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Characterization and Inhibition Effect of *Conyza* Leaf Extract (*Pluchea dioscoridis*) on Lipase Enzyme Produced by *Aspergillus niger*, Isolated from Otomycosis Disease

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ABSTRACT

This study reported the inhibition of *Aspergillus niger* lipase enzyme by *Conyza* leaf extract which considered an effective tool to prevent fungal invasion into the external ear canal causing otomycosis. Lipase enzyme was extracted from otomycotic *A. niger*, precipitated by ammonium sulphate and purified by Sephadex G-100 chromatography. The mode of *Conyza* leaf extract inhibitory action on lipase was detected by preparing both different substrate and *Conyza* concentrations. The results showed that 30% ammonium sulphate fractionation gave maximum lipase activity 59.2 U mL⁻¹ and the highest lipase activity of Sephadex G-100 chromatography purified sample was found to be 79.1 U mL⁻¹. The specific activity was 87.9 U mg⁻¹ proteins with purification fold of 8.7. Electrophoretic analysis for the purified enzyme indicated one subunit of molecular weight of 45 kDa. *Conyza* extract acted as irreversible competitive lipase inhibitor with inhibition constant (K_i = 6.2 mg mL⁻¹). The results obtained in the present study suggest that *Conyza* extract can be used to inhibit *A. niger* growth that causes otomycosis.

Key words: *A. niger*, lipase, *Conyza* extract, otomycosis

INTRODUCTION

Otomycosis is a superficial mycotic infection of the outer ear canal. It may involve to the middle ear causing some complications.

Aspergillus and *Candida* spp. are the most frequently isolated fungi from otomycosis patients (Vennewald *et al.*, 2003). Production and secretion of hydrolytic enzymes, such as proteases, lipases and phospholipases are very important as virulence factors as they play an important role in fungal pathogenicity and tissue damage (Karkowska-Kuleta *et al.*, 2009).

Some investigators attributed the characteristic lipase activity of *A. niger* as they are lipophilic, and propose that this lipase activity is related to the adaptation of *A. niger* to the host body regions rich in fatty acids under given conditions (Lydia *et al.*, 2011). Also, Mizuki *et al.* (1994) described the lipophilic activity of *A. niger* that involved in cellular growth process and related to pathogenicity mechanisms in aspergillosis.

A little information is available on the characterization of these enzymes from molds or yeasts and their role in the external ear canal and ear drum infection causing otomycosis. Only (Valentina *et al.*, 2004) attributed the characteristic protease activity of *Candida albicans* from immunocompetent patients with otomycosis and propose that this protease activity is related to pathogenicity mechanisms.

The aim of this work is to purify, characterize and inhibit lipase of *A. niger* as one of the most important enzymes used by a fungal pathogen to invade ear canal causing otomycosis.

MATERIALS AND METHODS

Microorganism: The strain of *A. niger* used in this study was isolated from the outpatient clinic of otolaryngology department at Tanta University Hospital and maintained on agar slants and subcultured for every month.

Inoculum preparation: The fungal spore inoculum was prepared by adding 10 mL of sterile distilled water to Sabouraud's Dextrose Agar slants (SDA). The spores were dislodged using a sterile inoculation loop under aseptic conditions. One milliliter of spore suspension (5×10^6 spores mL⁻¹) was used as the inoculum.

Lipase production process: *A. niger* was cultured according to (Aires-Barros *et al.*, 1994) on liquid medium containing g L⁻¹ : peptone 5, yeast extract 10.0, NaCl 5.0, olive oil 1% (v/v) as inducer. The initial pH of the medium was adjusted to 7 then divided into 100 mL fractions in flasks (250) and autoclaved. The spore suspension was inoculated into 250 mL Ehrlenmeyer flasks containing 100 mL sterilized liquid medium and incubated at 25°C for a period extended to 12 days. The biomass was separated by filtration through Wattman No. 1 filter paper, then filtrate was centrifuged at 6,000 rpm for 20 min after which the supernatant was used as a crude enzyme preparation.

Lipase assay: Lipase activity was determined according to Burkert *et al.* (2004). One unit of the enzyme activity was defined as the amount of the enzyme necessary to produce 1 μ mol of free fatty acids per min under assay conditions.

The optimum incubation period giving the highest lipase production was determined and a curve was plotted to illustrate the rate of lipase production from *A. niger*.

Enzyme purification: The following step is to purify lipase enzyme from *A. niger*.

- **Precipitation of lipase by ammonium sulphate:** Crude enzyme obtained from culture filtrate was precipitated with solid ammonium sulphate at various concentrations (30-90% saturation) overnight at 4°C (Pabai *et al.*, 1995). After fractionation with ammonium sulphate, all precipitates were collected and dialyzed against 0.02 M sodium phosphate buffer pH = 7.2 for 48 h using dialysis bag. The dialyzed enzymatic fractions were subjected to protein and lipase activity determination. Then the precipitate of the highest ammonium sulphate fraction was applied to gel filtration chromatography
- **Gel filtration chromatography on Sephadex G-100:** The highest ammonium sulphate fraction was applied to the Sephadex G- 100 column (with dimensions 1.5 cm diameter and 20 cm length). The column was equilibrated with 0.02 M sodium phosphate buffer pH = 7.2. Then the sample was eluted with the same equilibration buffer on the column. Twenty fractions were collected at a flow rate 1 mL min⁻¹ constant intervals. The most active fractions (7-10) were dialyzed against 0.02 M sodium phosphate buffer pH=7.2 at 4°C. Then enzyme activity was calculated to these fractions (Bodhankar *et al.*, 1998) and protein concentration was determined for the most active fraction
- **Protein assay:** The protein concentrations in different stages of enzyme purification were estimated according to Bradford (1976) using bovine serum albumin as a standard protein

- **Electrophoretic analysis:** Small parts of lipase samples came from crude culture supernatant and the highest fraction activity of the enzyme from the two steps of purification were frozen and stored to be run in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis to confirm the level of purification in each step. All were analyzed against a standard marker protein mixture with known molecular weights according to the protocol (Bradford, 1976)

Effect of *Conyza* extract on fungal growth and lipase activity: The antifungal activity of ethanol *Conyza* extract was assayed by the hole-plate method (Igbinosa *et al.*, 2009). Freshly prepared spore suspension of isolated *A. niger* (0.5 mL of about 5×10^6 cells mL⁻¹) was mixed with 9.5 mL of sterile Sabouraud's dextrose medium at 45°C, poured on sterile Petri dishes and left to solidify at room temperature. Regular wells were made in the inoculated agar plates by a sterile cork borer with 0.8 cm diameter. Each well was aseptically filled up with 0.2 mL from each concentration of *Conyza* extract (0, 5, 10, 15, 20, 25 mg mL⁻¹) using a dropping pipette under aseptic conditions. The plates were incubated at 25°C for 3-4 days for the observation of inhibition zones. Three replica were made for each tested extract and all plates were incubated at 25°C for 3-4 days. Then the average diameters of inhibition zones were recorded and compared by all plates.

Mode of inhibition of *Conyza* on *A. niger*: In order to detect if *Conyza* extract is a lipase inhibitor or not; This could be obtained by culturing *A. niger* on liquid lipase dependent medium as mentioned previously according to Aires-Barros *et al.* (1994). Then the media was divided into 5 mL fractions in test tubes and sterilized by autoclave. Different concentrations of leaf extract of *Conyza* (50 µL of 5, 10, 15, 20, 25 mg mL⁻¹) were separately added to each test tube. Spore suspension of *A. niger* (50 µL of 10^6 cells mL⁻¹) was inoculated in each tube and incubated at 25°C for 8 days (the optimum time for lipase production) and one tube without leaf extract used as control. Then lipase activity was measured quantitatively for each mixture as described previously (Burkert *et al.*, 2004). Three replica were made for each tested concentration.

Other parameter should be tested to elucidate the mode of inhibition on *A. niger* by *Conyza* extract, two factors were studied. The first is the concentration of *Conyza* extract (as a lipase inhibitor) and the second is the concentration of olive oil (as a substrate for lipase), to detect if *Conyza* extract is a reversible lipase inhibitor or not, arising the mode of its competition with olive oil. This could be obtained as follows:

Purified lipase dissolved in 0.02 M sodium phosphate buffer (pH = 7.2) was used. Different concentrations of *Conyza* leaf extract (0, 5, 10, 15, 20, 25 mg mL⁻¹) were prepared and incubated separately with enzyme added to it different concentrations of (substrate) olive oil (1, 2, 3, 4, 5, 6%) then the activity of lipase enzyme was determined for each substrate and extract concentration quantitatively according to Burkert *et al.* (2004). Results were represented graphically by a reciprocal plot between enzyme activity and substrate concentration for each *Conyza* extract concentration (Lineweaver and Burk, 1934).

Statistical analysis: It was carried out for the studied data of *A. niger* lipase enzyme production and the effect of *Conyza* leaf extract on enzyme activity through analysis of variance (ANOVA) one way tests by SPSS V17, to evaluate the variation among concentrations of *Conyza* leaf extract on fungal growth and its enzyme activity.

RESULTS

Production of lipase: The production of lipase enzyme by *A. niger* was studied during a period of 12 days. The highest significant rate of lipase production ($30.8 \text{ unit mL}^{-1}$) was quantitatively reported in the culture filtrate of *A. niger* on the 8th day of growth at $p < 0.001$ as in Table 1.

Purification of lipase: Lipase was precipitated by gradual saturations of ammonium sulphate with *A. niger* culture filtrate, then lipase activity was measured quantitatively in the precipitate of each ammonium sulphate saturation.

The most efficient saturation of ammonium sulphate precipitated the highest percentage of lipase yield (59.2 U mL^{-1}) was 30%. After the dialysis of the highest ammonium sulphate precipitate against 0.02 M sodium phosphate buffer pH = 7.2 it was carried out successfully by chromatography on Sephadex G-100 column and it revealed a single peak for the maximum lipase activity. Figure 1 showed that fractions (7-10) were the most active fractions of *A. niger* lipase activity. This was confirmed by appearance of a single protein band at molecular weight 45 kDa during the SDS-PAGE analysis as shown in Fig. 2. After purification, lipase enzyme has indicated specific activity of $87.9 \text{ unit mg}^{-1}$ from $10.3 \text{ unit mg}^{-1}$ with a purification fold of nearly 8.6 times the enzyme crude as shown in Table 2.

Table 1: Time course of lipase production by *A. niger*

Growth age (days)	Lipase enzyme activity ($\mu \text{ mL}^{-1}$)
1	5.0±0.11
2	6.7±0.12
3	9.1±0.11
4	10.8±0.11
5	13.3±0.12
6	16.6±0.13
7	27.5±0.11
8	30.8±0.13
9	22.5±0.12
10	12.5±0.11
11	5.8±0.09
12	1.7±0.07
ANOVA	
F	25295.159
p-value	<0.001*

*p-value is statistically highly significant at the 0.001 level

Table 2: Purification profile of *A. niger* lipase

Purification parameter	Purification stage		
	Crude culture filtrate	30% amm. sulphate ppt.	Sephadex G-100 fraction of highest activity
Volume (mL)	150	20	5
Protein conc. (mg mL^{-1})	3	1.8	0.9
Enzyme activity ($\mu \text{ mL}^{-1}$)	30.8	59.2	79.1
Total enzyme activity (μ)	4620	1183	395.8
Total protein (mg)	450	36	4.5
Specific enzyme activity ($\mu \text{ mg}^{-1}$)	10.3	32.9	87.9
Purification fold	1	3.2	8.6
Yield (%)	100	25.6	8.5

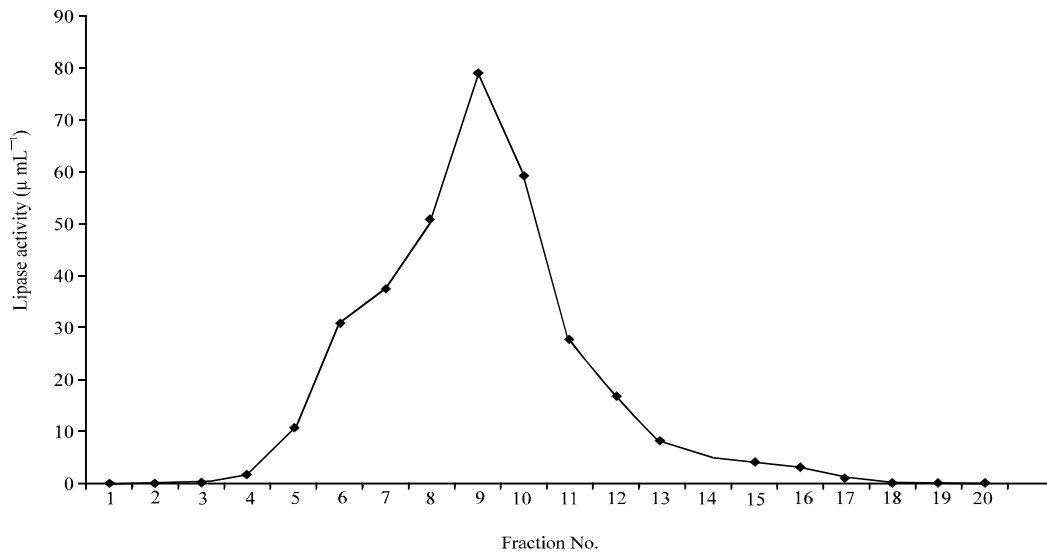


Fig. 1: Elution profile of *A. niger* lipase on sephadex G-100 column

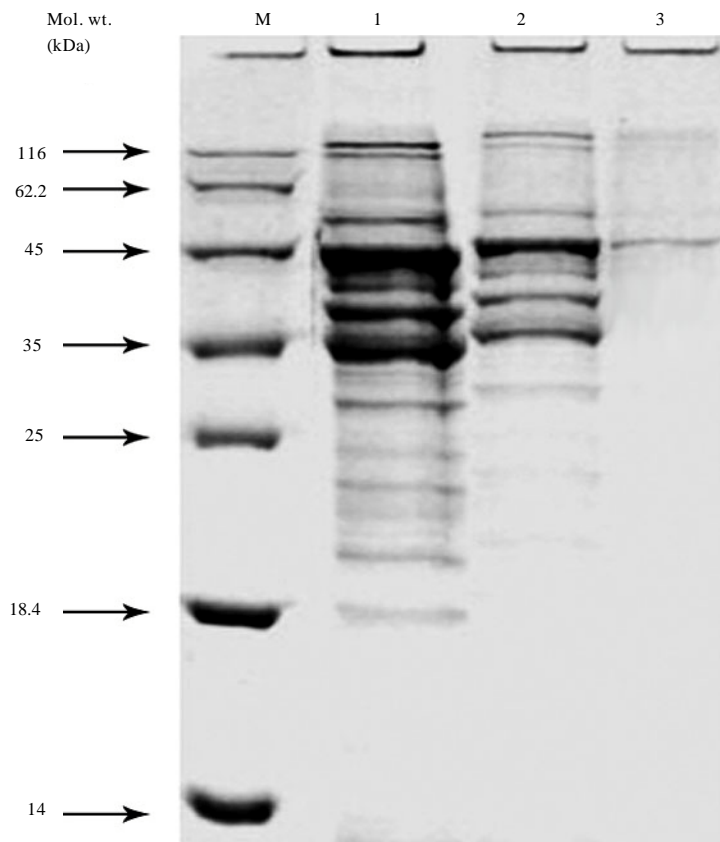


Fig. 2: SDS-PAGE for different stages of lipase purification, M: Standard protein marker, 1: Crude culture filtrate, 2: Amm. sulphate precipitate, 3: Sephadex G-100 fraction of most lipase activity

Effect of *Conyza* on fungal growth and lipase activity: A trial was carried out to find a relationship between high inhibition of *A. niger* growth by *Conyza* leaf extract and lipase activity as an important offensive force for the fungi to establish otomycosis infection.

Table 3 showed significant differences at $p < 0.001$ on the effect of *Conyza* on the growth of *A. niger* as concentration of 20 mg mL^{-1} was found to be the lowest concentration of *Conyza* leaf extract giving the highest inhibitory effect on *A. niger*. Also, a great decrease in lipase activity by increasing the concentration of *Conyza* leaf extract, as lipase activity decreased from 30.8 to $2.8 \mu \text{ mL}^{-1}$ as shown in Table 3. Also, statistical analysis revealed that the variation in the effect of different concentrations of *Conyza* on lipase activity was highly significant at $p < 0.001$.

Figure 3 showed that *Conyza* leaf extract was an irreversible competitive lipase inhibitor, as its activity decreased by arising its substrate concentrations which was confirmed with different *Conyza* leaf extract concentrations. K_m (Michaelis constant) = 7.14 mg mL^{-1} . Plotting the slope of double reciprocal plot of *Conyza* leaf extract inverse against the inverse of lipase initial activity indicated an inhibition constant (k_{is}) of 6.2 mg mL^{-1} due to the effect of *Conyza* leaf extract on enzyme reaction slope as shown in Fig. 3.

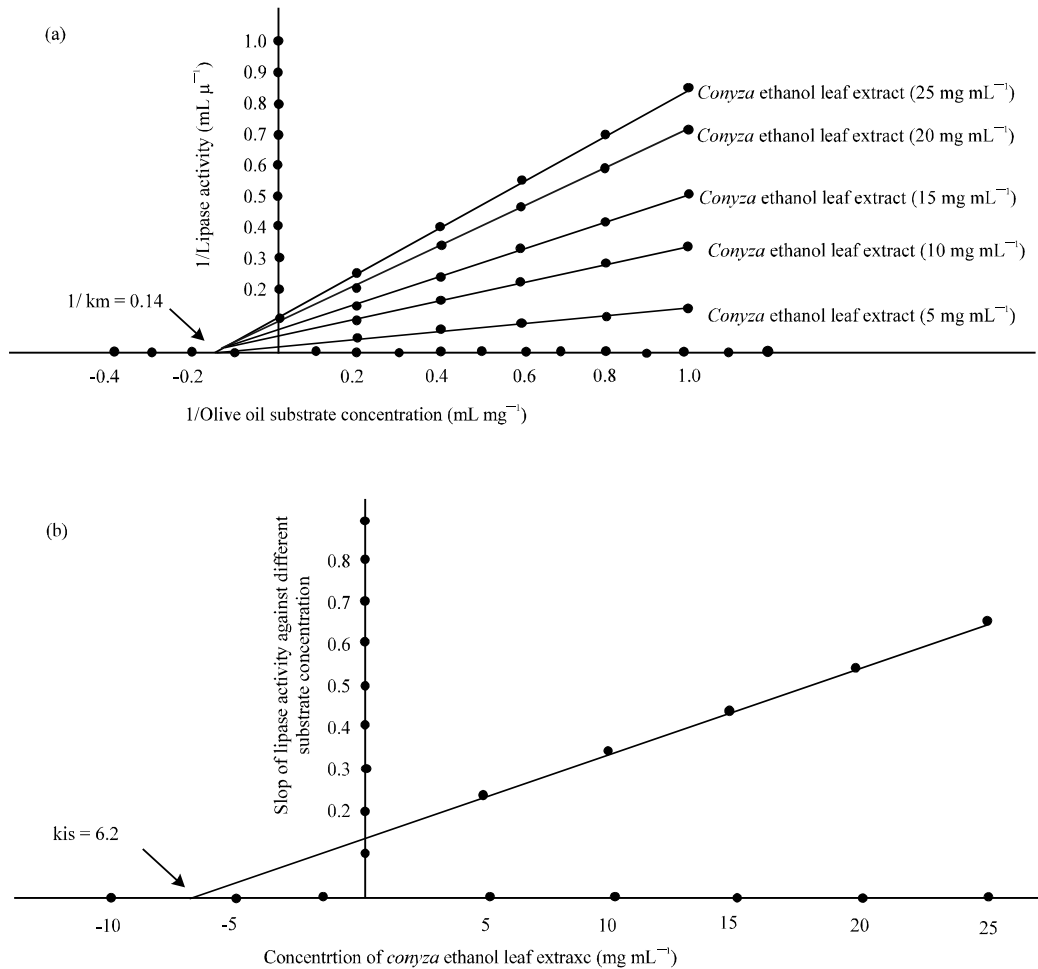


Fig. 3(a-b): Inhibition mechanism of *Conyza* ethanol leaf extract against lipase of *A. niger*, (a): Lineweaver Burk plot and (b): Replot of reciprocal lipase activity slope against different concentrations of *Conyza* ethanol leaf extract

Table 3: Inhibitory effect of *Conyza* ethanol leaf extract on growth and lipase activity of *A. niger*

Concentration (mg mL ⁻¹)	Diameter of inhibition zone (cm)	Lipase activity (U mL ⁻¹)
0	0	30.8±0.1
5	2.0±0.11	14.1±0.1
10	3.9±0.12	7.5±0.1
15	6.0±0.11	4.2±0.1
20	8.0±0.13	2.8±0.1
25	9.0±0.12	0.0
ANOVA		
F	4392.6	39263.1
p-value	<0.001*	<0.001*

*p-value is statistically highly significant at the 0.001 level

DISCUSSION

Fungal growth can be established within skin tissues of the ear canal by strong offensive forces, such as secretion of extracellular enzymes.

According to several predisposing factors such as humidity, moisture, high temperature, entrance of water inside ear due to swimming or sweat secretions, increased use of topical antibiotics, weak immune function, cleaning of ear wax and absence of the protective coating of cerumen, some changes may appear swelling of stratum corneum of external ear canal and inflammation of the mucous membrane in the middle ear (Dyckhoff *et al.*, 2000), lead to destruction of protective barriers that include secretions from sweat, sebaceous and cerumen glands. This results in a disturbance of the continuous drainage of fluids from the middle ear cavity to the auditory tube at which mucous discharge serves as a substrate for secreting lipase enzyme (Vennewald *et al.*, 2003) that support air fungal spores colonization of the external ear causing otomycosis.

So, a part of the present study focused on detecting the ability of isolated otomycotic fungi to produce this enzyme, which may facilitate the passage of fungi to stratum corneum of skin by physical barriers and other skin layers which could be a reason for otomycosis infection.

In the present work, the highest rate of lipase production was recorded on the 8th day growth of *A. niger*. The major lipase yield was precipitated from culture filtrates of *A. niger* with ammonium sulphate (30%) saturation. This result was supported by the results of Rifaat *et al.* (2010) for *Fusarium oxysporum* with ammonium sulphate (30% saturation). Also, Borkar *et al.* (2009) precipitated lipase with ammonium sulphate at 30%. However, Stocklein *et al.* (1993) for *Penicillium expansum* with ammonium sulphate (80% saturation). Also, El-Shora and Metwally (2006) reported that lipase in the culture supernatant of *Bacillus thuringensis* was precipitated with 75% ammonium sulphate saturation.

In the present work, purification of lipase by gel filtration chromatography on Sephadex G-100 matrix revealed that lipase gave elution profile with a single peak and was also confirmed by (SDS-PAGE) analysis representing lipase as a single protein band at molecular weight ranging at about 45 kDa. Other SDS-PAGE analytical studies of lipase revealed that it possess a single protein band with molecular weight of 40 kDa. This was reported for *Bacillus licheniformis* by Sangeetha *et al.* (2010). Also, El-Shora and Metwally (2006) reported that purified lipase from *Bacillus thuringensis* showed only one protein band at 49 kDa on SDS-PAGE analysis. However, Macedo *et al.* (1997) represented lipase of *Geotrichum* sp. as a single band with molecular weight of 29 kDa upon SDS-PAGE analysis. Moreover, the results of Karadzic *et al.* (2006) recorded a molecular weight of 54 kDa for lipase protein band isolated from *Pseudomonas aeruginosa*.

Inhibition of lipase by *Conyza* leaf extract as an important offensive force against *A. niger* leads to very low fungal growth, so it will decrease the ability of the fungi to invade the ear canal causing otomycosis. In accordance with our results, Slanc *et al.* (2009), detected a potential lipase inhibitory activity of *Pisum sativum*. Moreover, El-Zawawy (2010) reported that *Anethum graveolens* seed extract had moderate lipase inhibitory activity. While, Gholamhoseinian *et al.* (2010) reported the inhibition of lipase enzyme by *Eucalyptus galbe*.

In a trial to explain the ability of *Conyza* leaf extract to manage otomycosis it was observed that *Conyza* leaf extract has strong antilipase activity. Our study determined that *Conyza* extract kinetics properties acted as irreversible competitive inhibitor for lipase enzyme. In contrast to our results, Zhao and Ki (2004) inhibited the pancreatic lipase activity in a non-competitive manner by *Levisticum officinale* extract.

However, in accordance with our results, some studies reported an irreversible-competitive inhibition of lipase (Won *et al.*, 2007; Huerta *et al.*, 2007; Han *et al.*, 2001). Also, Gholamhoseinian *et al.* (2010) represented the irreversible-competitive inhibition of lipase by *Eucalyptus galbie*.

CONCLUSION

Lipase enzyme was found to be the most important offensive force of isolated fungi which facilitate their invasion into ear canal. It was purified and characterized from *A. niger* with molecular weight of 45 kDa. Also, *Conyza* leaf extract had high inhibitory action against *A. niger* growth and lipase enzyme with irreversible competitive action. *Conyza* leaf extract can be used as anti-otomycosis fungi.

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