ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

A Survey on the Effect of ethanol *Pluchea dioscoridis* Leaf Extract on Lipase Gene Expression in otomycotic *Aspergillus niger* via Real-time PCR

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Manuscript Info

Manuscript History:

Received: 18 March 2015
Final Accepted: 22 April 2015
Published Online: May 2015

Key words:

P. dioscoridis, A. niger, Real-time PCR and Biocontrol.

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Abstract

The present study focused on the inhibitory effects of *Pluchea dioscoridis* ethanol leaf extract on growth, survival and lipase gene expression of otomycotic *Aspergillus niger*. There is no report regarding the effects of *P. dioscoridis* or its mechanism of action on pathogenic *Aspergillus* lipase gene. Ethanol leaf extract of *P. dioscoridis* was assessed for its antifungal activity against *A. niger*, also the minimal inhibitory concentration (MIC) for *P. dioscoridis* extract was measured. After harvesting the fungi, its mRNA was extracted and cDNA synthesized by using two specific primers. The quantitative change in the lipase expression was analyzed via real-time PCR. The fungal growth decreased with increasing concentrations of *P. dioscoridis* extract and it recorded a low minimal inhibitory concentration value (MIC=20mg/ml). At this concentration, lipase gene expression was reduced from 100 % to 7% and 19% than that obtained from untreated *A. niger* as documented by quantitative RT-PCR analysis. Overall we concluded that the *P. dioscoridis* could inhibit the lipase gene expression and consequently the lipase enzyme activity that play a role in the pathogenecity of *A. niger* in otomycosis infection.

INTRODUCTION

Otomycosis is a subacute or chronic fungal infection of the external auditory canal with some complications involving the middle ear. The molds mostly isolated from the ear causing otomycosis are *Aspergillus niger, and Penicillium* species (Loh, *et al*.,1998). Production and secretion of hydrolytic enzymes, such as proteases and lipases are very important as virulence factors. These enzymes not only play a role in fungal nutrition, but also in tissue damage (Kuleta, *et al*., 2009).

There are four main classes of drugs for the treatment of fungal otomycosis: polyenes, triazoles, nucleoside analogues, and echinocandins. The azoles family appears to be the most effective one. Although these antifungal agents are effective against otomycosis, they have several major drawbacks as itraconazole and fluconazole also cause adverse toxic effects including nausea, vomiting, skin rashes, hepatotoxicity, inhibiting cytochrome P_{450} isozymes, also cause gynecomastia and are not safe during pregnancy and lactation (Patel, 2000). So there is no single treatment gives high satisfactory results in controlling signs and symptoms of otomycosis without any side effects. (Wang, *et al*., 2005).

Medicinal plants represent an interesting source for finding novel antimicrobial compounds. They are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava, *et al*., 1996). A wide range of medicinal plant parts are used for extracting raw drugs and possess varied drugs which are still largely unexplored. (Uniyal, *et al*., 2006).

Microorganisms resistant to most antibiotics are rapidly spreading. Consequently, there is an urgent need for novel antibiotics. The World Health Organization (WHO) (1976) describes a medicinal plant as any plant in which one or more of its organs contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs.

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, phenolics and flavonoids, which have been found *in vitro* to have antimicrobial properties (Okwu, 2001; Ano and Ubochi, 2007). Many active constituents have been reported in the different parts of *Pluchea dioscoridis*, as it has a good reputation in folk medicine, its marketing is local and depends on collection from the wild and it is used in popular medicine for rheumatic pains (Boulos and El-Hadidi 1989).

The application of genome-wide expression profiling to determine how drugs achieve their therapeutic effect has provided the pharmaceutical industry with an exciting new tool for drug mode-of-action studies. The use of polymerase chain reactions (PCR) for the amplification of specific mRNA after a reverse transcription enables the studying of gene expression (Chelly, *et al*., 1990, Fiorenza and Mangia, 1998). It is a sensitive method for the detection of mRNA expression levels as mRNA tells us about the response of microorganisms to surrounding environment. Backer, *et al*. (2001) used mRNA and RT-PCR to study the effect of itraconazole on the *C. albicans* by analyzing the level of gene expression from in several genes from the *C. albicans* genome prior to and after treatment of *C. albicans* with this compound. The current study is the first to describe the virulence factors of pathogenic *Aspergillus niger* giving new safe approaches for its treatment as biocontrol agent. Moreover, the mode of action of the medicinal extract on the pathogenic fungi could be studied using the specific RT-PCR.

Material and methods:

The fungal isolate of *A. niger* used in this study was isolated from otomycosis patients at outpatient clinic of otorhinolaryngology department at Tanta University Hospital and maintained on agar slants.

- **Plant material and plant classification**

Fresh leaves of *P. dioscoridis* were collected from eight habitats which were identified in Nile Delta according to Ahmed (2003) as highways, railways, waste lands, abandoned fields, orchards, canal banks, drain banks and Lake Burullus. The plant identification was carried out in Tanta Herbarium, Tanta University (TANE) based on authentic materials. The leaves were washed thoroughly 2-3 times with running water and once with sterile distilled water, and then leaves were air-dried under shade (Satish, *et al.*, 2007).

- **Preparation of crude extracts and its antifungal activity**

Extract was prepared in ethanol at room temperature by simple extraction method according to Deshpande, *et al*., (2004). The antifungal activities of ethanol extract of *P. dioscoridis* with different concentrations (0, 5, 10, 15, 20, 25 mg/ml) were assayed by the hole-plate method (Igbinosa, *et al*., 2009). Freshly prepared spore suspension of isolated *A. niger* (0.5 ml of about $5x10^6$ spores /ml) was mixed with 9.5 ml of sterile Sabouraud's dextrose medium at 45 $^{\circ}$C, poured in sterile Perti dishes, and left to solidify at room temperature. Regular wells were made in the inoculated agar plates by a sterile cork borer with 0.8cm diameter. Each well was aseptically filled up with 0.2 ml of each concentration. The plates were incubated at 25°C for 3-4 days for the observation of inhibition zones. Three replicas were made for each tested extract and all plates were incubated at 25 $^{\circ}$C for 3-4 days.

- **Minimal Inhibitory Concentration:**

Different concentrations (0, 5, 10, 15, 20, 25 mg/ml) were prepared from *P. dioscoridis* leaf extract. Spore suspension of *A. niger* (0.5 ml of $5x10^6$ spores /ml) was mixed with 9.5 ml of each concentration in sterile test tube and incubated for 24 h at 25°C. Then 0.2 ml of each mixture was spread on the surface of previously sterile Sabouraud's dextrose agar plates for 2 days at 25°C. Colony forming units were counted, represented graphically and MIC was recorded for both antifungal materials. (Shadomy, *et al*., 1985, Brown, 1994 and Radhika, *et al*., 2008).Three replica were made for each tested suspension and compared by all plates.

- **RNA isolation and c DNA synthesis**

Two total RNAs were isolated from 72 h mycelia of *A. niger* (one sample treated with 20 mg (MIC) of *P. dioscoridis* leaf extract and one sample control "untreated") in liquid lipase dependent medium as mentioned previously, using Trizol (Gibcol) according to the manufacturer's protocol. First strand cDNA was synthesized using Advantage RT-for-PCR Kit (Clontech, alo Alto, CA, USA).

Primer name	Sequence 5`-`3	Reference
Lipase F1	CTCATATGGCTCCAGCACCTGCTCCGA	Yang, *et al*., 2010
Lipase R1	CTGAATTCCTACGAGCATTCACTAA	
Lipase F2	GATGCGCTTCCTCTCCGGCTTCG	Yu, *et al*., 2003
Lipase R2	TTAGCGGAAGGGCAATCCATGAC	

Table (1) Primers used in detection of lipase gene

- **Conventional RT- PCR amplification**

PCR was carried out using the primers (Table 1) according to (Williams, *et al*., 1990) in a 50 µl reaction. The PCR reaction consists of; a 100 ng of the synthesized cDNA, 200 mM dNTP, 0.1 mM of each primer, 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase (Takara), the volume was completed up to 50 µl using sterile H_2O. The PCR program was as follows: denaturation at 94°C for 4 min, 30 cycles of 94°C for 30 s, 56°C for 50 s and 72°C for 1 min, and final elongation at 72°C for 6 min. PCR products were separated in 2% agarose gel in TAE buffer [40 mM Tris–acetate, pH 7.6 and 1 mM Na acetic acid (EDTA)]. After electrophoresis, gels were stained with Ethidium Bromide (0.5 mg /mL), and visualized under UV light. Sizes were estimated by comparison with a DNA standard length (GeneRuler TM 100 bp DNA ladder, MBI Fermentans, Vilnius, Lithuania).

- **Real time PCR amplification conditions:**

The cDNA of the two samples (control and treated) were subjected to semi-quantitive PCR using the two primers presented in Table (1). The Real time PCR reaction consist of 12.5 µl of 2x Quantitech SYBR® Green RT Mix (Fermentase com.), 1µl of 25 pm/µl forward primer, 1 µl of 25 pm/µl reverse primer,1 µl of the cDNA (50ng), 9.25 µl of RNase free water for a total of 25 µl. Samples were spun before loading in the Rotor's wells. The real time PCR program was as follows : initial denaturation at 95 °C for 10 min.; 40 cycles of 95°C for 15 sec.; annealing at 60°C for 30 sec and extension at 72 °C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene- 6000-system (Qiagen, USA). The 18S rRNA gene was used as housekeeping gene (reference gene) in this test. Threshold cycle (C_T) values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred, for each amplification plot.

- **Data analysis:**

Comparative quantification analysis was done using Rotor-Gene-6000 Series Software according to (Rasmussen, 2001).

Results:

The inhibitory effect of ethanol *P. dioscoridis* leaf extract on the radial growth of pathogenic *A. niger* was studied. Data shown in figure (1) revealed the high inhibitory effect of *P. dioscoridis* leaf extract (15mg/ml) on radial growth of *A. niger* comparing with the rate of untreated fungal growth. Table (2) represented that inhibitory effect of *P. dioscoridis* leaf extract increase by increasing concentration. To detect the suitable concentration of *P. dioscoridis* leaf extract, minimal inhibitory concentration (MIC) of *P. dioscoridis* leaf extract was defined as concentration of 20 mg/ml that was found to be the lowest concentration of *P. dioscoridis* leaf extract giving the highest inhibitory effect on *A. niger*. Significant differences at $P<0.001$ were observed on the effect of *P. dioscoridis* on the growth of *A. niger* by oneway Anova test as in Table (3).

An evaluation of *A. niger* lipase gene expression under treatment with *P. dioscoridis* leaf extract was carried out by using Reverse transcriptase-PCR. Figure (2) shows differentially expressed lipase gene in treated fungi in comparison with control by using 2 primers. Primers lipase one gave amplicone with molecular size about 1100bp but lipase primer two gave amplicone with molecular size about 370bpBoth primers showed repression to lipase gene bands as they decreased the bands intensity which detected the expression in lipase gene. These results confirm the inhibitory effect of *P. dioscoridis* leaf extract on *A. niger* lipase enzyme.

So, real-time PCR was assayed to demonstrate the quantitative difference in expression levels for lipase selected gene between treated and treated *A. niger*.

Table (4) and figure (3) showed that *P. dioscoridis* had a bearing effect on the ratio of lipase gene expression that quantified by real-time PCR, by comparing the threshold (C_T) and amplification values of amplified cDNA extracted from *A. niger* treated with *P. dioscoridis* with those obtained from untreated *A. niger*. The ratio of expressed lipase gene extracted from *A. niger* treated with Conyza was more variable and significantly reduced up to 7 and 19% than that obtained from untreated *A. niger* from 100% to 7% and 19% as in figure (3).

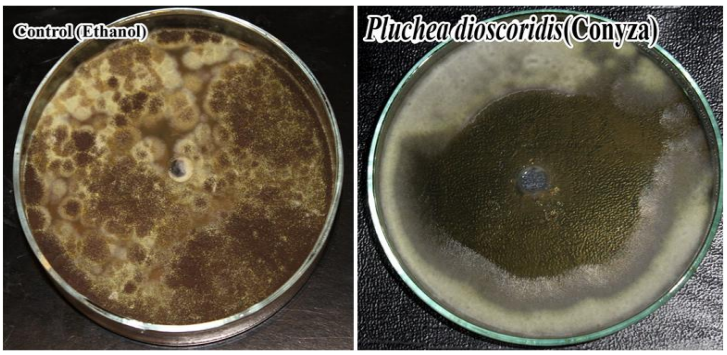

Fig. (1): Inhibitory effects of *P. dioscoridis* ethanol leaf extract (15mg/ml) against *A. niger*, isolated from otomycotic cases

Table (2): Effect of *P. dioscoridis* leaf extract on radial growth of *A. niger*

Concentration (mg/ml)		Diameter of inhibition zone (cm)
		P. dioscoridis extract
0		0.0
5		2.0±0.11
10		3.9±0.12
15		6.0±0.11
20		8.0±0.13
25		9.0±0.12
ANOVA	F	4392.6
	P-value	< 0.001*

* P value is statistically highly significant at the 0.001 level.

Table (3): Minimal inhibitory concentration (MIC) of *P. dioscoridis* leaf extract against *A. niger*

Concentration (mg/ml)		Number of surviving cells (C.F.U)
		P. dioscoridis extract
0		35±1.14
5		12±1.13
10		3±1.11
15		1±0.0
20		1±0.0
25		0
30		0
35		0
40		0
45		0
50		0
55		0
ANOVA	F	1049.0
	P-value	< 0.001*

* P value is statistically highly significant at the 0.001 level. C.F.U= Colony-Forming Units.

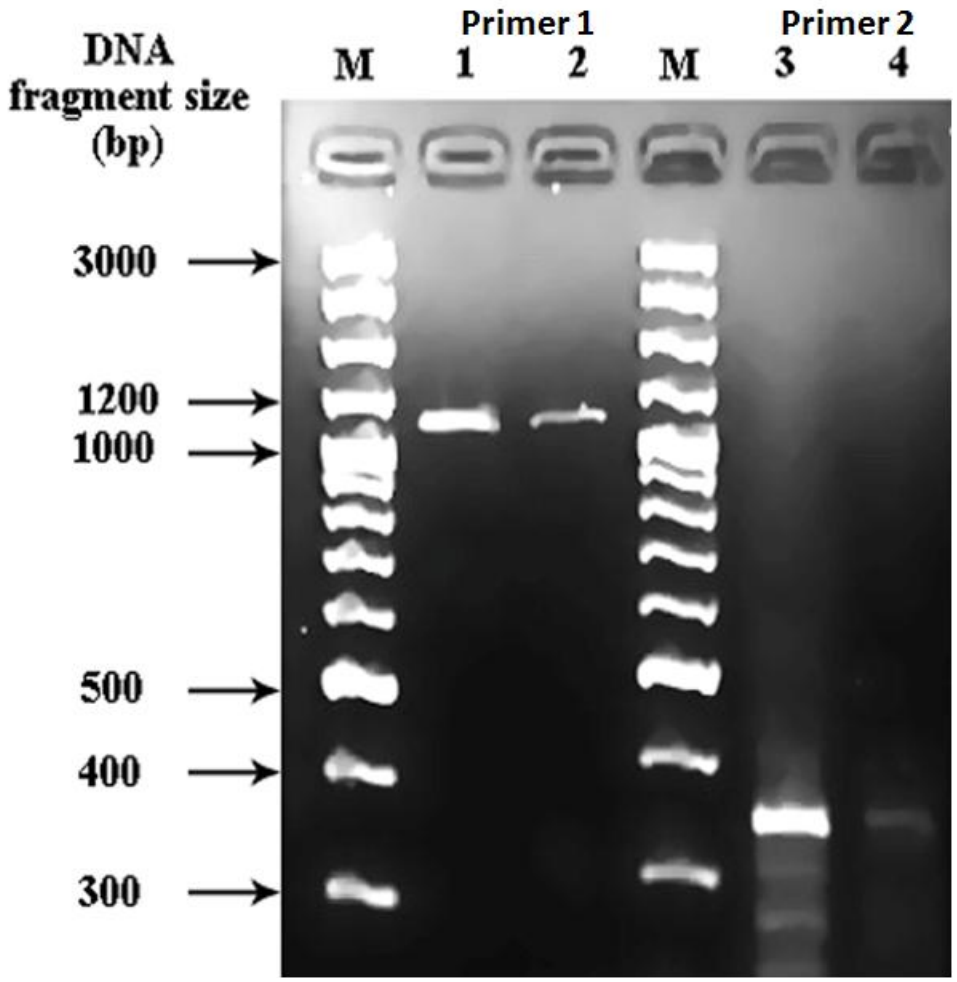

Fig. (2): Convential RT-PCR amplification for the lipase gene using the two primers (P1&P2) from the genome of *A. niger*. Lanes; M:3Kbp Ladder. Lane 1: Untreated *A. niger*. Lane 2: *A. niger* treated with *P. dioscoridis* extract. Lane3: Untreated *A. niger*. Lane 4: *A. niger* treated with *P. dioscoridis* extract .

Table (4): The fold change and the ratio of target lipase gene expression of isolated *A. niger*, using Real Time PCR

Sample	Primer	Amplification	Fold change in reference gene expression	Fold change in target gene expression	Percentage of change in gene expression
1	all	1.67	0.71	1	100
2	P.1	0.095		0.05	7
3	P.2	0.25		0.14	19

Sample (1): Control (non treated *A. niger* isolate), Sample (2,3): *A. niger*, treated with 20 mg/ml of *P. dioscoridis* ethanol extract.

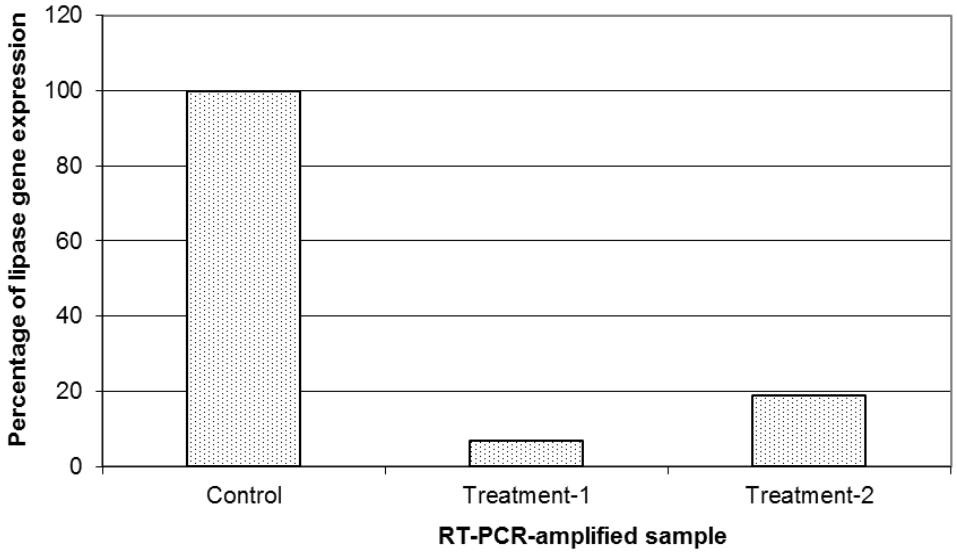

Fig. (3): Quantification of lipase gene expression during Real Time PCR of target lipase gene.

Discussion:

The present assay represent a trial to search for new antifungal agents suitable for treatment of otomycosis. *P. dioscoridis* possessed high antimicrobial activity which was tested against *A. niger* that was isolated in the present survey from otomycosis patients. The leaf extracts of *P. dioscoridis* using ethanol as extracting solvents presented a high inhibitory effect on the test organism This could be attributed to the concentration of the active substance causing the inhibitory effect which could have been higher in the leaves, also metabolites of the leaves are probably different from those of the other parts.

The use of ethanol as extracting solvent proved to be very efficient in extracting the active compounds in *P. dioscoridis*. This was in agreement with previous works which showed that ethanol is better than aqueous extracts in many plants especially in antimicrobial activities (Aiyegoro, *et al*., 2008 and Igbinosa, *et al*., 2009). Also, in our study, we were unable to use methanol as it is considered to be toxic for human, this is in agreement with (Kavet and Nauss, 1990).

The choice of the *P. dioscoridis* leaf extract was supported by its observable activity at low concentrations, at which a very high inhibitory action appeared against gram positive and gram negative bacteria. (Zain, *et al*.,2012). Moreover, Atta and Abo El-Soud (2004) examined the antinociceptive effect of the leaf ethanolic extract of *Pluchea dioscoridis* on mice. While, Atta and Mouneir (2004) studied the antidiarrheal activity of the same extract on rabbits. Both studies indicated significant effects under certain conditions.

In the present study, a great decrease of the *A. niger* growth rate by *P. dioscoridis* leaf extract was observed. This was in agreement with antibacterial activity of *P. dioscoridis* leaf extract against all gram positive bacteria and yeasts recorded by Paerkh and Chanda (2008). A high effect of the *P. dioscoridis* leaf extract was revealed by its low minimal inhibitory concentration (MIC) which was recorded at (20 mg/ml) against *A. niger.* This implies that the antimicrobial activity of a substance is concentration dependent. This is in concordance with the report of Oboh and Abulu (1997) who reported that antimicrobial activity is a function of the active ingredient with a definite concentration reaching an organism.

The application of genome- expression profiling to determine how drugs achieve their therapeutic effect has provided the pharmaceutical industry with an exciting new tool for drug mode-of-action studies. So, we used mRNA to identify lipase gene of *A. niger* whose expression is changed after treatment with *P. dioscoridis* leaf extract as anti-otomycotic drug.

In this study; firstly we have used RT-PCR to determine if *P. dioscoridis* leaf extract causes changes in gene expression in *A. niger* or not. This is to our knowledge, is the first report to use RT-PCR for characterization of *A. niger* lipase gene whose expression is changed after treatment with *P. dioscoridis* leaf extract. In accordance with our results, Gretty, *et al*., 2009 used RT-PCR to determine differential gene expression of lignocellulotic enzymes in *A. niger* biofilms. Also, RT-PCR was used to study other interactions between microbes and organisms, e.g fungi-plants (Bentio, *et al*., 1996).

Secondly, real-time PCR was used to enable the quantification of *A. niger* lipase gene in both *P. dioscoridis* treated and untreated samples. It is considered the first reported study on the detection and quantification of expressed lipase gene of these two different samples using quantitative real-time PCR, which is in accordance with what other studies have recently reported (Cullen, *et al*., 2001 and Winton, *et al*., 2002). In our present study, suppressing of expressed lipase gene in treated sample up to 7 and 19% than untreated one refers to the effect of *P. dioscoridis* on lipase gene that illustrated its inhibitory effect on lipase enzyme activity. This was in agreement with Mohseni, *et al*., 2012 who represented that Licorice extract affect *aflR* gene expression of *A. parasiticus* which was suppressed up to 40% by quantitative RT-PCR analysis.

Conclusion:

All these results in changes in fungal surface growth, metabolic and molecular parameters attribute to become unable to cause infection. So, changes in lipase gene expression may further help our understanding of fungal invasion and how *P. dioscoridis* treatment can inhibit fungal growth by stopping lipase activity and blocking its gene.

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