdulwahab et al., 2011) with some modifications. The stock solution of 1 ml DPPH was prepared by weighting 0.07mg of the DPPH reagent and dissolved in 100ml methanol. The reaction mixture consisting of 1ml methanol solution and 1ml DPPH reagent were placed in different test tubes ,to each of these tubes different concentrations

from extract was added (25µ,50µ,100µ) (Blois, 1958). Negative control tube have 2ml methanol only, positive control tube have 1ml methanol and 1ml DPPH reagent.
After incubation at room temperature for 30 min, the amount of DPPH remaining was determined by measuring absorbance at 517nm (Hossain et al. , 2009). All the tests are carried out in triplicate. The extract concentration providing 50% of inhibition (IC_{50}) is calculated from the graph of inhibition percentage plotted against extract concentration mean values were obtained from triplicate experiments.

2.4.1. Assaying methods

All measurements of absorbance were carried out using Shimadzu UV-1601 PC spectrophotometer. All experiments were done in triplicate. The % inhibition for DPPH assay was calculated according to the formula: [(AB –AA)/AB] x 100 Where AB is absorption of blank sample, AA is absorption of sample/standard extract.

2.5. Determination of total antioxidant capacity

The assay was done according to (Prieto et al.,1999) . the tubes containing extract and reagent solution (0.6 M sulfuric acid, 28Mm sodium phosphate and 4mM ammonium molybdate) were incubated at 90° for 90 min. the antioxidant capacity was expressed as Gallic acid equivalent (GAE).

2.6. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability of the extract was determined according to the method of Ruch et al. (1989). By weighting (10.0mg) of each extract and dissolved in (1ml) methanol solution we have the extract soluble, then prepare the reagent solution(0.1M phosphate buffer (PH 7.4) mixed with H2O2 solution (0.6ml,40mM). Different concentrations of extract in tubes with the phosphate buffer mixed with H2O2 solution. The absorbance value of the reaction mixture was recorded at 230nm. For each concentration, a separate blank sample was used for background subtraction. The percentage of H2O2 scavenging of the extracts and standard compounds was calculated.

2.7. Reducing power activity

The reducing power of the extracts was determined according to the method of (Oyaizu, 1986). Briefly, dissolving 10 mg from each extract per one ml methanol and different concentrations of the extracts were mixed with 2.5 ml of 1% potassioum ferricyanide solution. After incubation at 50° for 20 min, the mixtures were mixed with 2.5 ml of 10% trichloroacetic acid followed by centrifugation at 650 g for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance of this solution was measured at 700nm. Ascorbic acid act as positive control.

2.8. α-Amylase assays in vitro

α-Amylase activity was carried out using the starch-iodine method. The reaction mixture contained 10 µl of α-Amylase solution (10mg/ml), phosphate buffer (0.02M, PH 7.0) with 0.006 M NaCl (0.4ml) and 1% starch solution (0.1 ml). After incubation at 37° for 10 min, the starch solution was added, then the mixture was re-incubated for 1 h. Here after, 0.1ml of 1% iodine solution was added, and after the adding of 5ml distilled water the absorbance was taken at 565 nm. Substrate and α-amylase blank determinations were undertaken under the same conditions. The above experiment was conducted using different starch solutions.

3 Results

3.1. Phytochemical screening of crude methanolic extracts

Samples revealed the presence of some secondary metabolites such as alkaloids, Terpenoids, phenolic and flavonoid contents as shown in table (1) The phytochemical compounds detected are known to have medicinal importance. For example, alkaloids have been reported as powerful poison and many alkaloids derived from medicinal plants show biological activities like, anti-inflammatory(Augusto et al.2012), antimalarial (Due et al. 2013), antimicrobial (Benbott et al. 2012), cytotoxicity, antispasmodic and pharmacological effects (Ameyaw and Duker-Eshun, 2009). Similarly, terpenoids derived from endophytes are known to have cardiotonic effect and also possess antibacterial and insecticidal properties (Alexei et al. 2009). They are very often used in medicines due to their well-known biological activities. The results in (Table 1) revealed that the methanolic extract of Phoma only has a flavonoid content, flavonoids are of great importance because they help human body to fight against diseases. The ability of flavonoids to act as potent antioxidants depends on their molecular structures the position of the hydroxyl group and other features in its chemical structure (Iqbal et al. 2015).

3.2. DPPH radical scavenging activity

The DPPH radical scavenging activities of the different extracts are shown in table 2. The level of free radical (DPPH) scavenging activity ranged from 17.8 to 5.0% at 25 µg/ml of extract. At this concentration, Phoma showed the highest DPPH radical scavenging activity (17.8%) followed by Emericella (10.4%), Chaetomium (5.0%)had the lowest IC50, which means that among the tested extracts, it had the strongest radical scavenging activity. The level of free radical (DPPH) scavenging activity ranged from 43.4 to 15.2% at 50 µg/ml of extract. At this concentration, Phoma showed the highest DPPH radical scavenging activity (43.4%) followed by Emericella (30.1%), Chaetomium (15.2%) had the lowest IC_{50}, which means that among the tested extracts, it had the strongest radical scavenging activity.

Table 1. Phytochemical screening of extracts from endophytic fungus (Phoma eupyrena – Emericella nidulans lata – Chaetomium olivaccium), and total phenolic and flavonoid contents of extracts.

Test	phoma extract	Emericella extract	Chaetomium extract
Alkaloids:			
(a) Dragendorff's test	+++	-	-
(b) Mayer's test	++	-	-
(c) Wagner's test	++	-	-
Terpenoids:			
(a) Salkowski test	++	++	+++
(b) Liberman-Burchard test	++	++	+++
Saponins	-	-	-
Phenolics	-	-	-
Flavonoids	++	-	-

The level of free radical (DPPH) scavenging activity ranged from 63.3 to 26.8% at 100µg/ml of extract. At this concentration, *Phoma* showed the highest DPPH radical scavenging activity (63.3%) followed by *Emericella* (56.2%), *Chaetomium* (26.8%) had the lowest IC_{50}, which means

that among the tested extracts, it had the strongest radical scavenging activity.

3.3. Total antioxidant capacity (TAC)

Total antioxidant capacities, which are expressed as the gallic acid equivalents (GAE) of the different extract are shown in table.3. The capacity was highest in phoma followed by Chaetomium, whereas the lowest in Emericella.

3.4. Hydrogen peroxide scavenging activity

The H2O2 scavenging ability of the extract is shown in Table 4. At a concentration of 50 µg/ml in phosphate buffer, Phoma extract showed the highest activity (39%) followed by chaetomium (35%), the lowest activity showed by emericella extract (33%). At a concentration of 100 µg/ml in phosphate buffer, Phoma extract showed the highest activity (66%) followed by chaetomium (43%), the lowest activity showed by emericella extract (39%). At a concentration of 150µg/ml in phosphate buffer, Phoma extract showed the highest activity (85%) followed by chaetomium (54%), the lowest activity showed by emericella extract (52%).

Table 2. DPPH radical scavenging activity of the different methanolic fungal extracts.

Name of extracts	% at 25µg/ml	% at 50µg/ml	% at 100µg/ml
Emericella nidulans lata	10.4	30.1	56.2
Chaetomium olivaccium	5.0	15.2	26.8
Phoma eupyrena	17.8	43.4	63.3

Table 3. Total antioxidant capacity of fungal extracts

Samples	Absorbance
Emericella nidulans lata	0.071
Chaetomium olivaccium	0.123
Phoma eupyrena	0.518

Table 4. H2O2 scavenging activity (%)

Extract	% at 50µg/ml	% at 100µg/ml	% at 150µg/ml
Emericella nidulans lata	33	39	52
Chaetomium olivaccium	35	43	54
Phoma eupyrena	39	66	85

3.5. Reducing power

Reportedly, the activity of antioxidants is concomitant with the development of reducing power (Duh et al.,1999) Table 5 shows the reducing power of the extracts determined using the potassium ferricyanide reduction method. Since Phoma had the high reducing activity among the extracts, at 25µg/ml (2.795). Also phoma had the highest reducing activity at 50µg/ml (2.699). And at 100µg/ml phoma had the high reducing activity (2.615).

Table 5. Reducing power of the different methanolic fungal extracts

Extract	25µg/ml	50µg/ml	100µg/ml
Emericella nidulans lata	2.675	2.363	2.081
Chaetomium olivaccium	2.735	2.560	2.340
Phoma eupyrena	2.795	2.699	2.615

3.6. α-amylase activity in vitro

In the present study, all extracts were found to possess significant ($P< 0.01$) Inhibitory effects on starch break-down in vitro as shown Table 6. At a concentration of 1mg/ml in phosphate buffer, in table 6 IC_{50} showed the highest inhibitory activity of α-amylase was in *Phoma* (0.203) at 100 µg/ml, followed by *Emericella* (0.056), *Chaetomium* had the lowest inhibitory activity of α-amylase (0.046). Also Phoma had the highest inhibitory activity (0.108) at 50 µg/ml. Phoma had the highest inhibitory activity (0.083) at 25 µg/ml.

Table 6. In vitro α-amylase inhibition activity of different fungal extracts .

Extract	20µg/ml	50µg/ml	100µg/ml
Emericella nidulans lata	0.033	0.044	0.057
Chaetomium olivaccium	0.027	0.036	0.046
Phoma eupyrena	0.083	0.108	0.203

4 Discussion

Endophytic fungi, a potential source of medicinal compounds, have attracted more and more attention in the last years. It is reported that special eco-environmental microorgan-isms may produce special activated metabolites (Stierle et al., 1993). According to this notion, we isolated endophytic fungi from Ocimum basilicum. Fungal endophytes and their host plants interact through physical or chemical signals and the former can promote host-plant growth through the production of phytochemicals, including antioxidants, without leading to biotic stress when they invade or live inside host plant tissues. Fungal endophytes from *Ocimum basilicum* may be also beneficial to their host plant through the production of antioxidant compounds without obvious side effects. To determine the antioxidant activities of these fungal endophytes, we determined the antioxidant activities of the fermentation broth of the isolated fungal endophytes in our study. Most fungal endophytes demonstrated some antioxidant activity(Pan et al., 2017).

This study deals with the isolation of endophytic fungi and their bioactive potentials from *O. basilicum*. A total of 3 endophytic fungal strains belonging to three genera, Phoma epyorena, Emericella nidulans lata and Chaetomium olivaccium were identified. The isolates were identified morphologically (mycelial characters, spore morphology), All three strains were tested for the TPC, TFC, and antioxidant capacities. The presence of phenolic, flavonoid and alkaloids phytochemicals in leaf , stem and root extracts of *O. basilicuim* were reported, there were not any results for the TPC of extracts. The TFC is only found to be of significantly higher concentration in intracellular-phoma extract than other extracts. Alkaloids are already known to have spasmolytic, antifungal, antimicrobial and antitumor activities. the intracellular-phoma extract and

extracellular-phoma extract have high alkaloid content. DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants(Singh et al., 2016). Among all the isolated strains, Intracellular-Phoma eupyrena was found to have potentially high antioxidant capacity. The DPPH radical scavenging capacity of extract Intracellular-Phoma eupyrena (IC50 17.8% at 25µg/ml), (IC_{50} 43.4% at 50µg/ml), (IC_{50} 63.3% at 100µg/ml). Quercetin was also reported to have genoprotective effect against H2O2 induced DNA damage (Szeto et al., 2002). Various oxidase enzymes, such as superoxide dismutase produce H2O2 in vivo. It is toxic to cells because of the production of hydroxyl radicals, which oxidized a number of molecules including DNA. Thus, removing H2O2 is very important for protecting cell components from oxidation. In this study, α-Amylase activity was significantly ($P < 0.01$) inhibited by all extract. In dose-dependent effects, at high concentrations saturation of components may have occurred there by causing no further increase in inhibition. These extracts probably non-competitively bind to the active site of the enzyme. Promising anti-oxidative and anti-α-amylase activities of Intracellular-phoma extract indicate that it might be used to prevent onset of various diseases including diabetes, cancer, inflammation. Reportedly, various natural products inhibit α-amylase and α-glucosidase activities (Hossain et al., 2008) such as flavone and flavonoids.

5 Conclusion

The results of this study shows the presence of some phytochemicals such as alkaloids, terpenoids and flavonoids in methanolic extracts of endophytes isolated from *Ocimum basilicum*. Finally, we concluded that *Ocimuim basilicium* plant has strong scavenging activity and confirm this plant as possible good cancer preventer and also is very useful for management of other diseases. We suggest and recommend to pay attention to this plant for using in various important applications in food and medicine.

Conflict of interests

There are no conflicts of interest between the authors.

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