Saudi Pharmaceutical Journal 27 (2019) 401–405

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Evaluation of antimicrobial activity of secondary metabolites of fungi isolated from Sultanate Oman soil

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ARTICLE INFO

Article history:
Received 2 October 2018
Accepted 31 December 2018
Available online 3 January 2019

Keywords:
Aspergillus flavus
Phytochemical contents
Intra cellular
Extra cellular
Fusarium chlamydosporum
Candida albicans
Antimicrobial
Soil microbes
Aspergillus
Fusarium

ABSTRACT

Seven fungal species were isolated from soil samples collected from the University of Sultan Qaboos, Muscat, Sultanate of Oman. The fungal isolates were identified as *Aspergillus athecius*, *A. terreus* var. *africans*, *A. flavus*, *A. terreus*, *A. foetidus*, *Fusarium chlamydosporum* and *F. nygamai*. Phytochemical and chromatographic investigation showed variety of secondary metabolites in all of the fugal extracts (extra and intra cellular). The antimicrobial activity of internal and external extracts of the isolated fungal species were screened against *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus*, *Streptococcus gordonii*, *S. mutans*. The antimicrobial activity of external secondary metabolites was generally better than the internal metabolites. The highest antimicrobial activity (32 mm, 30 mm and 29 mm) was obtained from external secondary metabolites of *Aspergillus flavus* against *Candida tropicals*, *Candida parapsilosis* and *Candida albicans*, respectively.

1. Introduction

Antibiotic is a natural substance of biological origin used to treat diseases caused by microbes to eukaryotic organisms including human being (Pannapa and Pattra, 2017; Hacioglu and Dulger, 2011; Nikodinovic et al., 2003). According to their mode of action, antibiotics are known as wide- or narrow-spectrum. These include cell wall, protein and nucleic acid inhibition (Tortora et al., 2007; Brooks et al., 2001).

The necessity for new antibiotics increasing daily due to the emergence of drug-resistant pathogens. Despite the huge potential sources of antibiotics including medicinal plants, soil still the most important reservoir for novel antibiotics with pharmaceutical and biological activity (Rajaperumal et al., 2013). There are more than 500 antibiotics are discovered every year, however, almost 60% are obtained from the soil (Molinari, 2009). Interestingly, a few grams of soil contain numerous microorganisms (Makut and Owolewa, 2011).

Numerous species of fungi including *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium* and Yeasts are able to produce enzymes and secondary metabolites including antibiotics (Hyde, 2001). Approximately 20% of antibiotics have been obtained from fungi isolated from soil (Berdy, 2005). Those antibiotics, produced by fungi, including fusidic acid (Akpotu et al., 2017), cephalosporin and penicillin are widely used for treatment of many diseases (Sethi et al., 2013) and they are the most important source of potentially significant pharmaceutical drugs (Wasser, 2002). The objective of this study was to isolate and identify soil fungi and to determine their ability to inhibit the growth of microorganisms.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2018.12.009
1319-0164/

2. Materials and methods

2.1. Fungal isolation and identification

2.1.1. Isolation

2.1.1.1. Soil samples collection. The soil samples were collected from different sites at Sultan Qaboos University, Muscat, Sultanate Oman. The direct inoculation method was used for sampling and isolation of fungal isolates. Soil samples were separately transferred onto the surface malt agar media and incubated for 5–7 day at 25 °C.

2.1.1.2. Fungal isolation. For isolation of all fungi four different media were used (yeast extract sucrose potato dextrose, czapek's dox and yeast malt extract agar malt extract) were uses. The technique used was described by Al-Enazi et al. (2018).

2.1.2. Identification of fungal isolates

For identification of the isolated fungi, each fungal isolate was microscopically examined be by transferring fungal mycelia onto a glass slide containing a drop of lactophenol cotton blue stain, and then covered with the cover slip and was viewed under microscope. Also, the macroscopic observation was determined by comparing the fungal isolate characters with the Pictorial atlas of soil and seed fungi (Casero et al., 2017). However, identification of the obtained fungal isolates was confirmed by the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

2.2. Extraction

2.2.1. Internal fungal secondary metabolites extraction

The mycelial mat for each fungus (250 g) was obtained, washed with distilled water, extracted by refluxing in 2 liters of boiled ethanol for 120 min and re-extracted again for three times (as mentioned before). The obtained filtrates were then concentrated together and dried from ethanol under reduced pressure at low temperature and kept in the fridge for the investigation.

2.2.2. External fungal secondary metabolites extraction

One liter of each fungal growth medium was separately extracted using 2 L of n-butanol (saturated with water), and re-extracted again for four repetitive times. The obtained butanol extract was filtered using Buchner funnel contains anhydrous sodium sulphate. The collected butanol extracts was dried from solvent using rotatory evaporator at temperature not exceeding 35C and the dry extract was then kept in fridge for investigation (Zain et al., 2012).

2.3. Phytochemical screening and chromatographic investigation

2.3.1. Phytochemical screening were carried out for all both extra and intra fungal extracts to investigate their phytochemical

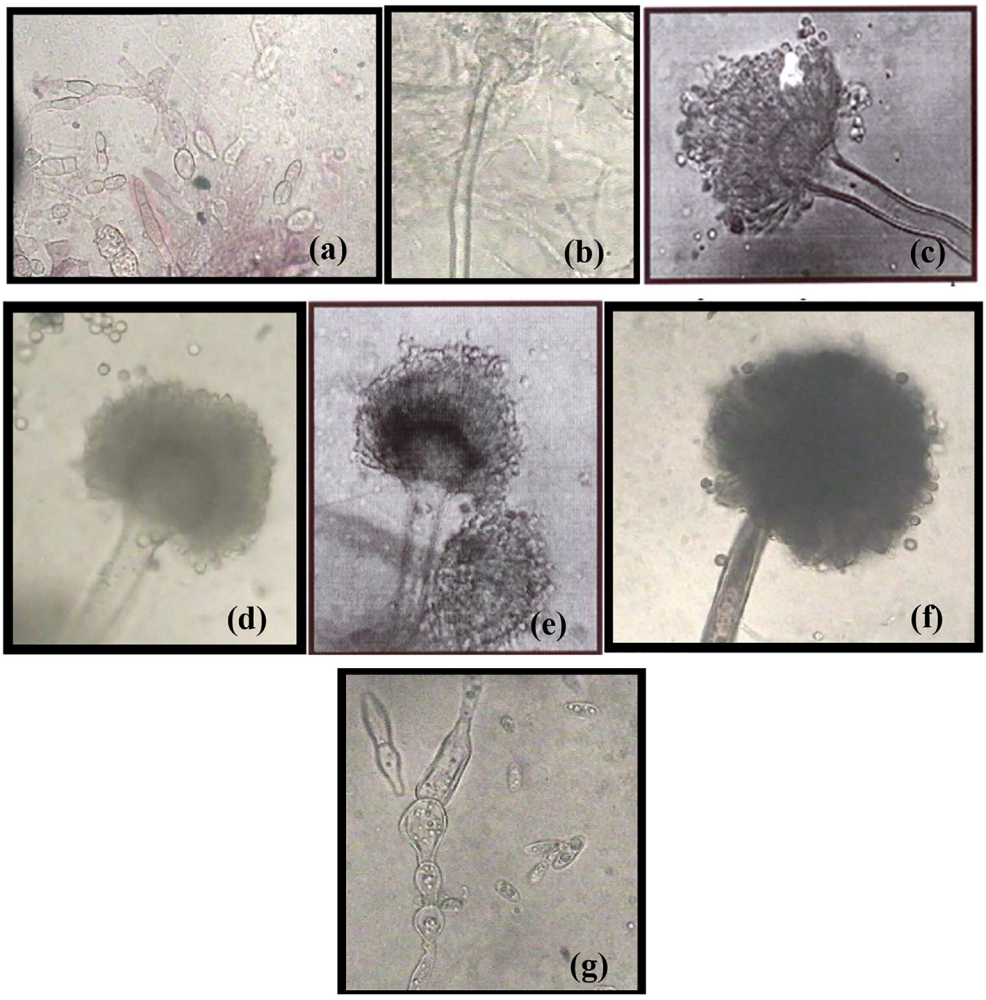

Fig. 1. The microscopic structures of fungal isolates; *Aspergillus athecius* (a), *Aspergillus terreus* var. *africans* (b), *Aspergillus flavus* (c), *Fusarium chlamydosporum* (d), *Aspergillus terreus* (e), *Aspergillus foetidus* (f) and *Fusarium nygamai* (g).

contents of secondary metabolites, these were done according to the standard published methods (Tiwari et al., 2011).

All of Each fungal extracts (intra and extra) was tested chromatographically using three systems a- c [(a) ethyl acetate: methanol: water 90: 5: 4 v/v/v, (b) chloroform: methanol 95: 5 v/v & (c) benzene: ethyl acetate 86: 14 v/v]. Visualization of the spots were carried out under UV before and after spraying with Antimony trichloride ($SbCl_3$).

2.4. Antimicrobial activity

2.4.1. Test organisms

The test organisms used were *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Lactobacillus acidophilus*, *Pseudomonas aeruginosa*, *Streptococcus gordonii*, and *Streptococcus mutans* were obtained from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt as test organisms.

2.4.2. Antimicrobial screening

The antimicrobial activity of internal and external secondary metabolites of the isolated fungal strains grown on malt extract, yeast extract sucrose, and yeast-malt extract media were determined using disc-diffusion method (Al-Enazi et al., 2018). Petri dishes containing 20 mL of agar medium were seeded with test organisms. Standard discs (6 mm in diameter) loaded with 50 μL of fungal extract were placed onto the agar, and incubated at 37 °C for 24–48 h. The antimicrobial activity was recorded as the diameter of the inhibition zone formed around the disc.

Table 1
Phytochemical Screening of all fungal extracts (extra and intra cellular).

Test for	*Aspergillus athecius*		*Aspergillus terreus* var. *africans*		*Aspergillus flavus*		*Fusarium chlamydosporum*		*Aspergillus terreus*		*Aspergillus toetidus*		*Fusarium nygama*	
	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra
Alkaloids	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Carbohydrates and /or glycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Saponins	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Tannins	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Unsaturated sterols and/orTriterpens	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anthraquinones	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteins and/or amino acids	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coumarins and lactones	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Cardenolides	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(+): present; (–) absent, (±) trace.

Table 2
TLC results for phytochemical Screening of all extracts (extra and intra cellular).

R_f values			Colour			*Aspergillus athecius*		*Aspergillus terreus* var. *africans*		*Aspergillus flavus*		*Fusarium chlamydosporum*		*Aspergillus terreus*		*Aspergillus toetidus*		*Fusarium nygama*	
a	b	c	UV	NH_3	$SbCl_3$	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra
–	0.86	–	bl.	bl.	bl.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	0.86	–	br.	br.	br.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	–	0.73	bl gr.	bl gr.	bl gr.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	–	0.64	bl gr.	bl gr.	bl gr.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	0.85	0.55	bl gr.	bl gr.	bl gr.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
–	0.83	0.49	bl gr.	bl gr.	bl gr.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	–	0.43	bl gr.	bl gr.	bl gr.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	–	0.43	br.	br.	br.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	–	0.35	bl.	bl.	bl.	±	+	±	+	±	+	±	+	±	+	±	+	±	+
–	–	0.31	bl.	bl.	bl.	_	±	_	±	_	±	_	±	_	±	_	±	_	±
–	–	0.18	bl.	bl.	bl.	_	±	_	±	_	±	_	±	_	±	_	±	_	±
–	–	0.15	bl.	bl.	bl.	_	±	_	±	_	±	_	±	_	±	_	±	_	±
–	–	0.13	bl gr.	bl gr.	bl gr.	_	+	_	+	_	+	_	+	_	+	_	+	_	+
–	0.51	0.05	bl.	bl.	bl.	_	±	_	±	_	±	_	±	_	±	_	±	_	±
0.80	0.28	0.02	bl.	bl.	bl.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.77	0.03	0.18	y.	br.	br.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.71	0.04	0.02	y.	br.	br.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.66	0.22	–	bl.	bl.	bl.	+	_	+	_	+	_	+	_	+	_	+	_	+	_
0.59	0.21	–	bl.	bl.	bl.	±	_	±	_	±	_	±	_	±	_	±	_	±	_
0.53	0.14	–	bl.	bl.	vi.	_	_	_	_	_	_	_	_	_	_	_	_	_	_
0.40	0.12	–	bl.	bl.	bl.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.37	0.08	–	br.	br.	br.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.22	0.06	–	br.	br.	br.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.16	0.04	–	br.	br.	br.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.13	–	–	y.	y.	y.	±	±	±	±	±	±	±	±	±	±	±	±	±	±
0.08	–	–	y.	y.	y.	±	_	±	_	±	_	±	_	±	_	±	_	±	_
0.03	–	–	br.	br.	br.	+	±	+	±	+	±	+	±	+	±	+	±	+	±
–	0.86	–	bl.	bl.	bl.	_	+	_	+	_	+	_	+	_	+	_	+	_	+

(–): absent; (+): present; (±): trace; (a): ethyl acetate: methanol: water 90:5:4 v/v/v; $SbCl_3$: antimony trichloride; (b): chloroform: methanol 95:5 v/v; (c): benzene: ethyl acetate 86:14 v/v; bl: blue; br: brown; gr: green; y:yellow; NH_3: ammonia; ex: extra cellular; In: intra cellular.

3. Results and discussion

3.1. Isolation and identification of fungal strains

Fungal isolates were obtained from the analyses of different soil samples collected from the University of Sultan Qaboos, Muscat, and Sultanate of Oman. Seven fungal strains were isolated from malt extract agar plates incubated at 28 °C for 7 days. All fungal isolates were obtained in pure cultures by single conidial transfer onto beer agar plates. The fungal isolates were identified as *Aspergillus athecius*, *A. terreus* var. *africans*, *A. flavus*, *A. terreus*, *A. foetidus Fusarium chlamydosporum* and *F. nygamai* (Fig. 1).

Similarly, Raja and others (Raja et al., 2017) isolated and identified different fungal strains from soil samples collected from Loyola College Campus, Chennai, India. Also, it was suggested that the number and frequencies of fungal species isolated from soil depend on the moisture content and/or level of organic carbon in the soil (Salar and Aneja, 2006).

3.2. Phytochemical and chromatographic screening

All extracts of each fungi (extra & intra cellular) were found to be quite similar to each other, they contain; Proteins and/or amino acids, unsaturated sterols and/or triterpens, carbohydrates and /or glycosides in addition to traces of coumarins and lactones, saponins, tannins > no alkaloids, flavonoids, Anthraquinones and Cardenolides were detected (Table 1).

Thin Layer Chromatography (TLC) investigation of all fungal extracts (extra and intra) showed that all extra cellular extracts of each fungi were similar to each other, and that all intra cellular extracts of each fungi were similar to each other. However, the intra cellular extracts contain more compounds than extra cellular extracts (Table 2).

3.3. Antimicrobial activity

The internal and external secondary metabolites of the obtained fungal species were investigated for their antimicrobial activity against *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus*, *Streptococcus gordonii*, *S. mutans*. Each fungal strain was grown on Malt Extract (ME), Yeast Extract Sucrose (YES) and Yeast-Malt Extract media at 28 ± 2 °C for 14 days. Both the intra- and extra-cellular metabolites of the fungal species were screened for their antimicrobial activity (Tables 3–5). The isolated fungal species exhibited anticandidal and antibacterial activity against almost all the investigated test organisms. However, activity of secondary metabolites of fungi grown on malt extract media was the highest (Table 3). Moreover,

Table 3
Antimicrobial activity of cell extract of fungal isolates grown on malt extract medium.

Test organism	Diameter of inhibition zone (mm)													
	Aspergillus athecius		*Aspergillus terreus* var. *africans*		*Aspergillus flavus*		*Fusarium chlamydosporum*		*Aspergillus terreus*		*Aspergillus foetidus*		*Fusarium nygamai*	
	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.
Unicellular fungi														
Candida albicans	17.0	19.0	21.0	22.0	16.0	16.0	14.0	14.0	27.0	29.0	17.0	18.0	15.0	16.0
Candida glabrata	15.0	15.0	19.0	19.0	14.0	17.0	13.0	15.0	26.0	25.0	16.0	16.0	14.0	17.0
Candida parapsilosis	17.0	18.0	18.0	17.0	15.0	17.0	13.0	13.0	28.0	30.0	17.0	17.0	16.0	21.0
Candida tropicalis	16.0	19.0	20.0	21.0	17.0	17.0	14.0	15.0	27.0	32.0	17.0	19.0	14.0	19.0
Gram-Negative Bacteria														
Pseudomonas aeruginosa	15.0	18.0	16.0	20.0	16.0	18.0	10.0	10.0	22.0	24.0	16.0	18.0	17.0	17.0
Gram-Positive Bacteria														
Lactobacillus acidophilus	15.0	15.0	17.0	17.0	19.0	20.0	12.0	15.0	21.0	27.0	19.0	20.0	14.0	15.0
Streptococcus gordonii	16.0	17.0	17.0	19.0	17.0	19.0	13.0	14.0	23.0	26.0	18.0	18.0	17.0	17.0
Streptococcus mutans	14.0	16.0	15.0	16.0	17.0	18.0	14.0	16.0	22.0	25.0	17.0	21.0	15.0	17.0

Int., intracellular metabolites; Ext., extracellular metabolites.

Table 4
Antimicrobial activity of cell extract of fungal isolates grown on yeast extract sucrose medium.

Test organism	Diameter of inhibition zone (mm)													
	Aspergillus athecius		*Aspergillus terreus* var. *africans*		*Aspergillus flavus*		*Fusarium chlamydosporum*		*Aspergillus terreus*		*Aspergillus foetidus*		*Fusarium nygamai*	
	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.
Unicellular fungi														
Candida albicans	12.0	12.0	11.0	10.0	10.0	10.0	00.0	00.0	13.0	11.0	09.0	09.0	10.0	09.0
Candida glabrata	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
Candida parapsilosis	14.0	15.0	12.0	13.0	13.0	13.0	10.0	11.0	15.0	16.0	11.0	12.0	12.0	14.0
Candida tropicalis	10.0	12.0	10.0	10.0	11.0	10.0	07.0	00.0	12.0	12.0	00.0	00.0	10.0	11.0
Gram-Negative Bacteria														
Pseudomonas aeruginosa	10.0	10.0	10.0	12.0	11.0	11.0	11.0	11.0	14.0	13.0	12.0	12.0	11.0	10.0
Gram-Positive Bacteria														
Lactobacillus acidophilus	11.0	12.0	11.0	12.0	10.0	11.0	00.0	00.0	13.0	12.0	00.0	00.0	13.0	16.0
Streptococcus gordonii	11.0	11.0	12.0	10.0	00.0	00.0	00.0	00.0	12.0	15.0	09.0	10.0	11.0	11.0
Streptococcus mutans	10.0	10.0	10.0	11.0	10.0	11.0	00.0	00.0	11.0	13.0	00.0	00.0	10.0	11.0

Int., intracellular metabolites; Ext., extracellular metabolites.

Table 5
Antimicrobial activity of cell extract of fungal isolates grown on yeast malt-extract medium.

Test organism	Diameter of inhibition zone (mm)													
	Aspergillus athecius		*Aspergillus terreus* var. *africans*		*Aspergillus flavus*		*Fusarium chlamydo-sporum*		*Aspergillus terreus*		*Aspergillus foetidus*		*Fusarium nygamai*	
	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.	Int.	Ext.
Unicellular fungi														
Candida albicans	12.0	13.0	11.0	11.0	10.0	10.0	08.0	10.0	12.0	13.0	00.0	00.0	10.0	11.0
Candida glabrata	11.0	10.0	12.0	12.0	09.0	10.0	07.0	07.0	12.0	12.0	00.0	00.0	10.0	10.0
Candida parapsilosis	12.0	12.0	12.0	11.0	10.0	11.0	00.0	00.0	11.0	11.0	00.0	00.0	12.0	12.0
Candida tropicalis	00.0	00.0	12.0	12.0	11.0	11.0	08.0	09.0	11.0	11.0	00.0	00.0	11.0	11.0
Gram-Negative Bacteria														
Pseudomonas aeruginosa	00.0	00.0	10.0	11.0	09.0	09.0	00.0	00.0	10.0	10.0	00.0	00.0	00.0	10.0
Gram-Positive Bacteria														
Lactobacillus acidophilus	12.0	12.0	10.0	12.0	10.0	11.0	10.0	09.0	13.0	13.0	00.0	00.0	10.0	11.0
Streptococcus gordonii	13.0	12.0	12.0	12.0	09.0	10.0	00.0	00.0	11.0	11.0	00.0	00.0	12.0	10.0
Streptococcus mutans	13.0	13.0	00.0	09.0	11.0	10.0	00.0	00.0	10.0	11.0	00.0	00.0	12.0	12.0

Int., intracellular metabolites; Ext., extracellular metabolites.

the antimicrobial activity of external secondary metabolites was generally better than the internal metabolites.

The internal and external secondary metabolites of all the isolated fungi grown on malt extract media showed anticandidal and antibacterial activity against all the investigated candidal and bacterial species. However, the highest antimicrobial activity (32 mm, 30 mm, 29 mm) was obtained from external secondary metabolites of *Aspergillus flavus* against *Candida tropicals*, *C. parapsilosis* and *C. albicans*, respectively (Table 4).

On the other hand, when grown on yeast extract sucrose media, all the isolated fungi showed no activity against *Candida glabrata* (Table 4). Also, there was no antimicrobial activity against any of the test organisms for internal or external secondary metabolites of *Aspergillus foetidus* grown on yeast malt extract media (Table 5).

The differences in the antimicrobial activity between intra and extra cellular extracts of the investigated fungi are somewhat similar to each other's but different in the concentration of the secondary metabolites (according to their TLC) in each of them.

Previous studies mentioned that different culture media could lead to variation in morphology, physiology and growth rate of the same fungal strain according to the components and nutrients of the growth media (Magaldi et al., 2004). Several studies have been carried out on the antimicrobial activity of fungi and their secondary metabolites and proved that the activities is depend on the nature of the secondary metabolites present in the fungi (Akpotu et al., 2017; Zain et al., 2014; Wang et al., 2013; Shen, 2013; Zhang, 2008; Effendi, 2004).

Acknowledgment

This research project was supported by a grant from the "Research Center of the Female Scientific and Medical Colleges", Deanship of Scientific Research, King Saud University.

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