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Protein Patterns and Mycelial Growth of Dermatophytic Fungi Affected by Desert Plant Extracts

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ABSTRACT

The effect of extracts (at 0.5, 1.0, 1.5 & 2.0 mg/ml) of six desert plant species commonly used in curing the dermatophytic disease was studied on mycelial growth and protein patterns of three dermatophyte fungi namely; *Microsporom gypseum, Microsporum canis* and *Aspergillus niger*. Dermatophyte fungi were isolated from skin and hairs of skin diseased patients. Plant species [*Mentha pulegium* L., *Gnaphalium luteo-album*, *Adenis microcarpus*, *Halophyllum tuberculatum* (Forssk), *Melilotus indicus* & *Mentha microphylla* C. Kock] were collected from New Valley Governorate, Egypt. The occurrence of surface fungi in each plant part used for extract preparation was also studied. Low concentration (0.5 mg/mL) of some plant extracts generally favoured mycelial growth of *Microsporum canis* and *Aspergillus niger*. In contrast, drastic inhibition in mycelial growth of *Microsporum gypseum* and *Aspergillus niger* was observed at high concentrations (1.5 & 2.0 mg/mL) of all plant extracts. *Microsporum canis* was less affected by plant extracts than the other two fungi. Plant extracts suppressed the expression of ~ 120 kDa protein band in *Microsporum gypseum* and enhanced the ~ 199 kDa protein band in *Microsporum canis*. Reduction of protein band in conjunction with the inhibition of mycelial growth suggested that the extracts of the tested plant species seem to contain active fungicidal chemical substances.

Key Words: Protein patterns; Mycelial growth; Dermatophytic fungi; Desert plant extract

INTRODUCTION

Dermatophytic fungi parasitize the humans, animals and plants. Human infections, particularly those involving the skin and mucosal surfaces constitute a serious problem, dermatophytes and *Candida* sp. being the most frequent pathogens. Furthermore, the number of reported cases of immuno-compromised patients, which frequently develop opportunistic and superficial mycoses, such as candidiasis, cryptococcosis and aspergillosis, has increased dramatically in recent years (Caceres *et al.,* 1991; Rahalison *et al.,* 1991). Although several antimycotic drugs are valuable at present, its use is limited by a number of factors, such as low potency, poor solubility, emergence of resistant strains and drug toxicity. Therefore, there is a distinct need for the discovery of new, safer and more effective antifungal agents (Fromtling & Rahway, 1987). Plants used in traditional medicine usually constitute an important source of new biologically active compounds. So in the present investigation, the antifungal effects of extracts of six desert plant species commonly used in curing the dermatophytic disease were studied on mycelial growth and protein patterns of the dermatophytic fungi *Microsporum gypseum, Microsporum canis* and *Aspergillus niger*. Information on medicinal uses of plants was obtained by personal contact with traditional healers and from the literature (Schultes & Raffauf, 1990).

MATERIAL AND METHODS

Plant material. A list of 6 plant species used in traditional medicine for the treatment of several diseases, mainly fungal skin infections, were elaborated on the basis of information obtained from the people in New Valley. The field work was carried out between March and August (2003). All samples were identified by the botanists and special keys (Täckholm, 1974). Botanical name, family, plant parts used to obtain the extracts are summarized in (Table I).

Occurrence of surface fungi. The occurrence of surface fungi in each plant part used for extract preparation were determined. Czapek's agar media was prepared, sterilized, allowed to cool to 45ºC and rose Bengal (1/15000) was added as a bacteriostatic agent (Smith & Dawson, 1944). Then the medium was poured into sterile Petri dishes and the method of Johnson and Curl (1972) was used for the estimation of mycoflora. Three plates were used for each part of each plant species and incubated at 25ºC for 1 - 2 weeks. The developing fungi were examined, identified (Domch & Gams, 1972; Moubasher, 1993) and counted.

Preparation of plant extracts. Powdered air-dried plant material from each plant species was extracted with water, dichloromethane and methanol (Vesonder, 1986; Portillo *et al*., 2001). Dichloromethane extract was maintained in a Soxhlet apparatus over 12 h, filtered and concentrated in vacuum to dryness. The extracted plant material was dried and later left to soak in 95% methanol

at room temperature and shaken three times over a 12 h period. The crude methanol extract was filtered and evaporated to dryness. Aqueous extracts were obtained by decoction in distilled water over 30 min and adjusted to give 0.5, 1.0, 1.5 and 2.0 mg/mL concentrations.

Isolation of dermatophytic fungi. Samples from skin and hairs of dermatophytosis (Tinea) diseased patients were brought from the hospital to the laboratory on slides in clean plastic pages. Then the samples were cultured on Sapouraud's Dextrose agar media (Moss & Mcquown, 1969). After 4 weeks, grown fungi were examined and identified (Connole, 1965; Moss & Mcquown, 1969; Dominik, 1970; Frey *et al.,* 1979; James & Katherine, 1984). Each fungal species was purified in Sapouraud's Dextrose agar media.

Antifungal assay. Antimicrobial activity of aqueous, dichloromethane and methanol extracts of each plant species was evaluated by the paper disk –agar diffusion method (Barry & Thornsberry, 1991). Test plates (15 cm dia.) were prepared with Sapouraud's Dextrose Agar medium (Difco) and inoculated in surface with 1.0 mL spore suspension.

Three dermatophytic fungi, *Microsporum gypseum* (Bodin) Guiart & Grigorakis, *Microsporum canis* and *Aspergillus niger* (van Tieghem) were used to study the antifungal effect of plant extracts. Equal disks (1 cm) from each purified culture of the three fungi were introduced into 250 mL conical flasks containing 100 mL of the Sapouraud's liquid media. The cultures were incubated at 25°C for 15 days. After then, the cultures were supplied with plant extracts. Five concentrations (0.0, 0.5, 1.0, 1.5 & 2.0 mg/mL) from each plant extract were used for each fungus in three replicates. The cultures were incubated at 25°C for one week, filtered and the dry weight was determined.

SDS-PAGE analysis. Mycelia of *Microsporum gypseum*, *Microsporum canis* and *Aspergillus niger* grown on the control and 1.5 mg/mL of each plant extract were collected, filtered through Whatman No.1 and washed twice with chilled (4°C) sterilized distilled water. The cells of three treated-fungi were fractionated by SDS-PAGE (10%) following Laemmli (1972). After electrophoresis at 30 m A for 45 min, the gels was stained with Coomassie blue (Sigma).

RESULT AND DISCUSSION

Occurrence of surface fungi. Twenty seven fungal species belonging to 18 genera in addition to yeast spp. were recovered from six plant species collected from New Valley Governorate in Egypt. The most common species were *Alternaria alternata, Cladosporium herbarum, Aspergillus flavus, A. niger, Curvullaria tetramera, Fusarium moniliforme, Mucor hiemalis, Penicillium notatum* and *Rhizopous nigricans*. The number of isolated fungi varied with plant species. In this instance, the maximum number of fungi (16 species + Yeast spp.) was recovered from *Halophyllum tuberculatum* and *Mentha microphylla,* while the minimum number (7 species + Yeast spp.) was isolated from *Gnaphalium luteo-album.* However, moderate numbers of isolated fungi were recovered from *Mentha pulegium* (10 + Yeast spp.*), Melilotus indicus* (10 species) and *Adenis microcarpus* (9 species).

Alternaria alternata was isolated in high frequency of occurrence and recovered from all plant species, except *Adenis microcarpus*. It represented 78 isolates constituting 9.07% of the total fungi. *A. alternata was* recovered from stem of *Mentha pulegium* (19 isolates), stem and leaves of *Gnaphalium luteo-album* (7, 3 isolates) and *Melilotus indicus* (3, 3), stem, leaves and roots of *Halophyllum tuberculatum* (10, 15, 7) and *Mentha microphylla* (1, 1, 9).

Aspergillus spp. was isolated in high frequency of occurrence (110 isolates, 5 plant species) constituting 12.79% of the total fungi. It was represented by 4 species namely; *Aspergillus niger* (64 isolates, 4 plant species), *A. flavus* (16 isolates, 4 plant species), *A. ustus* (15, 2) and *A. fumigatus* (15, 2). These species of *Aspergillus* has already been isolated previously, but with variable densities and frequencies from different beans, peas and other type of seeds in Egypt (Moubasher *et al*., 1977; Mazen *et al.,* 1990).

Curvullaria spp. was of high occurrence (41 isolates, 4 plant species) and was represented by *Curvullaria tetramera* (22 isolates, 4 plant species) and *Curvullaria lunata* (19 isolates, 1 plant species). *Fusarium* was recovered in high occurrence (6 plant species) and it was represented by 74 isolates giving rise to 8.6% of the total fungal counts. It was represented by two species namely; *F. moniliforme* (37 isolates, 4 plant species) and *F. tricinctium* (37, 2). *Fusarium* has been isolated from different plant species including rye (Garifoliana & Dolgova, 1996) and onion (Abdel-Sater & Eraky, 2002; Abd-Elaah & El-Aref, 2005).

Mucor spp. was of high occurrence, which recovered from 5 plant species and represented by 207 isolates constituting 24.07% of the total fungi. It was represented by *M. hiemales* (121 isolates, 4 plant species), *M. circinelloides* (70, 3) and *M. racemoses* (16, 2). *Penicillium* was of high occurrence, which isolated from 5 plant species and represented by three species. These species were *P. notatum* (12 isolates, 4 plant species), *Penicillium citrinum* (9 isolates, 3 plant species) and *P. purpurogenum* (6, 3). *Cladosporium herbarium* (107 isolates, 5 plant species) and *Rhizopus nigricans* (21 isolates, 4 plant species) were also recovered in high occurrence. These species were previously isolated, but with variable densities and frequencies, from different seeds (El-Maraghy, 1989; Mazen *et al*., 1990).

Five species in addition to yeasts were recovered in moderate occurrence. These species were *Mammaria echinoulatum* (45 isolates, 3 plant species), *Circinella* (21, 3), *Epicocum nigrum* (6, 3), *Dersheslera halodes* (4, 2) and *Chaetomium nigricum* (12, 2). The remaining species

Table I. Plant species used for preparation of plant extracts

Species	Family	Plant part
Mentha pulegium L.	Criuciferae	Stem
Gnaphalium luteo- album	Asteraceae	Stem and leaf
Adenis microcarpus	Papaferaceae	Leaf and root
Halophyllum tuberculatum (Forssk)	Rutaceae	Stem, leaf& root
Melilotus indicus	Criuceferae	Stem, leaf &root
Mentha microphylla C. Kock	Criuceferae	Stem, leaf &root

(*Absidia coerulea, Pythium intermedium, Stemphyllium botroyosum, Torula herbarium* & *Trichoderma viride*) were isolated in rare occurrence and each of these was recovered from one plant species (Table II).

Isolation of dermatophytic fungi. Seven filamentous fungi were recovered from skin and hairs of skin diseased patients namely, *Microsporum gypseum* (Guiart), *M. canis* (Bodin), *Trichophyton mentagrophytes* (Robin), *T. rubrum* (Bodin), *Cladosporium verrucosum* (Link ex Fr.), *Aspergillus fumigatus* (Fresenius), *A. niger* (van Tieghen) and *Cunninghamella elegans* (Kuwabara et Hoshino). Most of these fungi were isolated from farmer patients with a fungal infection of skin. *Microsporum audouinii* has been found responsible for dermatophyte infections among the school children (Soyinka, 1978; Lindemann & Bhm, 1994; Spiewak & Szostak, 2000). Dermatophyte fungi (*Microsporum gypseum*, *Trichophyton mentagrophytes* & *T. terrestre*) have been isolated from human and animal mycosis (Abdel-Hafez, 1991). Similarly, *T. mentagrophytes* and *T. verrucosum* were found among the agent of ringworm diseases on different parts of the body of the school children (Ogbonna *et al.*, 1985).

Three fungi, *Microsporum gypseum*, *Microsporum canis* and *Aspergillus niger*, were chosen and used to study the antifungal effect of six plant extracts on them. The two dermatophytic fungi (*M. canis* & *M. gypseum*) are common agents for "tinea capitis" which refers to dermatophytosis of the scalp (MacKenzie *et al.*, 1986; Kwon-Chung & Bennet, 1992; Richardson & Warnock, 1993).

Table II. Fungal species isolated from six desert plant species collected from New Valley, Egypt

Fungal Species	Plant species														Total		
	M.p.	G.l.		A.m.		H.t.			M.i.			M.m.					
	S	S	L	L	R	S	L	R	S	L	R	S	L	R	TC	%TC	O.R
Absidia coerulea (Bain)			4												4	0.47	1R
Alternaria alternata (Fries) Keissler	19	7	3			10	15	7	3	3		1	1	9	78	9.07	5H
Aspergillus	28			20	3	6	8	17	5	1	7	7		8	110	12.79	5H
Aspergillus flavus (Link)	7					1	2	2			2			2	16	1.86	4H
Asp. niger (van tieghem)	21					5	6	8	5	1	5	7		6	64	7.44	4H
Asp. ustus (Bainier)				12	3										15	1.74	2M
Asp. fumigatus (Fresenius)				8				7							15	1.74	2M
Chaetomium nigricum(Ames)						3						9			12	1.40	2M
Circinella nigra (Bainier)		4	3	2								10		2	21	2.44	3M
Cladosporium herbarum (Pers.) Link ex Fr	7	10	20	10	1	15	17	15	8	4					107	12..44	5H
Curvullaria lunata Wakker (Boedijn)												19			19	2..21	1R
*Curvullaria tetramera(*Mckinney) Boedijn	5			2		2							3	10	22	2..56	4H
*Dersheslera halodes (*Dershesler) Suberamanian et Jain	4														4	0.47	2M
Fusarium	6	4	2	10	6	5			10		10	10		11	74	8.60	6H
Fusarium moniliforme (Schlecht ex Fr.)	6	4	2			5			10		10				37	4.30	4H
Fusarium tricinctum (Corda)				10	6							10		11	37	4.30	2M
Epicocum nigrum(Link)												6			6	0.70	3M
Mammaria echinoulatum(Cesati)				4	10				15				1	15	45	5.23	3M
Mucor		13	14	14	14	16	17	13	27	11	41	20	1	6	207	24.07	5H
Mucor circinelloides (Fresenius)				14	14		17		4		21				70	8.14	3M
Mucor hiemalis (Wehmer)		13	14					13	23	11	20	20	1	6	121	14.07	4H
Mucor racemosus (Fresenius)						16									16	1.86	2M
Penicillium	2			2		3	6	3			2			9	27	3.15	5H
Penicillium citrinum (Thom)	1			1										7	9	1.05	3M
Penicillium notatum (Westiling)	1			1		3	6							1	12	1.40	4H
Penicillium purpurogenum (Stoll)								3			2			1	6	0.70	3M
Pythium intermedium (de Bary)						35									35	4.07	1R
Rhizopous nigricans (Ehrenberg)	2	8				4								7	21	2..44	4H
Stemphyllium botroyosum (Wallroth)						3									3	0.35	1R
Torula herbarium(Person) Link												25			25	2..91	1R
Trichoderma viride Pers. Ex S.F.Gray									10						10	1.16	1R
Yeast spp.	3	7					15	20						5	30	3.49	4M

M.p.: *Mentha pulegium, G.l.*: *Gnaphalium luteo-album*, *A.m.*: *Adenis microcarpus*,
H.t.: *Halophyllum tuberculatum*, *M.i.*: *Melilotus indicus* and *M.m.*: *Mentha microphylla*
S: stem, L: leaf, R: root, TC: Total count, O.R.: Occurrence remarks.
H: High occurrence, between 3-6 plant, M: Medium occurrence, between 2-3 plant,
R: Rare occurrence, one plant only.

Antifungal effect of plant extracts on mycelial growth. Mycelial dry weights of *Microsporum gypseum, Microsporum canis* and *Aspergillus niger* treated with 0.0, 0.5, 1.0, 1.5 and 2.0 mg/mL extracts of *Mentha pulegium*, *Gnaphalium luteo-album*, *Adenis microcarpus*, *Halophyllum tuberculatum, Melilotus indicus* and *Mentha microphylla* is summarized in Table III and illustrated in Fig 1.

In *Microsporum gypseum*, mycelial growth was significantly inhibited at all concentrations (0.5 to 2.0 mg/mL) of the extracts of *Mentha pulegium* and *Gnaphalium luteo-album.* While, mycelial dry weight of *M. gypseum* was affected by 1.0 to 2.0 mg/mL extracts of *Adenis microcarpus*, *Halophyllum tuberculatum* and *Mentha microphylla.* With respect to *Melilotus indicus* extract, mycelial growth of *M. gypseum* was significantly enhanced by low concentration (0.5 mg/mL), while it was significantly inhibited at higher concentrations (1.5 & 2.0 mg/mL).

Mycelial growth of *Microsporum canis* was less affected by extracts of *Mentha pulegium, Gnaphalium luteo-album*, *Adenis microcarpus*, and *Halophyllum tuberculatum* than the other tested fungi. The low concentration (0.5 mg/mL) of all plant extracts favored mycelial growth of *Microsporum canis,* while significant enhancement was found in the case of *Microsporum gypseum* and *Halophyllum tuberculatum.* Mycelial dry weight was decreased significantly with increased concentration of *Melilotus indicus* and *Mentha microphylla* extracts (from 1.0 to 2.0 mg/mL). While, no inhibitory effect of *Mentha pulegium* extract was observed on mycelial growth of *Microsporum canis* at tested concentrations.

In case of *Aspergillus niger,* low concentration (0.5 mg/mL) extracts of *Mentha pulegium, Gnaphalium luteo-album*, *Adenis microcarpus* and *Halophyllum tuberculatum* enhanced the fungus growth, being significant with *Gnaphalium luteo-album* extract. In all plant extracts, mycelial growth of *A. niger* was decreased with the increase of extract concentration.

Generally, the results revealed that low concentration (0.5 mg/mL) of some plant extracts favoured mycelial growth of *Microsporum canis* and *Aspergillus niger*. In contrast, drastic inhibition in mycelial dry weight of *Microsporum gypseum* and *Aspergillus niger* was observed at high concentrations (1.5 & 2.0 mg/mL) of all plant extracts. *Microsporum canis* was less affected by plant extracts than the other two fungi. It was only affected by moderate concentrations (1.0 mg/ml) of *Melilotus indicus* and *Mentha microphylla* and higher level (2.0 mg/mL) of *Gnaphalium luteo-album*, *Adenis microcarpus* and *Halophyllum tuberculatum.*

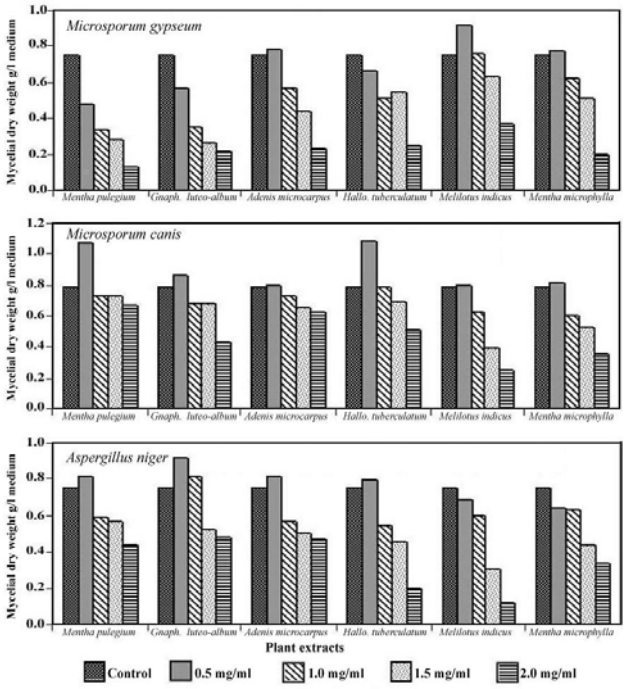
The extracts of the tested plant species seem to contain active fungicidal chemical substances because of their inhibitory influence on the mycelial growth of *Microsporum canis, M. gypseum* and *A. niger*. The higher concentration of the plant extracts was responsible for more inhibition of the mycelial growth, implying lower dry weight of the fungal mycelium. Fungicidal and bactericidal properties of plant extracts have already been reported (Aggarwal & Mehrotra, 1988; Liu *et al*., 2001). Leaf extracts of *Aloe arborescens* has been found to inhibit the growth of the human fungal pathogen *Trichophyton mentagrophytes* (Fujita *et al*., 1979). Inhibition of fungal growth by natural products extracted from peppermint (*Mentha piperita*), penny royal (*Mentha pulegium*), jasmine (*Jasminum grandiflorum*), wintergreen (*Gaultheria procumbens*) and hyssop (*Hyssopus officinalis*)

Table III. The effect of different concentrations of six plant extracts on mycelial dry weight of three fungal species

Fungal species	Plant extract mg/ml	Plant species					
		Mentha pulegium	*Gnaphalium luteo-album*	*Adenis microcarpus*	*Halophyllum tuberculatum*	*Melilotus indicus*	*Mentha microphylla*
Microsporum gypseum	0.0	0.755	0.755	0.755	0.755	0.755	0.755
	0.5	0.475**	0.570**	0.780	0.665	0.910**	0.770
	1.0	0.335**	0.350**	0.560**	0.507**	0.760	0.620*
	1.5	0.285**	0.265**	0.430**	0.540**	0.630*	0.515**
	2.0	0.132**	0.220**	0.230**	0.255**	0.370**	0.201**
	LSD 0.05 = 0.1032						
	0.01 = 0.1372						
Microsporum canis	0.0	0.785	0.785	0.785	0.785	0.785	0.785
	0.5	1.075**	0.855	0.800	1.083**	0.790	0.807
	1.0	0.735	0.680	0.725	0.785	0.625*	0.595**
	1.5	0.728	0.672	0.655	0.695	0.388**	0.525**
	2.0	0.670	0.430**	0.625*	0.510*	0.245**	0.350**
	LSD 0.05 = 0.1346						
	0.01 = 0.1791						
Aspergillus niger	0.0	0.745	0.745	0.745	0.745	0.745	0.745
	0.5	0.810	0.915**	0.813	0.790	0.690	0.640*
	1.0	0.585**	0.820	0.567**	0.540**	0.595**	0.630*
	1.5	0.565**	0.519**	0.495**	0.455**	0.305**	0.440**
	2.0	0.435**	0.475**	0.470**	0.200**	0.115**	0.340**
	LSD 0.05 = 0.1032						
	0.01 = 0.1372						

*, and ** : Significant and highly significant as compared with its control treatment, respectively.

Fig. 1. The effect of different concentrations of six plant extracts on mycelial dry weight of three fungal species

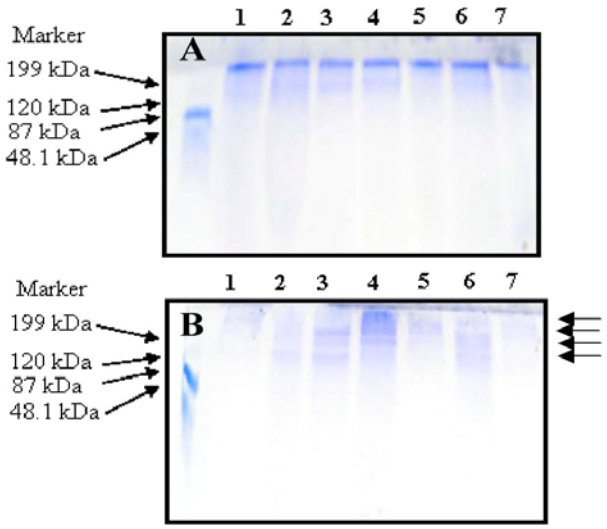


and rosemary (*Rosmarinus officinalis*) were also reported (Shimoni *et al*., 1993; Cutler *et al.*, 1996; Antonov *et al*., 1997). In addition, Wilson *et al.* (1987) and Caccioni and Guizzardi (1994) reported that volatile compounds derived from peach plant extracts inhibited germination and mycelial growth of a wide range of pathogenic fungi such as *B. cinerea, Monilinia laxa, M. fructicola, Mucor piriformis, Rhizopus stolonifer* and three *Penicillium* spp.

Protein analysis. The effect of various plant extracts on protein expression of *Microsporum gypseum*, *Microsporum canis* and *Asperigllus niger* is shown in Fig. 2. The patterns of protein expressed were different when the *Microsporum gypseum* was treated with plant extracts (Fig. 2A). Untreated fungus showed three protein bands of molecular weights > 199 kDa, ~ 199 kDa and ~ 120 kDa. However, the fungus lost the ~ 120 kDa band when it was treated with the extracts of *Mentha pulegium* L., *Ganphalium luteoalbum*, *Adenis microcarpus* and *Melilotus indicus*. While the fungus showed only one band of > 199 kDa in the presence plant extracts of *Halophyllum tuberculatum* (Forssk) or *Mentha microphylla* C. Kock. This result indicated that *Halophyllum tuberculatum* (Forssk) and *Mentha microphylla* C. Kock have more effect than other plant extracts on gene expression of this fungus.

The results of protein profiles showed that plant extracts suppressed the expression of ~ 120 kDa protein band as compared to the control treatment. The reduction of such protein band in conjunction with the inhibition of

Fig. 2. 10% SDS-PAGE analysis of *Microsoprum gypseum* (A), *Microsporum canis* (B) And *Aspergillus niger* (C) treated with 1.5 % extract from different plants. Lane 1, without treatment; lane 2, treated with *Mentha pulegium* L.; lane 3, *Ganphalium luteoalbum*; lane 4, *Adenis microcarpus*; lane 5, *Halophyllum tuberculatum* (Forssk); lane 6, *Melilotus indicus*; lane 7, *Mentah microphylla* C. Kock. Molecular weight standards (Biorad) are myosin; 199 kDa, B-galactosidase; 120 kDa, bovine serum albumin; 87 kDa and oval albumin; 48.1 kDa. The gel was stained with Coomassie blue. Arrows show respective protein bands.



mycelial growth (Table III) indicating that a modification in overall cell energy metabolism and protein synthesis occurred following treatment with the tested plant extracts (1.5 mg/mL). Similar conclusion was also reported by Niimi *et al*. (2002) Abd-Elaah and Ahmed (2005).

As demonstrated in Fig. 2B, the effect of plant extracts showed different protein profiles of *Microsporum canis.* Three protein bands were detected in untreated fungus, one of molecular weights ~ 199 kDa and other two of > 199 kDa. The band ~ 199 kDa was enhanced with *Ganphalium luteoalbum* and *Adenis microcarpus* extracts. However, the fungus with *Mentha microphylla* C. Kock demonstrated very weak bands. The same results were shown with *Aspergillus niger* but with different effects (data not shown). It is concluded that the plant extract showed high effect of gene expression in the three fungi as indicated by protein profiles.

The enhanced protein band (~ 199 kDa) suggested that such band may play a role in the defence mechanism against the toxic effect of plant extract. In *Aspergillus nidulans,* Del Sorbo *et al*. (1997) observed that the transcription of atrA and atrB genes were strongly enhanced by treatment with several drugs, including antibiotics, azole fungicides and plant defence toxins.

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