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Research Article

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Assessment of secondary metabolites as antioxidants from terrestrial fungi and bacteria

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ABSTRACT

Eighteen and thirty different isolates of terrestrial fungi and bacteria, respectively isolated from Egyptian environment (El-Fayoum, Egypt). All fungal isolates showed antioxidant activities in ethyl acetate and chloroform. Thirteen fungal isolates showed high antioxidant activities in case of using ethyl acetate as a solvent than using chloroform as solvents, five chloroform extracts showed high antioxidant activities than ethyl acetate extract. Fungal isolate No. 18(Penicillium lividum Westling)showed highest antioxidant activity (94.80%).Fifteen bacterial isolates showed antioxidant activities in case of using ethyl acetate and chloroform, eleven isolates have significant antioxidant activities in case of using ethyl acetate as solvents. Only four chloroform extracts show antioxidant activities. Bacterial isolate No. 20(Bacillus azotoformans) showed highest antioxidant activity (69.27%).

Key words: Terrestrial fungi and bacteria, microbial secondary metabolites, antioxidants.

INTRODUCTION

Natural products are defined as chemical substances produced by nature; they are not artificial or manufactured. These compounds are produced by microbes, plants, insects or other animals [1]. Bioactive compounds are mostly secondary metabolites produced by microorganisms in an active culture cultivation process.Secondary metabolites are excreted by microbial cultures at the end of primary growth and during the stationary phase of growth. Secondary metabolites represent some of the most economically important industrial products and are of huge interest. The most common are steroids, terpenes, alkaloids, cyclopeptides, and coumarins, and some of theseare mycotoxins. These bioactive compounds can be used as human drugs or can be chemically modified to modulate therapeutic activities [2].Antioxidant components are micro-constituents present in the diet that enables the scavenging of reactive oxygen species (ROS). Due to toxicities of the synthetic antioxidants such as butylatedhydroxyanisole (BHA), butylatedhyroxytoluene (BHT), there is an increasing attention towards natural antioxidants [3].Organisms are well known to have an abundance of antioxidant compounds that have been shown to be effective at removing ROS such as superoxide, anions, hydrogen peroxide, and hydroxyl radicals from the body. ROS are strongly associated with cardiovascular disease, cancer and various neurodegenerative disorders. The main sources of free radicals are oxidation reactions of polyenoic fatty acids [4,5].It is important to find an effective antioxidant from natural origin such as edible plants, spices and herbs because these natural substances have been eaten safely for long time and also microbes in general are considered a cheap source for antioxidants [6].Fungi play a major role in soil ecosystems along with bacteria, protists, small invertebrates and plants, through complex trophic interactions. Most soil fungi are regarded as saprobes, decomposing organic matter and contributing to nutrient cycling, while several species form mycorrhizal associations with plants or are plant pathogens [7]. Also recognized

as prolific secondary metabolite producers, fungi have provided several bioactive compounds and chemical models currently used as pharmaceuticals, and soils are traditionally the main source of fungal genetic resources for bio-prospection programs [8]. Bacteria are some of the smallest and most abundant microbes in the soil. Bacteria are an interesting source of bioactive secondary metabolites with interesting activity as antioxidant activity.

The aim of present work is to isolate and identify bacterial and fungal strains that produce secondary metabolites as antioxidant agents.

EXPERIMENTAL SECTION

1. Collection and isolation of bacteria and fungi

The present study was carried out on 48 isolates from soil samples collected from El-Fayoum governorate, Egypt. Soil samples were collected from patches free from roots according to the method described by Johnson [9].The isolates were obtained using standard serial dilution technique. Each individual bacterial isolate was cultivated on medium (g/l): glucose (20.0),$CaCO_3$ (1.0), NH_4NO_3(0.8), K_2HPO_4 (0.6), KH_2PO_4 (0.05), $MgSO_4.7H_2O$ (0.05), $MnSO_4.4H_2O$ (0.1) and yeast extract (0.1) and the plates were incubated at 37ºC for 24 h [10].The dilution-plate method was used to determine soil fungi. Fungal isolates were cultivated on Czapek-Dox medium and the plates were incubated at 28ºC for 7 days [11] and also Czapek's agar was used throughout the present investigation for identification of fungal isolates. The medium was supplemented with Rose-Bengal and chloramphenicol as bacteriostatic agents [12]. Media were sterilized by autoclaving at 121ºC for 15 min.

2. Production of Secondary Metabolites

Each individual bacterial isolate was cultivated on production medium (peptone 4.0 g/l, yeast extract 2.0 g/l, and sucrose 20.0 g/l) [13] and inoculated with 2 ml of 24 h old cultures. The cultures were incubated at 37^{o}C on rotary shaker (150 rpm) for 3 days while the fungal isolates were cultivated on synthetic medium (malt – yeast – glucose – peptonemedium; MYGP)[14]. The experimental cultures were grown in 250 ml Erlenmeyer flasks, each containing 50 ml of the synthetic medium and inoculated with 2 ml of 7-10 days old cultures. Fungal cultures were incubated at 28^{o}C on rotary shaker (120 rpm) for 7 days.

3. Microbial Extraction

Forty eight fungal and bacterial isolates were screened for production of bioactive secondary metabolites. The solvent extraction was the first step in the whole separation process. At the end of fermentation period, the content of each flask (medium and mycelium) was extracted with two different solvents (ethyl acetate or chloroform) as described by Serizawa [15]. The combined solvent, dried over anhydrous sodium sulphate, filtered, then distilled to give a semisolid extract (test material which used for further bioassay test (antioxidant bioassay).

4. Antioxidant activity

The stable free radical of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used to assay free radical scavenging activity [16]. The DPPH radical scavenging activity was measured according to Todaka[17]. Eight mg of DPPH was dissolved in 100 ml of chloroform and ethyl acetate and also samples dissolved in chloroform or ethyl acetate. Chloroform DPPH or ethyl acetate DPPH served as control. The tested material dissolved in ethyl acetate or chloroform (0.2 ml) was mixed vigorously with 3 ml of DPPH solution and was kept in the dark for 30 min. The medium itself was used as control and was treated in the same manner as the culture broth. Absorbance at 517 nm was measured using spectrophotometer and the radical scavenging activity was quantified as units/ml according to the following formula;

$$\text{Scavenging ability (\%)} = (A_{517 \text{ of control}} - A_{517\text{of sample}} / A_{517 \text{ of control}}) \times 100.$$

5. Microbial identification

a. Identification of fungi

Identification of the higher producer fungi was carried out using the morphological characteristics and microscopic features were examined by optical light microscope (10×90) Olympus CH40 according to the following references: Ainsworth [18] as a dictionary of the fungi, Ramirez [19] and Pitt [20,21], for *Penicillium* species.

b. Identification of bacteria

Cell morphology

Bacterial cells were stained with Gram's stain according to the method described by Shaffer and Goldin [22]. After staining, the morphology of bacterial cells; including shape and staining features; was examined by optical light microscope (10×90, Olympus CH40).

Biochemical tests

The pure isolated strain was identified according to the methods of Sneath[23] as described inBergey's Manual of Systematic Bacteriology to the genus level.

RESULTS AND DISCUSSION

1. Microbial isolation

Forty eight isolates were recovered from soil samples, 18 of them were fungal species and 30werebacterial species. Numbers of bacterial isolates(rate of isolation) was higher than numbers of fungal isolates (Figure 1).

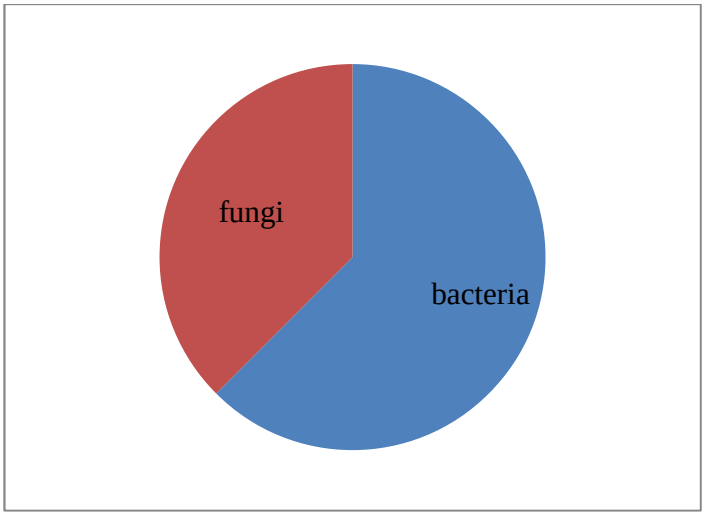

Figure 1. Distribution of tested terrestrial microorganisms

2. Radical scavenging activity for bacterial and fungal extracts (Antioxidant Bioassay)

Natural antioxidants are ginning importance, due to their benefits for human health, decreasing the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation [24,25].bacterial and fungal extracts act as hydrogen donors, When the free radical have been scavenged, will generate its color to yellow because as odd electron of the radical becomes paired off in the presence of a hydrogen donor, this discoloration due to the absorption intensity will be decreased which related to the number of electron captured**.** The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl group [26]. Eighteen fungal strains were screened for antioxidant activity by using ethyl acetate and chloroform; the antioxidant activities of ethyl acetate extracts were slightly greater than that of chloroform extracts. thirteen isolates have high antioxidant activities in the case of using ethyl acetate as a solvent than in the case of using chloroform as a solvent, isolates No. 1, 2, 3, 5, 8, 9, 10, 11, 13, 14, 15, 16 and 18 showed antioxidant activities of 85.22%, 84.24%, 75.43%, 80.98%, 87.77%, 87.13%, 80.04%, 91.91%, 84.41%, 84.41%, 56.04%, 86.61% and 94.80%, respectively, while only five chloroform extracts showed high antioxidant activities than ethyl acetate extract. In addition, isolates No. 4, 6, 7, 12 and 17 showed antioxidant activities of 78.33%, 77.01%, 84.19%, 67.00% and 79.22%, respectively (Table 1).

The active isolated fungal taxa in this study belong to the common isolated terrestrial fungi (soil fungi) and were reported as terrestrial fungi in previous studies [27-29]. There are many reports about biological active secondary metabolites produced by terrestrial fungi in cultures that were had different biological activity such as antioxidant, antiviral, antimicrobial and antitumor activity [4,30].

Thirty bacterial strains were screened for antioxidant activity using ethyl acetate and chloroform as solvents; the antioxidant activities of ethyl acetate extracts were slightly higher than that of chloroform extracts. Eleven isolates showed antioxidant activities in case of using ethyl acetate as a solvent, isolates No. 20, 21, 24, 25, 26, 28 and 35 showed significant antioxidant activities of 69.27%, 24.39%, 63.20%, 41.18%, 35.57%, 28.33% and 23.71%, respectively, while other isolates showed neglected antioxidant activities. Also, only four chloroform extracts showed antioxidant activities, isolates No. 20, 23, 25 and 26 showed antioxidant activities of 53.11%, 6.25%, 21.03% and 11.8%, respectively (Table 2).

Screening of bacterial extracts shows huge diversity of natural compounds with broad biological activities, such as antimicrobial, antiviral, immunosuppressive, and antitumor activities, that enable the bacterium to survive in its natural environment. These results enlarge the potential industrial importance of *Bacillus* spp.[31].

Table 1. Antioxidant activity of active fungal extract

Code number	Scavenging ability (%)							
	Ethyl acetate extract				Chloroform extract			
	Time (min)				Time (min)			
	30	60	90	120	30	60	90	120
1	21.30	45.19	55.95	85.22	18.90	45.22	54.34	83.56
2	35.65	59.10	77.17	84.24	34.50	49.12	71.23	82.56
3	25.24	40.00	55.67	75.43	20.00	48.14	67.98	75.00
4	35.44	40.00	65.78	78.29	35.00	49.92	66.23	78.33
5	40.99	58.94	78.12	80.98	40.00	55.15	67.77	78.44
6	42.23	49.74	66.17	76.55	41.14	50.00	68.98	77.01
7	40.43	55.19	60.07	84.15	40.00	54.14	61.34	84.19
8	40.94	59.49	77.77	87.77	45.00	58.00	65.4	78.05
9	58.23	70.00	75.34	87.13	56.17	60.01	78.16	86.09
10	37.94	58.24	76.87	80.04	36.01	45.95	67.87	78.90
11	78.00	85.14	88.12	91.91	72.24	78.00	85.22	90.01
12	51.16	55.45	58.23	60.09	51.17	60.15	65.22	67.00
13	68.13	74.71	81.98	84.41	45.28	47.46	47.50	48.72
14	3.29	57.47	77.54	84.41	76.57	78.80	80.12	81.29
15	50.46	51.57	53.76	56.04	48.96	50.79	51.11	53.06
16	42.85	81.60	88.10	89.61	82.60	83.91	83.94	83.97
17	53.84	70.11	72.54	74.12	62.63	67.81	76.34	79.22
18	84.61	89.65	91.54	94.80	39.36	39.91	40.65	42.40

Table 2. Antioxidant activities of active bacterial extracts

Code number	Scavenging ability (%)							
	Ethyl acetate extract				Chloroform extract			
	Time (min)				Time (min)			
	30	60	90	120	30	60	90	120
19	-	-	1.4400	1.9200	-	-	-	-
20	40.9300	55.3100	68.7200	69.2700	38.9900	42.1700	48.2900	53.1100
21	12.9400	17.6100	21.81000	24.3900	-	-	-	-
23	0.0010	0.7010	1.2900	1.8900	0.9000	2.9200	5.9200	6.2500
24	44.4300	53.9200	60.8200	63.2000	-	-	-	-
25	25.0900	33.5000	38.7500	41.1800	9.1800	16.4200	20.4000	21.0300
26	20.1100	27.0100	32.4100	35.5700	2.01020	7.7100	10.9000	11.8100
28	18.1200	24.0100	27.9000	28.3300	-	-	-	-
29	-	0.8200	1.1600	1.4300	-	-	-	-
35	9.1800	15.0100	20.8500	23.7100	-	-	-	
36	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-
39	-	0.0200	0.5570	0.7710	-	-	-	-

Ethyl acetate is often used as an extraction solvent with a significant selectivity in the extraction of low-molecular-weight phenolic compounds and high molecular- weight polyphenols [32]. On the other hand, Conde[33] have reported that ethyl acetate allowed the highest phenolic content and the selective removal of non-phenolic compounds. Therefore, it could be that the antioxidant activity of ethyl acetate extracts of the terrestrial isolates may be caused by the presence of phenolic compounds in the extracts.

3. Microbial identification

a- Fungal identification

According to the above results the fungal isolate No.18has high antioxidant activity.Czapek agar medium, 25°C, 7 days: colony diameters 20-24 mm, center deep wrinkled, subcenter raised sulcate, dense, velutinous, margin entire, more or less narrow; mycelium mustard yellow to Naples yellow, white; conidiogenesis abundant, greyish to dark green; exudate yellowish white to light yellow; soluble pigment light yellow to yellow ; reverse brownish orange, olive brown or olive. Malt extract agar medium, 25°C, 7 days: colony diameters 17-21 mm, plane, low, velutinous, margin entire, thin, effuse; mycelium white; conidiogenesis abundant, greyish to dark green , exudate and soluble pigment absent; reverse greyish green or yellowish grey to greyish yellow. Czapek agar medium, 37°C, 7 days: no germination. Conidiophores and conidia: conidiophores on MEA borne from superficial mycelium; stipesseptate, apices vesiculate, up to 7.9 μm, 24-138×2.4-4.0 μm, smooth or roughened to conspicuously roughened, thin walled, strictly monoverticillate, bearing verticils of 12-31 phialides; phialidesampulliform to slightly acerose, 7.2-9.5×2.4-3.2 μm, collula short, sometimes gradually tapering; conidia mostly spheroidal, thin walled, finely roughened to roughened, borne in compact, slender long well-defined columns.These characteristics indicate that strain No. 18 is *Penicillium lividum* Westling[20,21].

According to the obtained data, terrestrial isolate No.18, *Penicillium lividum* which have strong antioxidation activityin case of using ethyl acetate as a solventand observed result were in agreement with El-Sayed [4],they reported that *Penicillium lividum* No.159 which isolated from soil have strong antioxidant activities in case of using ethyl acetate and also chloroform as solvents.

b. Bacterial identification

According to the obtained results, the bacterial isolate No.20which showed the highest antioxidant activity, this isolate was subjected for identification. The identification results were; Gram-negative, peritrichously motile rods (0.5–0.8 μmby 3–7 μm), forming ellipsoidal, subterminal and terminal spores which swell the sporangia. Nitrate, nitrite and nitrous oxide are denitrified with the production of N2. For anaerobic growth, nitrate, nitrite, nitrous oxide, tetrathionateandfumarate act as terminal electron acceptors. Growth requirements are complex; non-fermentative, carbohydrates are not attackedand a range of organic acids is utilized as carbon sources. Colonies circular and partially translucent, with entire margins, on yeast extract agar. Oxidasepositive, catalase-negative. Gelatin, starch and Tween 80 not hydrolyzed. Maximum growth temperature 42–46 °C. These characteristics indicate that strain No. 20 is *Bacillus azotoformans*.

The genus Bacillus consists of a large number of diverse, rod-shaped aerobic bacteria that have special characteristics make them good candidates as biological control agents [34]. First, they are good antibiotic producers with antagonistic activity against fungal and some bacterial pathogens. Second, they form spores that can be easily formulated, and have high viability compared with vegetative cells. Third, they are commonly found in soils. Bacillus spp. secretes many secondary metabolites, including antibiotics, antifungals, antivirals, antioxidants and siderophores. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored. These secondary metabolites exhibit strong antibiological activities such as antioxidant activity and enable the bacterium to survive in its natural environment [35].According to the obtained data, terrestrial isolate No.20 *Bacillus azotoformans* which have strong antioxidative activity and proved to be pioneer isolate for antioxidant bioassay. All genes of *Bacillus azotoformans* essential for aerobic respiration were identified, including those for terminal oxidases and for detoxifying reactive oxygen species except Draft genome sequence of *Bacillus azotoformans* MEV2011, a (Co-) denitrifying strain unable to grow with oxygen [36].

The use of oxygen as the respiratory substrate is frequently reported to lead to the development of oxidative stress, mainly due to oxygen-derived free radicals, which are collectively termed as reactive oxygen species (ROS) [37]. The metabolic rearrangement leads to the cessation of growth and the synthesis of secondary metabolites in fungal and bacterial cells, many of which are antioxidants [38-40].

CONCLUSION

The data presented in this study demonstrated that extracts of terrestrial fungi and bacteria isolated from soil, have antioxidant activity, especially *Penicillium lividum* and *Bacillus azotoformans*. Thus, terrestrial fungi and bacteria play an important role in the search for natural compounds and also represent an alternative source for the production of therapeutic agents and bioactive metabolites that are not easily obtained by chemical synthesis.

Further examination can be done to learn if terrestrial may have the potential to serve as a biological control or as new pharmacological agents.

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