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ORIGINAL ARTICLE

ANTIFUNGAL ACTIVITY AND CHEMICAL COMPOSITION OF ENDOPHYTIC FUNGUS *PHANEROCHAETE* SP. MFLUCC16-0609

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Abstract

The fungal endophytes associated with the *O. basilicum* var. *thyrsiflora* and its potential for providing antifungal compounds were investigated. Fourteen fungal endophytes were isolated and cultured. Crude extracts of endophytes showed antifungal properties against *Trichoderma reesei* and *Lasiodiplodia theobromae*. The crude extract of MFLUCC16-0609 played a role as a great natural fungicide due to his inhibiting property against *T. reesei* and *L. theobromae* compared to other extracts. Two major constituents including sclareolide (42.03%) and muzigadial (19.44%) were detected as the key antifungal compounds. DNA-phylogeny-based and morphology-based genus analysis of strain MFLUCC16-0609 can be reconciled in the *Phanerochaete* genus. Our results suggest that *Phanerochaete* sp. MFLUCC16-0609 may be a source of bioactive compounds inhibiting or controlling plant disease pathogens.

Rezumat

Au fost investigați endofiții fungici asociați cu *O. basilicum var. tirsiflora*, precum și potențialul acestora de a furniza compuși antifungici. Au fost izolați și au fost cultivați patruzeci de endofiți fungici. Extractele brute au prezentat proprietăți antifungice asupra *Trichoderma reesei* și *Lasiodiplodia theobromae*. Extractul brut MFLUCC16-0609 a prezentat caracter fungicid natural din cauza proprietății lui de inhibare a *T. reesei* și *L. theobromae* în comparație cu alte extracte. Doi constituenți principali, incluzând sclareolid (42,03%) și muzigadial (19,44%), au fost detectați ca principali compuși antifungici. Analiza genei ADN-bazată pe filogenie și morfologia genetică a tulpinii MFLUCC16-0609 poate fi regăsită în genul *Phanerochaete*. Rezultatele noastre sugerează că *Phanerochaete sp.* MFLUCC16-0609 poate fi o sursă de compuși bioactivi care inhibă sau controlează fitopatogeni.

Keywords: antifungal, endophytic fungi, *Lasiodiplodia theobromae*, *Phanerochaete*, *Trichoderma reesei*

Introduction

Most plant diseases are appeared by various microbial pathogens such as bacteria, nematodes, fungi and viruses [1]. Fungi are found to be one of the major pathogens of many plant diseases reducing a growth of many economically important agriculteral products. Fungal plant pathogenic diseases are mainly eliminated by using synthetic chemicals. However, the resistance of fungicides by various plant fungal pathogens has been increased and become the major problem with regard to poor agricultural disease control [2]. An increasing number of plant fungal diseases and pathogens are becoming difficult to treat and more deaths [3]. Considerable research effort has focused on the antimicrobial potential of endophytes [4-6]. They have significant antimicrobial activities and can be used in many applications including agriculture, industry and medicine [7, 8].

Ocimum basilicum var. *thyrsiflora* (*Lamiaceae*), commonly known as *Horapha* in Thai, is a widely-cultivated plant in Thailand and other areas of Asia. They are also used in Thai traditional cuisine and medicine for treatment of headaches, coughs, diarrhoea, worms and kidney malfunctions and for the carminative effect. In addition, it is applied for remedy of galactagogue, stomachic and antispasmodic properties [9]. In this present study we aimed to isolate fungal endophytes colonizing in *O. basilicum* var. *thyrsiflora* leaves. Crude extracts of all fungal endophytes were screened for the antifungal activities against *T. reesei* TISTR3080 and *L. theobromae* MFU. The secondary metabolites of crude fungal extract with great antifungal properties were further identified by using gas chromatography-mass spectrometry (GC-MS).

Materials and Methods

Plant material

Ocimum basilicum var. *thyrsiflora* leaves with a voucher herbarium specimen (QBG No. 41462) was assigned and deposited at the Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai, Thailand. Aerial portions of this plant were collected from Chiang Rai Province Thailand in February 2016.

Isolation, culture of endophytic fungi and extraction of crude fungal extracts

Tissue samples of *O. basilicum* var. *thyrsiflora* healthy leaves were cleaned with distilled water for one minute. The tissue surface was further dipped in 70% ethanol, 1% sodium hypochlorite, respectively, prior washed with sterile demineralized distilled water. Tissue samples were cut into 0.5 cm^2 pieces. They were further placed in Petri dishes containing potato dextrose agar (PDA) with chloramphenicol. Petri dishes were incubated at room temperature (28ºC) for 3 - 5 days. Colonies of fungal endophytes were subsequently plugged and placed them onto new PDA plates for purifying. The plates were kept at room temperature (28ºC) for seven days. All pure isolates were further cultured in potato dextrose broth (PDB) at room temperature (28ºC) for thirty days by using of 6 mm diameter mycelial agar plugs from PDA cultured in 150 mL PDB. After thirty days, the culture broth of each isolate was filtered. The obtained mycelia filtrates were macerated in ethyl acetate for five days and subsequently partitioned with ethyl acetate. The ethyl acetate extracts from PDB and the mycelia culture broth were combined and concentrated by using a vacuum rotary evaporator at reduced pressure at 40°C.

Analysis of antifungal activity

T. reesei TISTR3080 and *L. theobromae* MFU were selected as plant pathogenic fungi in this study. The antifungal activity of crude extracts was evaluated by using the agar disc diffusion method [10]. Each crude extract was prepared by two-fold dilution method as a stock solution at 1000, 500, 250, 125 and 62.5 µg/mL, respectively, in ethyl acetate. Each pathogenic fungus was cultured on PDA media and incubated at room temperature for five days. A plug of fungal colony was placed at the centre of the sterilized PDA plates. Paper discs moistened with various fungal extracts were also placed in the same plates and all plates were incubated at room temperature for five days. Diameter of the fungal mycelium growth was monitored and calculated as a percentage of inhibition of a radical growth relative to the control by using a formula: Percentage of inhibition (%) = 100 × [(1- the radical growth of treatment (mm)/radical growth of control (mm)]. Minimal inhibitory concentration (MIC) value of each fungal extract was also investigated.

GC-MS analysis of chemical components of strain MFLUCC16-0609

The GC-MS analysis of the *Phanerochaete* sp. MFLUCC16-0609 fungal extract was performed on a Hewlett Packard model HP6890 gas chromatograph, coupled to a mass selective detector with quadrupole analyser, HP model 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with a fused silica capillary HP5MS (5 % phenyl-polydimethylsiloxane) capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies, USA). The oven program was started with an initial temperature of 60°C held for 3 min, and then the oven temperature was raised at 3°C/min to 220°C. Helium was used as carrier gas at a flow rate of 1 mL/min. For GC-MS detection, an election ionization system with ionization energy and electron multiplier voltage of 70 eV and 1150 V was used, respectively. The ion source and quadrupole temperatures were set at 230°C and 250°C, respectively. A scan rate of 0.6 s (cycle time: 0.2 s) was applied, covering a mass range from 30 to 300 amu (atomic mass units). The samples were injected in split mode as 100:1. The chemical composition was identified by comparing the mass spectra with data from NIST05 (National Institute of Standards and Technology, US) and WILEY 7N libraries. The retention indices were in relation to a homologous series of n-alkanes (C_{10}-C_{23}) on the HP5MS column under the same chromatographic conditions. Components' relative concentrations were obtained by peak area normalization and summarized in term of relative peak area percentage.

Genomic DNA extraction and PCR

Due to significantly high antifungal activities against tested plant pathogens, the morphology of strain MLUCC16-0609 was selected for complement identification. Their aerial mycelium was scraped from surface of PDA and pulverized with a pestle and mortar until a fine powder was obtained. The genomic DNA was extracted using cetyltrimethyl ammonium bromide. The barcode fragment ITS1-5.8S-ITS2 sequence was amplified using the universal primers, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The PCR conditions were: 95°C for 5 min, followed by 40 cycles of 95°C for 50 s, 52°C for 50 s, 72°C for 50 s, and a final extension at 72°C for 10 min on a PeqSTAR 2x thermal cycler (Peqlab, Germany). PCR products were checked on 1% agarose gels stained with ethidium bromide under UV light and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The purified PCR

products were sequenced in both directions at the 1st Base Company (Malaysia) using the same PCR primers. The acquired gene sequence, with accession number as MH253672, was submitted to the NCBI Genbank.

Phylogenetic analysis

The closest taxa of strain MFLUCC16-0609 with the highest antifungal activity was determined with standard nucleotide blast searches in National Centre for Biotechnology Information (NCBI) database. A combined dataset of nLSU, ITS and rpb1 representative sequence data in *Phanerochaetaceae* were selected following Miettinen *et al*. [11]. *Bjerkandera adusta* (HHB-12826-Sp) and *Terana caerulea* (FP-104073) were selected as the outgroup taxa. MAFFT v.7.036 was used to perform multiple alignments while BioEdit v.7.2 was used to adjust the alignments for necessary improvement. After trimming, 3,074 sites remained in the alignment. MODELTEST v.2.0 supporting with Akaike Information Criterion was chosen to investigate the best-fit model of evolution for each dataset. Maximum-likelihood analysis was performed by using RAxML in raxmlGUI v.0.9b2. One thousand nonparametric bootstrap iterations were employed with the available models of generalized time reversible (GTRGAMMAI model) and a discrete gamma distribution. Maximum likelihood bootstrap values equal or greater than 70% are provided.

Results and Discussion

Fourteen endophytic fungi were deposited at the Centre of Excellence in Fungal Research, Mae Fah Luang University, Thailand (Voucher No. MFLUCC16-0603-MFLUCC16-0616). All fungi contained heavily intertwining hyphae with whitish mycelium. The surfaces of their mycelia appeared cottony white in colour, which turned whitish grey in older cultures. The culture of isolate MFLUCC16-0603 and MFLUCC16-0615 appeared yellow and pale green in colour, respectively, but olivaceous white exudates appeared in most isolates. Over the last two decades, at least one or more fungal endophytes have been isolated from healthy plants [3, 12]. Plants in the genus *Ocimum* are indigenous to the tropical and warm temperate regions and present significant protection of biotic and abiotic organisms. Several species of fungal endophytes were isolated in the healthy *Ocimum* plants [13, 14]. As noticed, various number and diversity of fungal endophytes were resulted from geographic location. Crude extracts of endophytic fungi were examined for antifungal activity against tested plant fungal pathogens. Screening with different concentrations of ethyl acetate extracts of the endophytic fungal culture was conducted using the agar diffusion method against the fungi. MIC was determined for all tested fungal pathogens. Percentage of growth inhibition and MIC value of fungal pathogens by endophytic fungal extracts isolated from *O. basilicum* var. *thyrsiflora* leaves are shown in Table I and Figure 1, respectively.

Table I

Percentage of growth inhibition by endophytic fungal extracts isolated from *O. basilicum* var. *thyrsiflora* leaves at the concentration of 1000 μg/mL

Extract	**Radical growth inhibition (%)**	
	T. reesei **TISTR3080**	***L. theobromae*** **MFU**
MFLUCC16-0603	35.5 ± 4.2	–
MFLUCC16-0604	48.2 ± 1.7	–
MFLUCC16-0605	39.5 ± 2.3	–
MFLUCC16-0606	48.1 ± 3.0	57.4 ± 2.9
MFLUCC16-0607	–	–
MFLUCC16-0608	37.5 ± 4.2	–
MFLUCC16-0609	**74.2 ± 3.6**	**68.9 ± 5.3**
MFLUCC16-0610	65.9 ± 3.3	20.6 ± 4.1
MFLUCC16-0611	–	32.4 ± 3.3
MFLUCC16-0612	59.2 ± 2.7	44.9 ± 2.1
MFLUCC16-0613	45.3 ± 3.1	46.7 ± 3.7
MFLUCC16-0614	44.7 ± 3.1	39.8 ± 3.6
MFLUCC16-0615	55.1 ± 1.3	–
MFLUCC16-0616	52.9 ± 2.8	51.5 ± 2.5

– = Antifungal activity not detected; results are presented as mean ± standard deviation

Most crude extracts of endophytic fungi inhibited at least one of the tested fungal strains while fungal crude extract of strain MFLUCC16-0607 displayed no antifungal activity against the pathogens used in this study. Among the crude extracts, the strain MFLUCC16-0609 crude extract exhibited the highest activity inhibiting the growth of *T. reesei* TISTR3080 (74.2%) and *L. theobromae* MFU (68.9%) at the concentration of 1000 μg/mL. MIC values of MFLUCC16-0609 fungal extract including 125 and 250 μg/mL were detected against *T. reesei* TISTR3080 and *L. theobromae* MFU, respectively. However, bioactivity of isolated fungal endophytes from *O. basilicum* var. *thyrsiflora* has been examined sparingly according to the overall antifungal potency of crude extract or the high amount of active components in the crude extract. All identified compounds of *Phanerochaete* MFLUCC16-0609 crude extract with the relative area percentages and their retention indices are summarized in Table II.

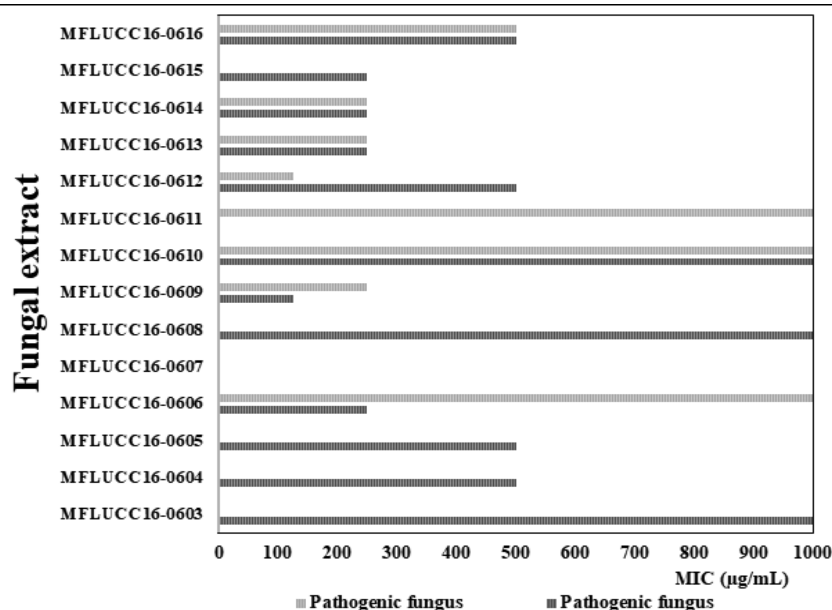

Figure 1.
MIC value by extracts of fungal endophytes isolated from *O. basilicum* var. *thyrsiflora* leaves

Table II
Identified constituents of *Phanerochaete* sp. MFLUCC16-0609 crude extract

No.	Compound	Retention index	%Peak area
1	2-ethyl-3-methyl-pyrazine	1002	0.06
2	2-isopropyl-4-methyl thiazole	1012	0.15
3	benzene acetaldehyde	1035	0.15
4	trans-decahydro-naphthalene	1053	0.09
5	2,3-diethyl pyrazine	1081	0.29
6	perillene	1102	0.19
7	6-camphenol	1111	0.07
8	stemone	1124	0.15
9	4E-ethyl octenoate	1184	1.55
10	4Z-decenol	1257	4.81
11	decanoic acid	1366	0.64
12	decyl acetate	1407	0.26
13	dodecyl acetate	1607	0.49
14	botrydiol	1690	0.32
15	melaleucol	1706	0.19
16	2E,6Z-farnesal	1713	0.23
17	neocnidilide	1722	0.21
18	E-isovalencenol	1793	0.12
19	benzoin	1803	0.43
20	acorone	1819	0.21
21	occidol	1837	0.78
22	Z,Z-farnesyl acetone	1860	0.18
23	flourensadiol	1870	0.41
24	totarene	1922	0.73
25	columellarin	1952	0.49
26	nootkatin	1959	0.49
27	tetrahydro-rimuene	1960	0.38
28	muzigadial	2046	19.44
29	sclareolide	2066	42.03
30	nootkatinol	2088	12.14
31	laurenan-2-one	2115	1.94
32	sandaracopimarinal	2184	0.92
33	phyllocladanol	2209	3.51
34	sclareol	2222	3.82

The extract of MFLUCC16-0609 contained 34 components representing 97.87% of the total extract. The major components were sclareolide, muzigadial, nootkatinol, 4Z-decenol, sclareol and phyllocladanol. GC-MS analysis of MFLUCC16-0609 fungal extract indicated that sclareolide and muzigadial had the high peak area and may be the main contributor to the significant antifungal properties [15, 16]. Other major compounds including sclareol and phyllocladanol were also identified. These detected major compounds have been reported to have antimicrobial and other pharmacological properties [17, 18]. Nonetheless, minor compounds such as perillene, benzoin, columellarin, totarene and stemone were reported as important antimicrobial agents [19]. These identified bioactive compounds were classified in a group of monoterpenoids, sesquiterpenoids and their derivatives which have been reported to have potency of antifungal properties [20, 21]. Higher antifungal activity of *Phanerochaete* MFLUCC16-0609 crude extract may be resulted from synergistic action of various active compounds rather than those obtained from individual active compounds [22, 23]. However, minor active components may present higher antifungal activity once purification is undertaken. Morphological features of strain MFLUCC16-0609 were analysed and compared with study of Miettinen *et al*. [11] describing of *Phanerochaete* genus. The colonies of strain MFLUCC16-0609 were white and their mycelia were slightly superficial and dipped whereas fructifications were mostly apparent. The hyphae were narrow and fused over the host cuticle. Phylogenetic methods with molecular information were applied to identify the genus of endophytic fungus MFLUCC16-0609. The tree topology of combined dataset derived from the maximum likelihood method, were very similar to study of Miettinen *et al*. [11]. The clade of *Phlebiopsis* contains 18 strains of *Phanerochaete* species, one strain of *Oxychaete cervinogilva* and *Riopa metamorphosa* with high boostrap support. The newly sequenced endophytic fungus MFLUCC16-0609 is placed within the genus *Phanerochaete*, and is costly related to the clade contains *P. australis*, *P. pseudomagnoliae*, *P. magnolia* and *P. chrysosporium* with high bootstrap support (Figure 2).

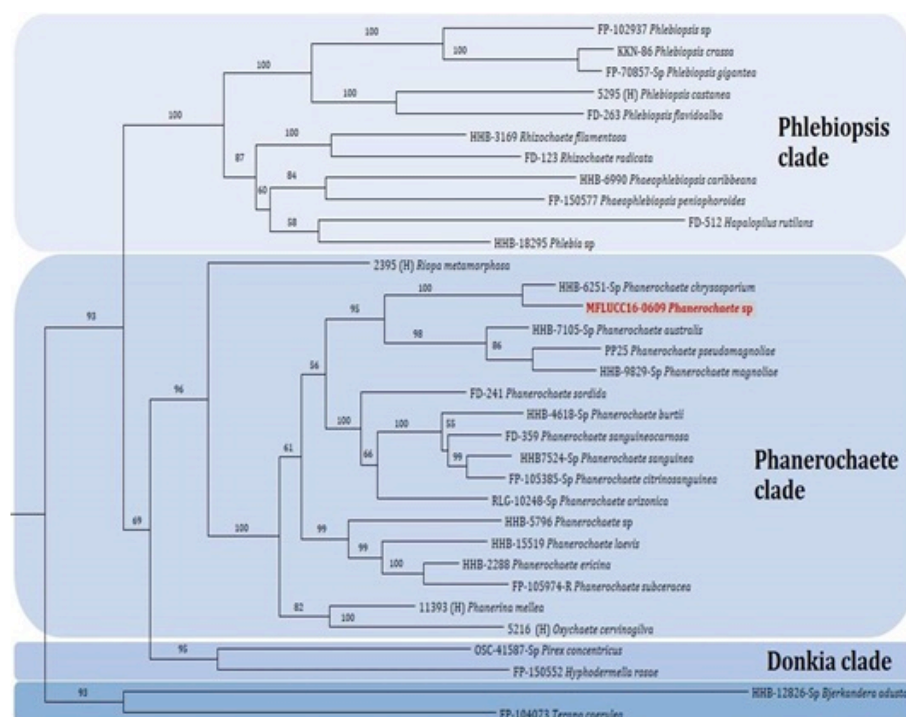

Figure 2.
Phylogenetic tree of the endophytic fungus *Phanerochaete* sp. MFLUCC16-0609 based on 5.8S and ITS regions sequences. *Bjerkandera adusta* and *Terana caerulea* are used as the outgroup

Conclusions

The endophytic fungal extract of *Phanerochaete* sp. MFLUCC16-0609 isolated from *O. basilicum* var. *thyrsiflora* leaves showed significant antifungal activity against plant pathogens including *T. reesei* TISTR3080 and *L. theobromae* MFU. The efficacy of fungal extracts of *Phanerochaete* sp. MFLUCC16-0609 against both tested fungal pathogens may be related mainly to the presence of sclareolide which was reported as important

antimicrobail compound. It can be concluded that the crude extract of *Phanerochaete* sp. MFLUCC16-0609 could be applied as alternative natural fungicides against plant diseases in line with its notable antifungal properties and can be exploited for the biocontrol of various agricultural plant pathogens.

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References

1. Montesinos E, Development, registration and commercialization of microbial pesticides for plant protection. *Int Microbiol.*, 2003; 6: 245-252.
2. Ishii H, Impact of fungicide resistance in plant pathogens on crop disease control and agricultural environment. *Jpn Agric Res Quart.*, 2006; 40: 205-211.
3. Strobel G, Daisy B, Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol Rev.*, 2003; 67:491-502.
4. Alvin A, Miller KI, Neilan BA, Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiol Res.*, 2014; 169: 483-495.
5. Kogel KH, Franken P, Huckelhoven R, Endophyte or parasite what decides?. *Curr Opin Plant Biol.*, 2006; 9: 358-363.
6. Porras-Alfaro A, Bayman P, Hidden fungi, emergent properties: endophytes and microbiomes. *Annu Rev Phytopathol.*, 2011; 49: 291-315.
7. Liang H, Xing Y, Chen J, Zhang D, Guo S, Wang C, Antimicrobial activities of endophytic fungi isolated from *Ophiopogon japonicus* (*Liliaceae*). *BMC Complement Altern Med.*, 2012; 12: 1-6.
8. Gherbawy Y, Gashgari R, Molecular characterization of endophytic fungi from *Calotropis procera* plants in Taif region (Saudi Arabia) and their antifungal activities. *Plant Biosyst.*, 2014; 148: 1085-1092.
9. Atiphasaworn P, Monggoot S, Gentekaki E, Brooks S, Pripdeevech P, Antibacterial and antioxidant constituents of extracts of endophytic fungi isolated from *Ocimum basilicum* var. *thyrsiflora* leaves. *Curr Microbiol.*, 2017; 74: 1185-1193.
10. Pripdeevech P, Chukeatirote E, Chemical compositions, antifungal and antioxidant activities of essential oil and various extracts of *Melodorum fruticosum* L. flowers. *Food Chem Toxicol.*, 2010; 48: 2754-2758.
11. Miettinen O, Spirin V, Vlasák J, Rivoire B, Stenroos S, Hibbett DS, Polypores and genus concepts in *Phanerochaetaceae* (Polyporales, Basidiomycota). *MycoKeys.*, 2016; 17: 1-46.
12. Tiwari R, Kalra A, Darokar MP, Chandra M, Aggarwal N, Singh AK, Khanuja SP, Endophytic bacteria from *Ocimum sanctum* and their yield enhancing capabilities. *Curr Microbiol.*, 2009; 60: 167-171.
13. Gangadevi V, Muthumary J, Endophytic fungal diversity from young, mature and senescent leaves of *Ocimum basilicum* L. with special reference to taxol production. *Ind J Sci Technol.*, 2007; 1: 1-12.
14. Chowdhary K, Kaushik N, Fungal endophyte diversity and bioactivity in the Indian medicinal plant *Ocimum sanctum* Linn. *PLoS ONE.*, 2015; 1-25.
15. Hayet E, Fatma B, Souhir I, Waheb FA, Abderaouf K, Mahjoub A, Maha M, Antibacterial and cytotoxic activity of the acetone extract of the flowers of *Salvia sclarea* and some natural products. *Pak J Pharm Sci.*, 2007; 20: 146-148.
16. Rabe T, van Staden J, Isolation of an antibacterial sesquiterpenoid from *Warburgia salutaris*. *J Ethnopharmacol.*, 2000; 73: 171-174.
17. Paun G, Zrira S, Boutakiout A, Ungureanu O, Simion D, Chelaruc C, Radu GL, Chemical composition, antioxidant and antibacterial activity of essential oils from moroccan aromatic herbs. *Rev Roum Chim.*, 2013; 58: 891-897.
18. Gouda S, Das G, Sen SK, Shin HS, Patra JK, Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. *Front Microbiol.*, 2016; 7: 1538.
19. Mousa WM, Raizada MN, The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. *Front Microbiol.*, 2013; 4: 65.
20. Burt S, Essential oils: their antibacterial properties and potential applications in foods – a review. *Int J Food Microbiol.*, 2004; 94: 223-253.
21. Silva-Hughes AF, Wedge DE, Cantrell CL, Carvalhoa CR, Panb Z, Moraes RM, Madoxxe VL, Rosa LH, Diversity and antifungal activity of the endophytic fungi associated with the native medicinal cactus *Opuntia humifusa* (*Cactaceae*) from the United States. *Microbiol Res.*, 2015; 175: 67-77.
22. Chapla VM, Zeraik ML, Leptokarydis IH, Silva GH, Bolzani VS, Young MC, Pfenning LH, Araújo AR, Antifungal compounds produced by *Colletotrichum gloeosporioides*, an endophytic fungus from *Michelia champaca*. *Molecules*, 2014; 19: 19243-19252.
23. Idris A, Ietidal A, Idris M, Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia africana*. *Egypt Acad J Biol Sci.*, 2013; 5: 1-9.