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Purification, characterization and antitumor activity of L-lysine alpha-oxidase from *Trichoderma harzianum* Rifai AUMC No 848

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Abstract L-lysine alpha-oxidase is an interested enzyme due to its potential applications in biotechnology and tumor therapy. Tumor cells are highly sensitive to the deficiency of growth factors including amino acids, L-lysine alpha-oxidase depletes L-lysine; thus the tumor cells die because of their inability to synthesize this amino acid. The L-lysine alpha-oxidase from *Trichoderma harzianum* Rifai AUMC No.848 has been purified to homogeneity through ammonium sulfate precipitation, anion exchange (Q FF column) and gel filtration chromatography (sephacryl-200). The enzyme has a molecular weight of approximately 118 kDa using native gel and found to be about 58 kDa that indicated the enzyme consists of two subunits identical in molecular weight. The parameters of purified L-lysine alpha-oxidase were optimized at reaction time 25 minutes, pH 8 and more stable at pH 9. Additionally, enzyme thermal stability was observed in the range of 40 to 50 $^{\circ}$C for four hours. L-lysine alpha-oxidase activity increased with increasing L-lysine concentration; 160 mM of L-lysine gave activity 154% and activity was increased by Ni^{2+} to 120 %. Whereas chelating agents such as EDTA had no effect on L-lysine alpha-oxidase, suggested that L-lysine alpha-oxidase was not metaloenzyme. The antitumor activity of the purified L- lysine alpha-oxidase was also investigated against the three carcinoma cell lines *in vitro*. The enzyme was more toxic and selective to cancer cells than normal cells. The apoptotic effect of L-lysine alpha oxidase was confirmed by detecting morphological changes in Caco-2, HEp-2 and HepG2 cells, nuclear staining by propidium iodide stain, assaying cell-cycle phase by flow cytometry and DNA fragmentation.

Keywords *Trichoderma* sp.; L-lysine alpha-oxidase, purification, *in vitro* anti- tumor, *in vivo* anti-tumor

Introduction

The enzymes treatment of cancer has a long history, first in 1902 with the work of Dr John Beard, a professor at the University of Edinburgh proposed that the pancreatic proteolytic enzyme trypsin represent a powerful anti-cancer tool. Later, there are several enzymes are used for cancer treatment like methionine syntheses [1]. L-Lysine alpha-oxidase enzyme was first isolated in the laboratory of Prof. K. Soda (Japan) from *Trichoderma viride* Y 244-2 [2].

L-lysine alpha-oxidase (LOX) is one of the L-amino acid oxidase. LOX is the first enzyme recognized of this enzyme family [3]. L-lysine alpha-oxidase was purified from a variation of tissues and species including chick cartilage, bovine aorta and lung, human placenta, and piglet skin [4]. L-lysine alpha-oxidase is composed of a four hundred and seventeen residue polypeptide in a four chained dimer structure with every portion of the dimer containing of two distinct chains. LOX is comprised of alpha helices that account for 17.9% of the structure, and beta sheets for 38.3% of the structure [5]. L-lysine alpha-oxidase found in mammals [6], snakes

[7], insects [8], marine organisms (mollusks and fish) [9], and microorganisms including bacteria [10], yeasts [11] and fungi [12].

The mechanism of LOX in cancer based on the specificity of cancer cell metabolism led to the elaboration of several original methods of cancer diagnostics and therapy [13]. It was determined that adenosine desaminase and 5'-nucleotidase regulating the intracellular level of adenosine play an important role in the normal functioning of macrophages, which are believed to be responsible for antimetastatic resistance [14]. Remarkable progress in human cancer therapy is based on the suppression of enzymes involved in the biosynthesis of polyamines, which are known to be mitogenic agents, and on the blockade of key enzymes of nucleic base synthesis [15]. The increase in intracellular reactive oxygen species detected by the 2,7-dichlorodihydrofluorescein assay suggests that the oxidative pathway is one of mechanisms underlying the cytotoxic LOX action; however, this does not rule out the involvement of other (previusly demonstrated) mechanisms of LOX effects on cell death [16].

Materials and Methods

Purification of L-lysine alpha-oxidase produced by *Trichoderma harzianum* Rifai AUMC No. 848 collected from Assiut University Mycology Center

Crude enzyme was collected after fermentation periods at optimum physical and chemical parameters, the clear supernatant was collected by centrifugation at 4000 rpm for 20 min and LOX was purified by ammonium sulfate fractionation, followed by QFF column chromatography and sephacryl-200 column chromatography according to [15] with slight modifications. All purification steps were carried out at 4 °C.

Ammonium sulfate precipitation

Ammonium sulfate was added to this crude enzyme solution in a quantity to reach 30% of the saturated concentration, whereupon soluble was separated, and the insoluble pellet was removed by centrifugation. Ammonium sulfate was further added to the supernatant to reach 60% of the saturated concentration to produce a precipitate. The separated precipitate was then dissolved in 500 ml of 0.02 M potassium phosphate buffer (pH 7.4), and the resulting solution was subjected to dialysis against the same buffer solution containing 2mM phenyl methane sulfonyl fluoride (PMSF) at 4 °C.

Anion exchange chromatography

The precipitate formed in the course of the dialysis treatment was removed by centrifugation, and the resulting supernatant was applied on QFF column (16/10 cm, Pharmacia, Sweden) which had been equilibrated with 50 mM phosphate buffer (pH 7.4). The column was washed with the same buffer, and then the bound proteins were eluted using gradient of NaCl from 0.0 to 1.0 M in the same buffer at flow rate 1.0 ml/min and fraction size 3.0 ml eluted with the same buffer solution containing 0.2 M sodium chloride by AKTA prime plus FPLC.

Gel filtration chromatography

From QFF column, the eluted active fractions were collected, dialyzed and concentrated, and then applied to gel filtration with Sephacryl-200 column (16/120 cm, Pharmacia, Sweden), equilibrated by 50 mM phosphate buffer (pH 7.4) containing 0.15M NaCl in same buffer at flow rate 0.8 ml/min and fraction size 4.0 ml. The active fractions were dialyzed by same buffer and lyophilized by Lyophilizer Model-08226.

Determination of the molecular weight of the purified enzyme Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The molecular weights of the purified L-lysine alpha-oxidase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by **[17]**.

Native gel electrophoresis

For native gel electrophoresis, the followed protocol was very similar to that for denaturing gel electrophoresis sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Native or non-denaturing gel electrophoresis was run in the absence of SDS and all reducing agents from the separating gel buffer, stacking gel buffer, tank buffer and sample buffer.

Dialysis of the purified proteins

The dialysis of the purified proteins solution was carried out using the dialysis tubes with an exclusion limit of 10-14 kDa (pores of 24A) against 2.0 L dialysis buffer (0.02 M potassium phosphate buffer (pH 7.4) containing 2 mM PMSF) at 4 °C, the procedure was repeated for at least five times.

Protein content determination

Protein concentration was determined either by measuring its absorbance at 280 nm or by the method of **[18]**. The method of Bradford utilizes the change in absorbance of Coomassie Brilliant Blue G-250, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm. A calibration curve was established using bovine serum albumin (BSA) as a standard.

Characterization of purified L-lysine alpha-oxidase

Optimum reaction time

The optimum reaction time of the purified LOX was estimated at different time intervals from 5 to 60 min and then assayed as described by [19].

Optimum pH and pH stability

The optimum pH was determined by measuring enzymatic activity using different buffers (0.1 M) with various pH values as follows: citrate–phosphate buffer (pH 2.0, 3.0, 4.0 and 5.0), phosphate buffer (pH 6.0 - 7.0), Tris-HCl buffer (pH 8.0 - 9.0) and glycine-NaOH buffer (pH 10.0, 11.0 and 12).

The pH stability was determined by incubation of the purified L-lysine alpha-oxidase at different pH values from 2 to 12 overnight and then detection of the activity.

Temperature and thermo stability of purified L-lysine alpha-oxidase

Optimum temperature was assayed by measuring the enzyme activity at varying temperature (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100) using 50mM phosphate buffer pH 7.4.

Heat stability of the purified LOX enzyme was determined by incubating the purified enzyme at various temperatures (40, 50 and 60) for (1, 2, 3, 4, 5 and 6 hours) and then the residual activity was determined at optimum pH and temperature.

Substrate specificity and determination of *Vmax* and Km

Some enzymes are adaptive and cannot be active without substrate and others are constitutive; can be active with or without substrate, so effect of different substrates on purified L-lysine alpha-oxidase activity such as lysine, glutamine, alanine, serine, methionine, histidine, glycine, asparagine, tryptophan and cysteine individually in the reaction mixture by concentration 100µM was assayed. The apparent *Vmax* and Km value of purified L-lysine alpha-oxidase was calculated from the Lineweaver-Burk plots relating 1 / V to 1 / [S] [20].

Effect of substrate concentrations

Purified L-lysine alpha-oxidase enzyme was incubated individually with different concentrations of L-lysine in the reaction mixture (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 mM) to find out the best substrate concentration for enzymatic assay under optimized assay conditions.

Effect of some chemical additives on enzyme activity

The effect of different chemicals on purified LOX was studied by incubating the enzyme with different metal ions including sodium, calcium, potassium, aluminum, magnesium, capper, barium, stannous and nickel at a final concentration of 1.0 mM and different concentration of EDTA (0.2, 0.4, 0.6, 0.8 and1 mole) separately. Residual activities in the presence of each chemical was assayed and compared with the control (without additions), which considered as 100% activity.

Spectral characterization of L-lysine alpha-oxidase

Absorption spectrum of L-lysine alpha-oxidase produced by *T. harzianum* Rifai AUMC No.848 was determined by dissolving 0.4 mg/ ml in 0.02 M potassium phosphate buffer, pH 7.4 at 25 °C.

***In vitro* Anti- tumor effect of L-lysine alpha-oxidase**

Human blood lymphocytes separation

The peripheral blood mononuclear cells (PBMCs) were isolated as reported by [21]. Briefly, peripheral blood samples were collected from a single healthy volunteer 31 years in heparinized vials. A 4 ml of anticoagulant-

treated blood were added to a 10 ml centrifuge tube. Ficoll Histopaque media (4 ml) was added to the centrifuge tube. The blood was gently layered onto the Ficoll Histopaque media solution (they should stay as two different layers) followed by centrifuging the tubes (without any delay) for 30 min at 400 *x g* at 4 °C in a swing-out bucket rotor; fixed angle rotors also can be used but would require more caution when separating cells in interphase. The whitish buffy coat (about 1 ml comprising PBMCs) formed at the interface was aspirated without delay. After centrifugation at 400 *x g* and 4 °C for 10 min twice, cells were resuspended in 10 ml of sterile PBS.

Cell culture

Caco-2 cell line and HEp-2 cells were plated in Dulbecco's modified Eagle (DMEM) medium supplemented with 20% and 10% v/v FBS, respectively. While, hepatoma cell line HepG2 was cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 2% v/v L-glutamine, 10% v/v FBS, and 1% v/v penicillin-streptomycin. The cultures were maintained at 37 ºC in a humidified atmosphere with 5% CO_2; 95% air in 25 cm^2 flasks. For subculturing, culture medium was discarded, and then the adherent cells were detached from the surface of the flask using 1 ml of Trypsin-EDTA solution (0.25% w/v Trypsin- 0.53 mM EDTA) for 5 to 15 min at 37 ºC. The action of Trypsin was stopped by the addition of 3 ml DMEM or RPMI-1640 medium. The cells were scraped and collected in a 15 ml conical tube, then washed twice by DMEM or RPMI-1640 supplemented medium, and centrifuged at 1200 rpm for 5 min at 4 °C after each wash. The pellet was resuspended in 3 ml DMEM or RPMI-1640 medium, and then appropriate aliquots of the cell suspension were added to new culture vessels.

PBMCs and fibro blast were cultured in RPMI-1640 medium supplemented with 10% v/v FBS, 100 U/ml of penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37 ºC in a humidified atmosphere with 5% CO_2; 95% air in 25 cm^2 flasks. The cells were passaged twice to three times weekly. For medium renewal, every 3 to 4 days; floating cells were collected, centrifuged at 1200 rpm for 5 min at 4 °C and added to the flask together with fresh medium.

Cell counting

The cells were collected by centrifugation at 1500 rpm for 5 min at 4 °C and resuspending the pellet in 3 ml DMEM or RPMI-1640 supplemented medium. Cells were mixed well by pipetting up and down to separate clumps. Ten microliters of the cell suspension were mixed with 10 µl trypan blue dye, then 10 µl of the previous mixture were applied to an improved Neubauer hemacytometer and examined under phase contrast optical microscope with 100x magnification. Dead cells were distinguished from viable cells by their uptake of trypan blue stain, which diffuses into dead cells but not live cells. The total number of cells per ml was calculated according to the following equation:

Cells per ml = Average count per square (the total number of cells found in the four large corner squares of the hemacytometer/ 4) x dilution factor $x10^4$.

Assaying L-lysine alpha-oxidase cytotoxic effect

The cytotoxic effect of L-lysine alpha-oxidase on Caco-2, HEp-2, HepG2, PBMCs and fibro blast was assayed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method [22 and 23]. Briefly, cells were seeded at density of $25x10^4$ cells/ml in 200 µl of suitable media per well in 96-well cell culture plates (flat-bottomed for Caco-2, HEp-2, and HepG2 cells and U-bottomed for PBMCs and fibro blast) and incubated at 37 ºC in a humidified atmosphere with 5% CO_2; 95% air. After 24 hours, cells were treated with various concentrations (10, 20, 30, 40 and 50 ng/ml) of the tested purified L-lysine alpha-oxidase in 4 replicates and incubated for 1, 2 and 3 days at 37 °C in 5% CO_2; 95% air. Twenty microliters of 5 mg/ml MTT solution were added to each well and incubated at 37 °C for 3 hours. The formed formazan crystals were dissolved with 180 µl of Dimethylsulfoxide. Optical density was measured at 560 nm. The percentage of viability compared to the untreated cells was determined with the following equation:

Cell viability (%) = Mean OD/Control (untreated cells) OD x 100

Calculations were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined.

Analysis of apoptotic effect of L-lysine alpha-oxidase on tested human cancer cells

Morphology of cancer cells.

The anti-tumor effect of L-lysine alpha-oxidase was studied by observing morphological changes in tested cancer cells (Caco-2, HEp-2, and HepG2 cells) treated with this enzyme. The cells were cultured at a density of 1.0×10^5 cells/well in 24-well culture plates at 37 °C in 5% CO_2; 95% air. After overnight incubation, the cells were treated with different enzyme concentrations (10, 20, 30, 40 and 50 ng/ml) for 24 h. The cellular morphology was investigated by inverted phase contrast microscopy (Olympus Optical Co., LTD., Japan) in comparison to untreated cells as negative control.

Nuclear staining

The apoptotic effect of L-lysine alpha-oxidase was analyzed using fluorescent nuclear dye propidium iodide (PI) [24] and dye mix of EB/AO consisted of 100 µg/ml ethidium bromide and 100 µg/ml acridine orange in PBS [25]. HepG2 cells were seeded at a density of 1.0×10^5 cells/well in 24-well culture plate at 37 ºC, 5% CO_2 overnight. HepG2 cells were treated with different enzyme concentrations (10, 20, 30 and 40 ng/ml) overnight, then washed three times with PBS, and fixed in 4% paraformaldehyde for 10 min. Subsequently, the cells were permeabilized with permeabilization buffer (3% paraformaldehyde and 0.5% Triton X-100) and stained with PI Dye and of EB/AO. After staining, cells were viewed with an inverted microscope at 40x magnification with an excitation filter (480/30nm), a dichromatic mirror cut-on 505 nm LP. Pictures were taken with an Olympus microscope equipped with a digital camera. All tests were done in triplicate. A minimum of 100 cells were counted.

Detection of apoptosis using flow cytometry

The HepG2 cells were treated with a concentration of 10, 20, 30 and 40 ng/ml of L-lysine alpha-oxidase for 24 h at 37 °C in 5% CO_2; 95% air. After 24 h incubation, the treated cells were harvested and washed two times with cold PBS and fixed in 70% ethanol. Cells were treated with RNase A (10 mg/ml) and stained with propidium iodide (50 µg/ml stock solution) for 30 min at room temperature in dark (cells were not washed after the addition of Propidium Iodide). Cell cycle reading was performed using the Cell Quest software within 3 h, stored at 2–8°C and protected from light until being ready for analysis. The propidium iodide fluorescence of individual nuclei was measured using a FACS-Calibur cytometer (BD Biosciences, Heidelberg, Germany). Data were analyzed with the CellQuest Pro V5.2.1 software (BD Biosciences). For each condition, at least three independent experiments were performed [26].

DNA-fragmentation

HepG2 cells were seeded at a density 1.0×10^5 cells/well in 24-well culture plates. After 24 hours incubation, cells were treated with L-lysine alpha-oxidase at different concentrations (10, 20, 30 and 40 ng/ml) at 37ºC, 5% CO_2, then harvested cells were washed with PBS three times, after that the oligonucleosome length DNA fragments in the cells and centrifugation , then precipitate was lysed in 5 mM, containing 20 mM EDTA, 0.5% Triton-X100, pH 8.0 at 4 °C overnight and centrifuged at 16000 × g for 20 min. DNA oligosomes in the supernatant were precipitated with ethanol overnight at –20 °C, treated with proteinase K (50 µg/ml) and RNase (50 µg/ml), and centrifuged at 16000 × g for 20 min. The precipitate was stained and detected by agarose gel as described by [27]; 1.8% agarose gels were used. Agarose gel electrophoresis was prepared by dissolving 1.8 g of agarose in 100 ml of 1x Tris/Borate/EDTA (TBE) buffer by heating the solution to boiling in the microwave. The gel was cooled to 50 ºC and stained with ethidium bromide then poured into a suitable gel tray. The gel was allowed to completely solidify at room temperature and then samples were loaded into the gel and let at 100 volts. The cDNA was visualized under UV-transilluminator and photographed by gel documentation.

Results and Discussion

Purification of L-lysine alpha-oxidase produced by *Trichoderma harzianum* Rifai AUMC No.848

L-lysine alpha-oxidase was purified from the culture liquid by successive steps including $(NH_4)_2SO_4$ fractionation, ion exchange QFF (fig.1) and gel filtration (Sephacryl S-200) chromatography. Extracellular L-

lysine alpha-oxidase from *T. harzianum* Rifai AUMC No.848 was precipitated using ammonium sulphate at a final concentration of 60% resulting in total activity of 24200 U, specific activity of 361.19 U/mg and 2.36 fold purification. Anion exchange QFF column chromatography exhibited total activity of 21000 U with specific activity of 420 U/mg and 2.74-fold increase in purity. While gel filtration resulted in total activity of 15166 U with specific activity of 583.33 U/mg and 3.81-fold increase in purity.

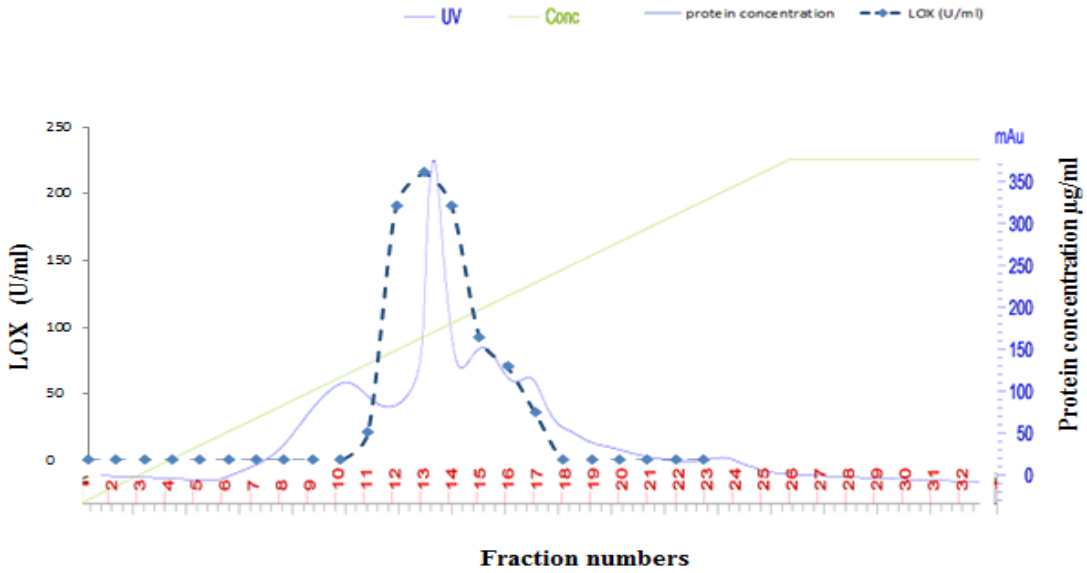

Figure 1: A typical elution profile for the chromatography of L-lysine alpha-oxidase on anion QFF column previously equilibrated with phosphate buffer pH 7.4. The elution was at flow rate 3.0 ml/fraction and absorbance recorded at 280nm.

Determination of the molecular weight of the purified enzyme

L-lysine alpha-oxidase produced by *Trichoderma harzianam* Rifai AUMC No.848 was analyzed using native gel electrophoresis and found to be 118 kDa band

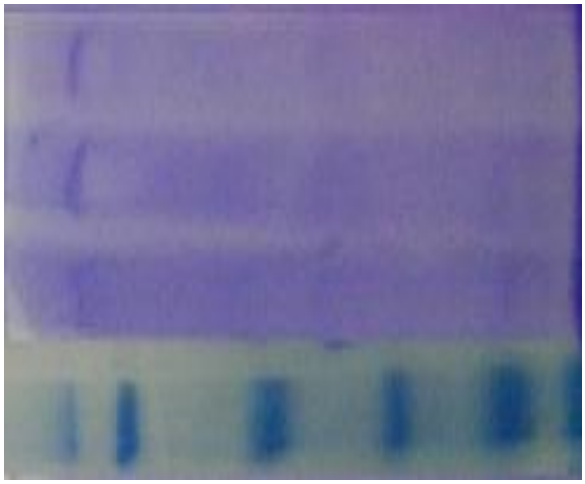

Figure 2: Native gel electrophoretic analysis of L-lysine alpha-oxidase at various steps of purification

Lane 1, molecular weight marker; lane 2, Crude-enzyme extract; lane 3, ion exchange chromatography on anion QFF; lane 4, purified L-lysine alpha-oxidase on Sephacryl-200.

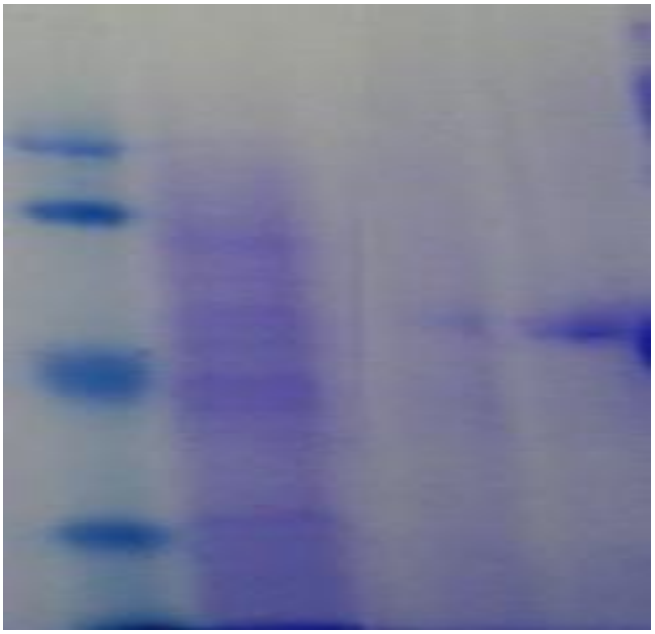

Figure 3: SDS-PAGE analysis of L-lysine alpha-oxidase during steps of purification

Lane 1, molecular weight marker; lane 2, Crude-enzyme extract; lane 3, ion exchange chromatography on anion QFF; lane 4, purified L- lysine alpha-oxidase on sephacryl-200.

Characterization of purified L-lysine alpha-oxidase activity

Effect of reaction time on the activity of purified L-lysine alpha-oxidase

The activity of purified L-lysine alpha-oxidase was tested at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min. Figure 4 showed that the activity of purified L-lysine alpha-oxidase increased with increasing of incubation time with a peak after 25 min, at which activity increased to become 135 % and became stable during the period started from 30 min until 60 min.

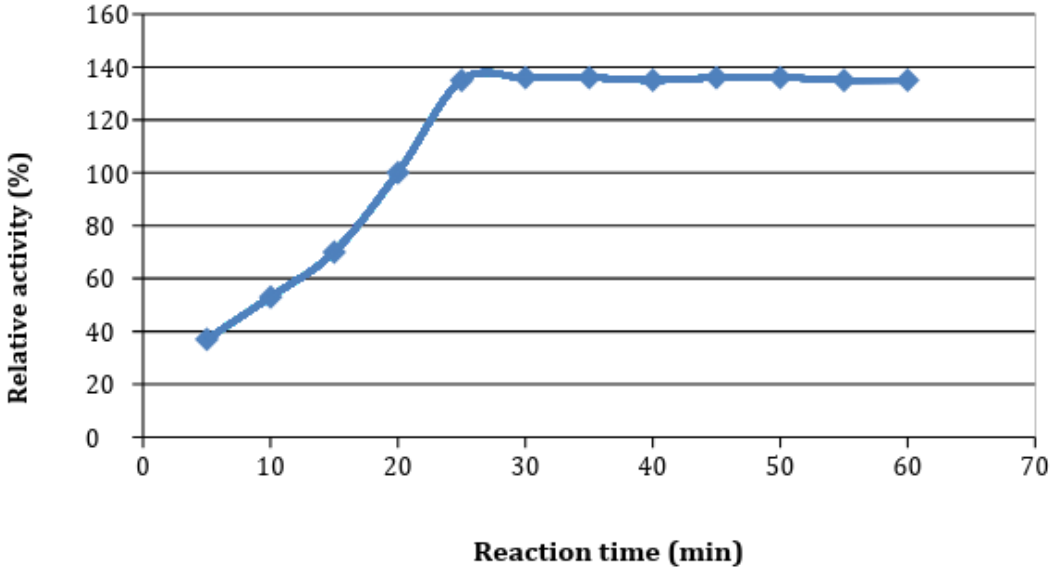

Figure 4: Effect of reaction time on purified L-lysine alpha-oxidase activity

Effect of different pH values and pH stability on purified L-lysine alpha-oxidase activity

The activity of purified L-lysine alpha-oxidase at different pH values from 2 to12 was determined. Results in figure 5, indicated that L-lysine alpha-oxidase exhibited the maximum activity at pH 7 and 8 of 97% and 100%, respectively. pH stability of L-lysine alpha-oxidase at pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 overnight was also examined and we found that the enzyme was more stable at pH 5, 6, 7, 8, 9 and 10 with activity of 81%, 93%, 95%, 100%, 101% and 87%, respectively.

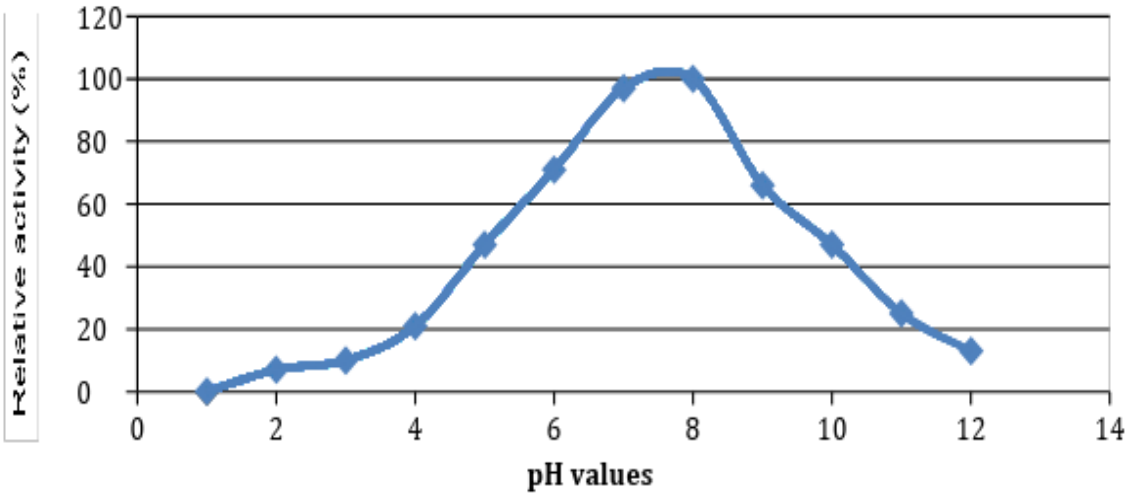

Figure 5: Effect of different pH values on purified L-lysine alpha-oxidase activity

Effect of different temperatures and thermo stability on L-lysine alpha-oxidase activity

The activity of purified L-lysine alpha-oxidase produced by *T. harzianum* Rifai AUMC No.848 at different incubation temperatures was showed in figure 6. Results demonstrated a wide range of enzyme activity from 30 to 50 °C. Thermo stability of purified L-lysine alpha-oxidase. The enzyme activity remained at 40, 50 and 60 °C for 4 hours and gradually decreased to 27, 15 and 7%, respectively.

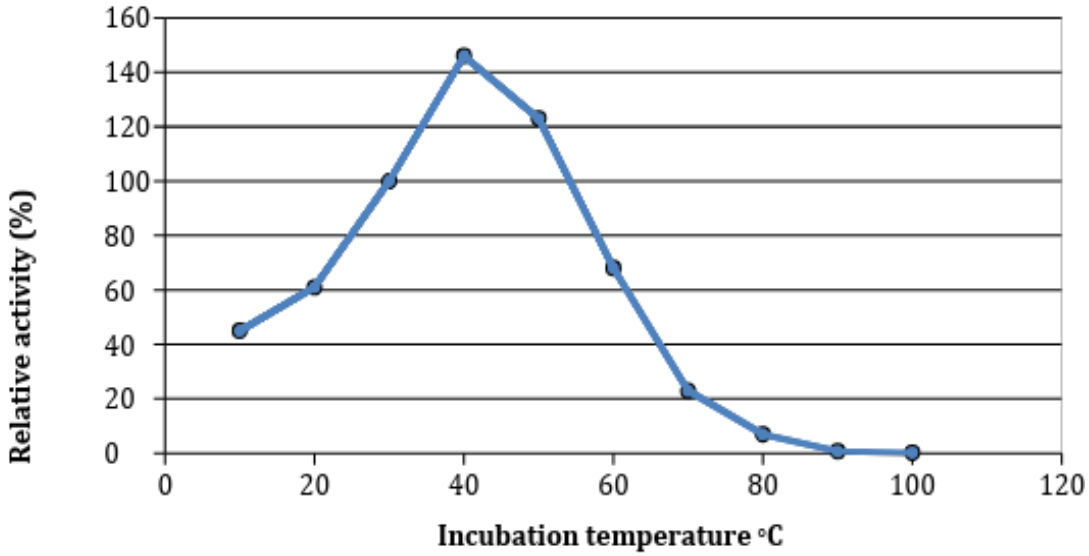

Figure 6: Effect of different incubation temperatures on purified L-lysine alpha-oxidase activity

Effect of different substrates on purified L-lysine alpha-oxidase activity

Figure (7) showed the effect of different substrates on purified enzyme activity, the maximum activity was observed using lysine, asparagine and glutamine with activity of 100%, 97% and 95%, respectively, while alanine, serine, methionine, histidine, glycine, tryptophan and cysteine showed activity of 50%, 49%, 87%, 77%, 51%, 50% and 14%, respectively. The activity of purified L-lysine alpha-oxidase increased with increasing substrate concentration (20, 40, 60, 80, 100, 120 and 140 mM) with activity of 30, 50, 62, 77, 100, 123 and 145% respectively, the LOX activity became stable at substrate concentrations of 160, 180 and 200 mM with relative activity around 156%. Based on the Lineweaver-Burk analysis presented in figure (8), the Km and *Vmax* values of L-lysine alpha oxidase were estimated to be 3.33 mM and 277.5 IU, respectively.

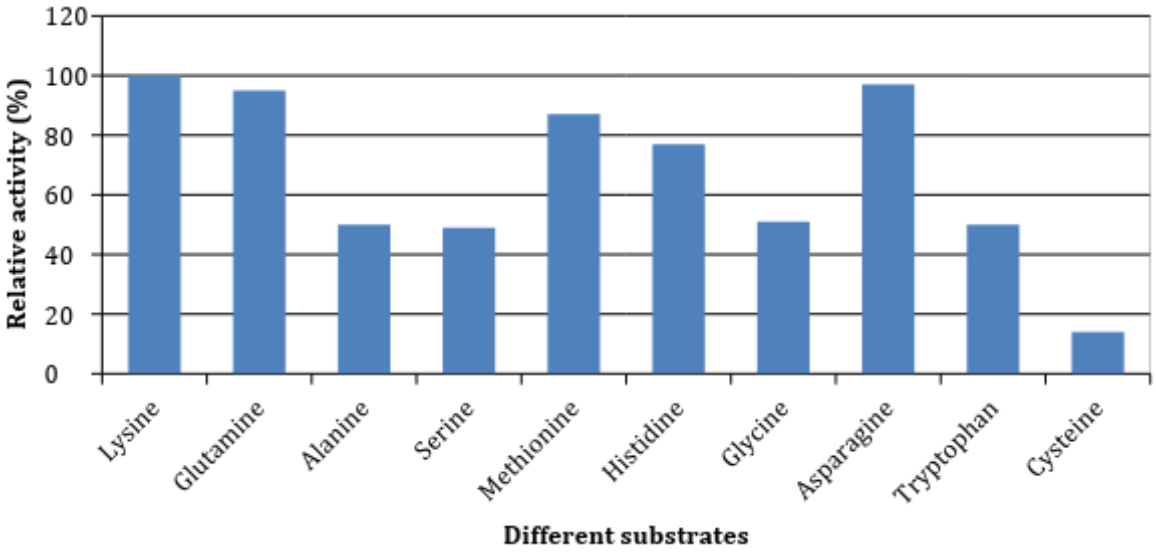

Figure 7: Effect of different substrates on the purified L-lysine alpha-oxidase activity

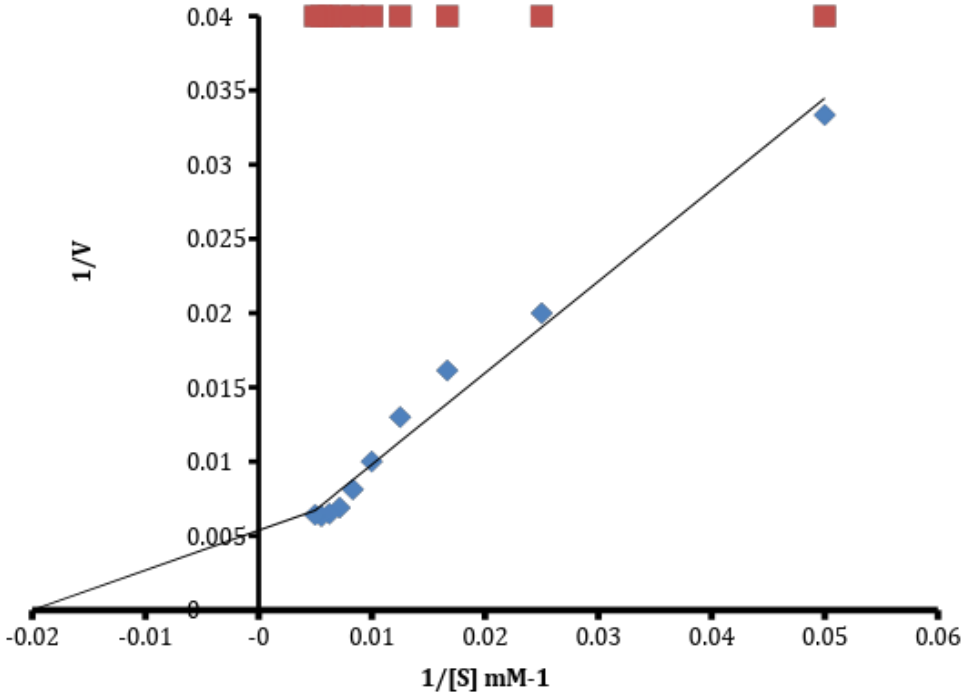

Figure 8: Lineweaver-Burk plot for different concentration of L-lysine as substrate for determination of Km and Vmax.

Effect of different metal cations on L-lysine alpha-oxidase activity

Figure (9) indicated the effect of different metals at concentration of 1.0 mg of each cation. nickel and potassium achieved increasing in the L-lysine alpha-oxidase activity by 20% and 10%, respectively, sodium did not affect L-lysine alpha-oxidase activity compared by control, however copper, calcium, barium, magnesium, aluminum and stannous cations inhibited enzyme activity by 80%, 90%, 20%, 70%, 89% and 55%, respectively. Furthermore, the effect of different concentrations of EDTA (0.0001, 0.001, 0.005, 0.01, 0.05, and 0.1 M) on the activity of purified L-lysine alpha-oxidase was studied. Results indicated that different EDTA concentrations have no effect on the enzyme activity which means that L-lysine alpha-oxidase enzyme was not belonging to the metalloenzymes.

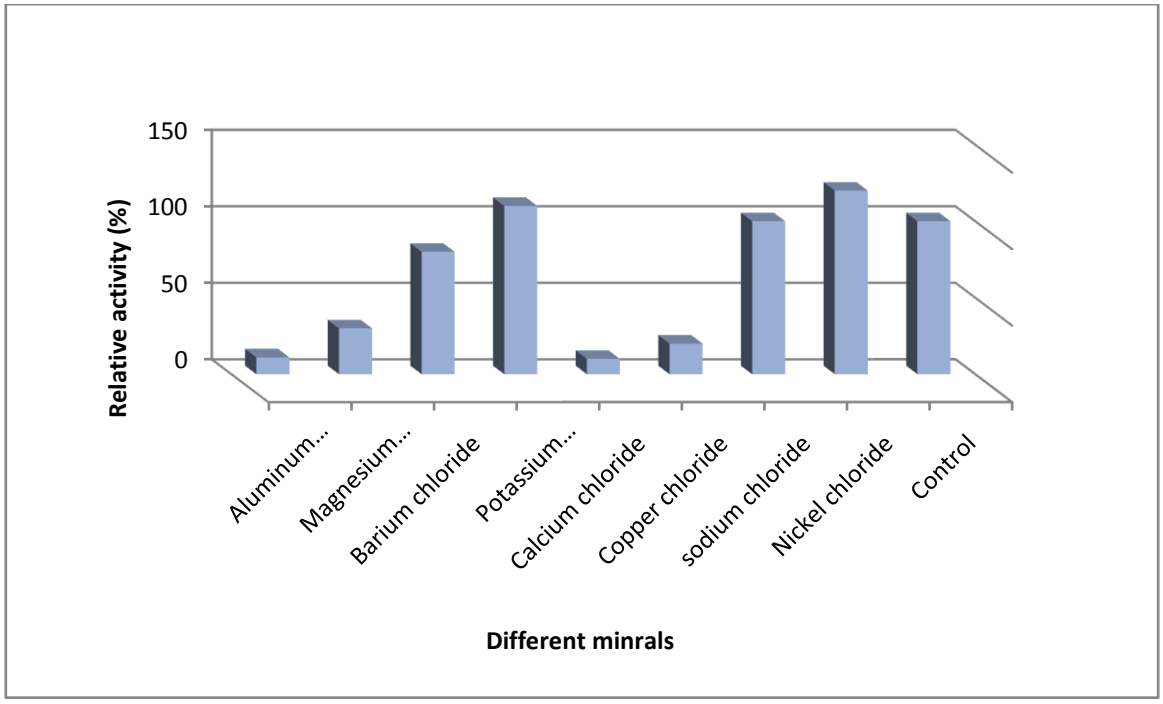

Figure 9: Effect of different metal cations on LOX activity

Spectral characteristics of the enzyme

The L-lysine alpha-oxidase absorbance spectrum was determined to detect flavoprotein peaks related to LOX enzyme as presented in Fig. 10. The spectrum was typical of a protein containing a flavoprotein which has maxima at 278, 390, and 465 nm; associated peaks in the visible region are well observed in LOX and slandered.

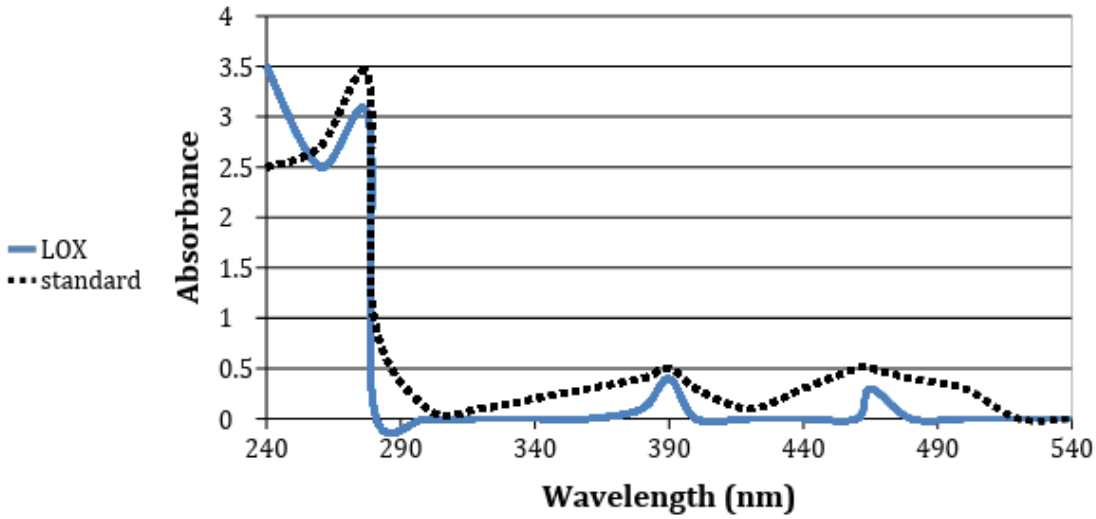

Figure 10: Spectral characterization of purified LOX

***In vitro* Anti-tumor effect of L-lysine alpha-oxidase**

Assaying of L-lysine alpha-oxidase cytotoxic effect using MTT method

After one, two and three days of incubation, percent of cell viability was determined by the MTT colorimetric assay. The enzyme induced cell cytotoxic effect on tested carcinoma cell lines in a concentration dependent manner as presented in Table 1. Enzyme was able to inhibit the proliferation of the cancer cells (Caco-2, HEp-2, and HepG2), while the enzyme showed slight effects on the normal PBMCs and fibroblasts viability as shown in table 2. IC_{50} values of enzyme was determined against three tested tumor cells, the lowest (40.82 ng/ml) being

obtained with enzyme in human Caco-2 cancer cell line while 42.55 and 52.63 ng/ml in HEp-2 and HepG2, respectively, selectivity index values of LOX on fibroblasts and PBMCs were calculated to be 4.12, 3.95 and 3.19 for fibroblasts against Caco-2, HEp-2 and HepG2, respectively, and 3.74, 3.59 and 2.9 for PBMCs against Caco-2, HEp-2 and HepG2, respectively (Table 3). Data in suggested that the enzyme was more toxic and selective to cancer cells than normal cells; thus considered a potential anticancer drug.

Table 1: Percent viability of Caco-2, HEp-2, and HepG2 cells after treatment with LOX at different concentrations determined by MTT assay

Enzyme concentration (ng/ml)	Incubation time								
	First day			Second day			Third day		
	Caco-2	HEp-2	HepG2	Caco-2	HEp-2	HepG2	Caco-2	HEp-2	HepG2
Control (untreated cells)	100	100	100	100	100	100	100	100	100
10	76±0.866	69±0.816	77.5±0.408	59.5±0.408	53±0.816	51.8±0.694	43±0.04	40.5±0.408	48.7±0.489
20	65.8±0.693	60±0.489	70.8±0.653	43.2±1.428	51±0.408	45.4±0.449	32.7±0.244	36.9±0.081	44.5±0.367
30	52.9±0.173	55±0.163	67.7±0.204	39.1±0.122	51±0.816	41.9±0.489	31.6±0.326	35±0.408	38.2±0.612
40	51±0.866	53±0.408	60.1±0.081	34.3±0.244	47±0.081	34.4±0.326	28.7±0.244	30.3±1.102	32.8±0.938
50	46.8±0.996	46±0.489	52.5±0.408	31.3±0.285	39±0.408	31.9±0.04	23.3±0.244	30±0.612	29.5±0.408

Table 2: Percent viability of PBMCs and fibroblasts after treatment with LOX at different concentrations determined by MTT assay

Enzyme concentration (ng/ml)	Incubation time					
	First day		Second day		Third day	
	Fibroblasts	PBMCs	Fibroblasts	PBMCs	Fibroblasts	PBMCs
Control (untreated cells)	100	100	100	100	100	100
10	98±0.816	96.6±0.285	96.9±0.734	95.9±0.898	95.9±0.081	94.1±0.816
20	97±0.326	95.1±0.163	95.6±0.326	95±1.633	95±0.408	93.8±1.102
30	96.4±0.489	94.9±0.204	95±0.816	94.8±0.285	49.6±0.816	93.5±0.816
40	95.7±0.204	94.4±0.816	94.9±0.898	93.4±0.816	94.3±0.408	93±0.326
50	95.5±0.408	94±0.122	94.1±0.734	93.1±0.081	94±0.204	92.8.±0.816

Table 3: IC_{50} and selectivity index values of LOX against Caco-2, HEp-2, HepG2, PBMCs and fibroblasts after an exposure time of 24

Tested cells	IC_{50} value (ng/ml)	Selectivity index (SI) (IC_{50} against normal cells/ IC_{50} against cancer cells)	
		Fibroblasts	PBMCs
Caco-2	40.82	4.12	3.74
HEp-2	42.55	3.95	3.59
HepG2	52.63	3.19	2.9
Fibroblasts	168.2	-	-
PBMCs	152.78	-	-

Apoptotic effect of L-lysine alpha-oxidase on human cancer cells

Morphological analysis

Figure 11(A)–(D) shows the comparative cellular morphology of untreated control and treated cell lines Caco-2, HepG2, HEp-2, and fibroblasts with different concentrations of L-lysine alpha-oxidase; 10, 20, 30, 40 and 50 ng/ml (b, c, d, e and f, respectively). These photomicrographs show that the cells have drastically changed in their morphology characterization in the treated groups. In the last stages of the process of apoptosis, it leads to cell reduction and pyknosis. The cells become reduced in size, numbers and cytoplasm becomes dense, due to cell shrinkage.

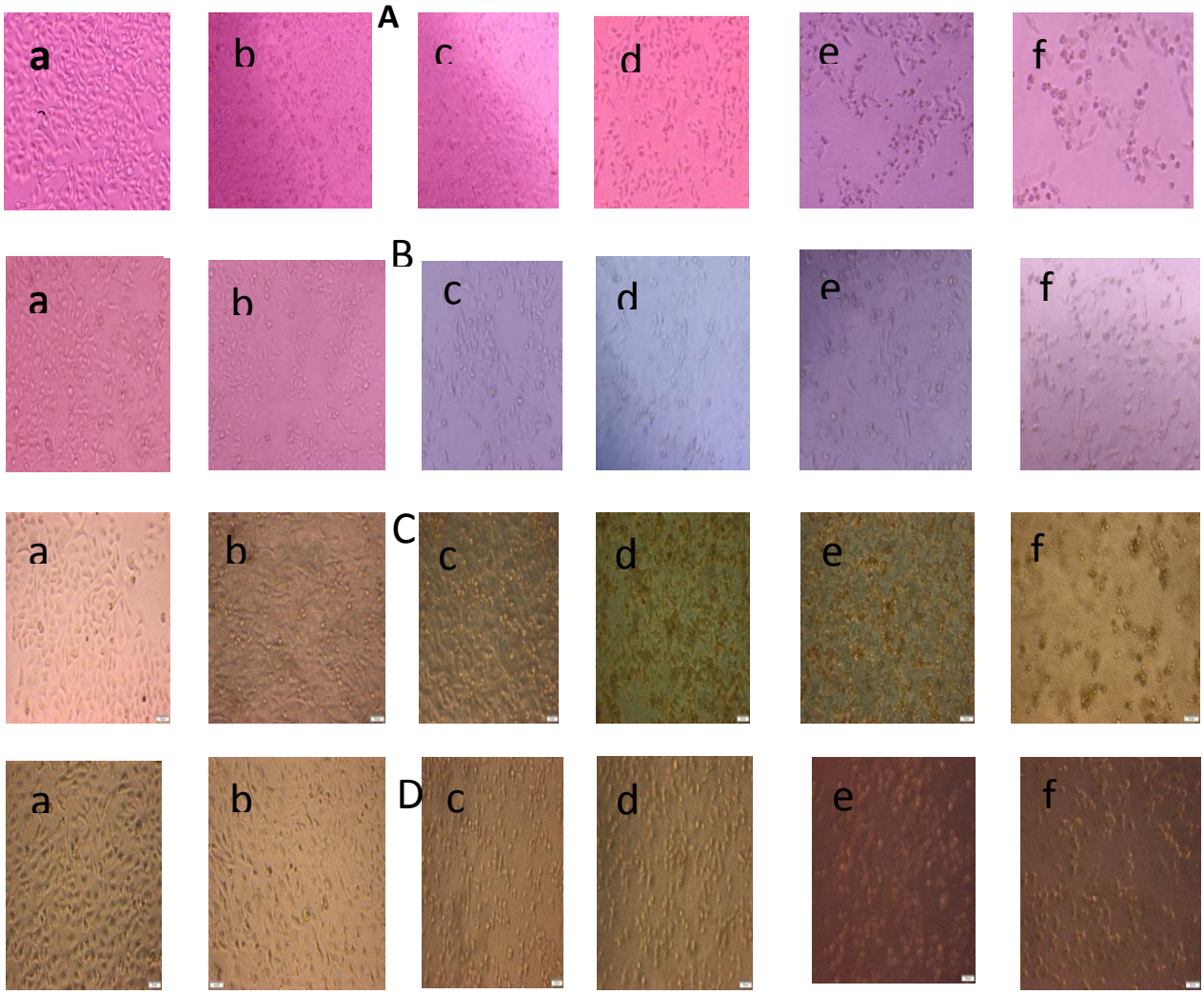

Figure 11: Photomicrograph (pictorial view) of (A) Caco-2, (B) HepG2, (C) HEp-2 and (D) normal fibroblast cells under phase contrast microscope. (a) Untreated cells, (b) cells exposed to 10 ng/ml, (c) cells exposed to 20 ng/ml, (d) cells exposed to 30 ng/ml, (e) cells exposed to 40 ng/ml and (f) cells exposed to 50 ng/ml of LOX

Nuclear staining

The apoptotic effect of different concentrations from L-lysine alpha-oxidase (10, 20, 30 and 40 ng/ml) was analyzed by nuclear morphological changes in HepG2 cell line using propidium iodide (PI), ethidium bromide and acridine orange (EB/AO) dyes. PI and (EB/AO) were hardy to normal cells with an intact plasma membrane; however, when the cell integrity becomes disturbed, it rises access to the nucleus where it complexes with the DNA, the nucleus become highly fluorescent, and it is commonly used for recognizing apoptosis It is detected from the photomicrograph (Fig. 12) that the cells became curved; the cell size and the nucleus have shrunk with increasing doses of L-lysine alpha-oxidase.. Furthermore, (EB/AO) dye was used for identifying early and late apoptotic effects of tested drug. Figure 13 shows live untreated healthy cells with normal green nuclei and early apoptotic cells exhibited bright green or yellow stained nuclei as it was shown in LOX treated cells. While late apoptotic cells whose nuclei were stained with orange.

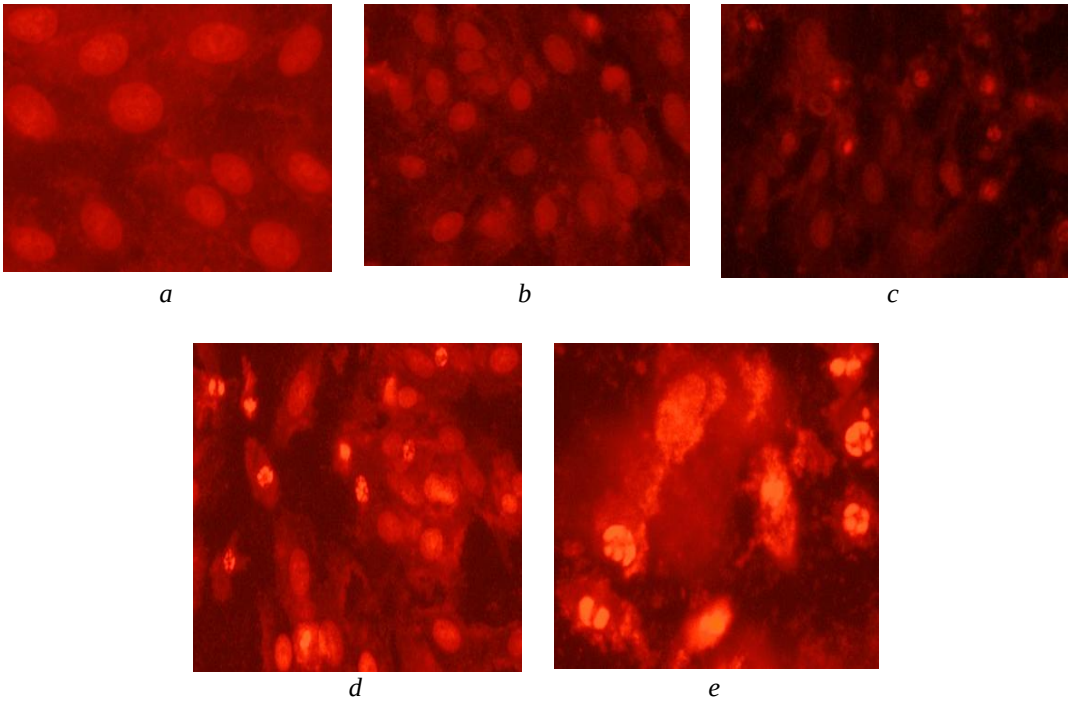

Figure 12: Photomicrograph of HepG2 cells stained with PI dye (A) control (untreated) cells, (B) cells exposed to 10 ng/ml, (C) cells exposed to 20 ng/ml (D) cells exposed to 30 ng/ml (E) cells exposed to 40 ng/ml of LOX

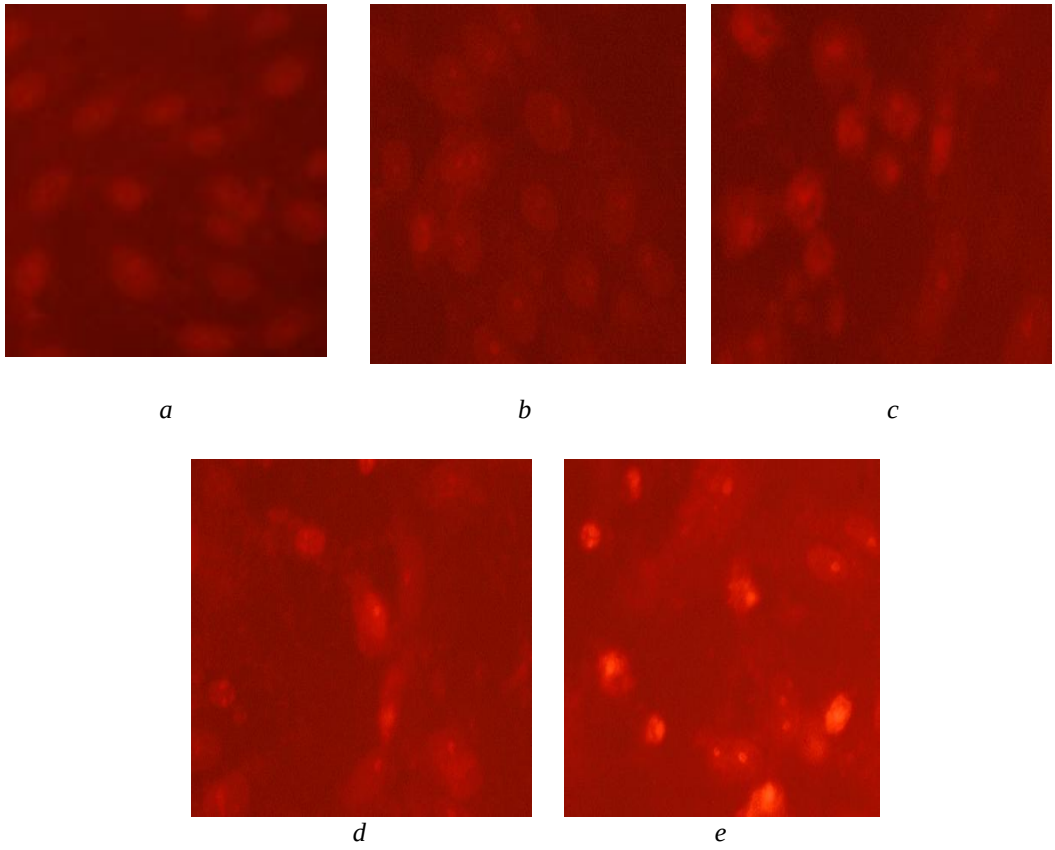

Figure 13: Photomicrograph of HepG2 cells stained with (EB/AO) dye. (A) control (untreated) cells, (B) cells exposed to 10 ng/ml, (C) cells exposed to 20 ng/ml (D) cells exposed to 30 ng/ml (E) cells exposed to 40 ng/ml of LOX

Detection of apoptosis using flow cytometry

Analysis of cell-cycle phase distribution was carried out to study the anti-proliferative mechanism of L-lysine alpha-oxidase on HepG2 cells. Figure 14 (A and B) showed that compared to the control, treatment resulted in increase of cells in subG1 phase by increased LOX concentration. G1 and G2 are the major check points, have an important role in cell cycle development, the G1 check point was interfered at 10, 20, 30 and 40 ng/ml of L-lysine alpha-oxidase. Cells in G1 phase have dramatically decreased, S phase was slightly increased by increasing the LOX enzyme concentration and G2 phase was reduced, when treated with 10, 20, 30 and 40 ng/ml of L-lysine alpha-oxidase as compared to untreated cells. These results indicated that L-lysine alpha-oxidase arrests the cells in G2 phase of cell cycle and also cause cell apoptosis in a dose dependent manner.

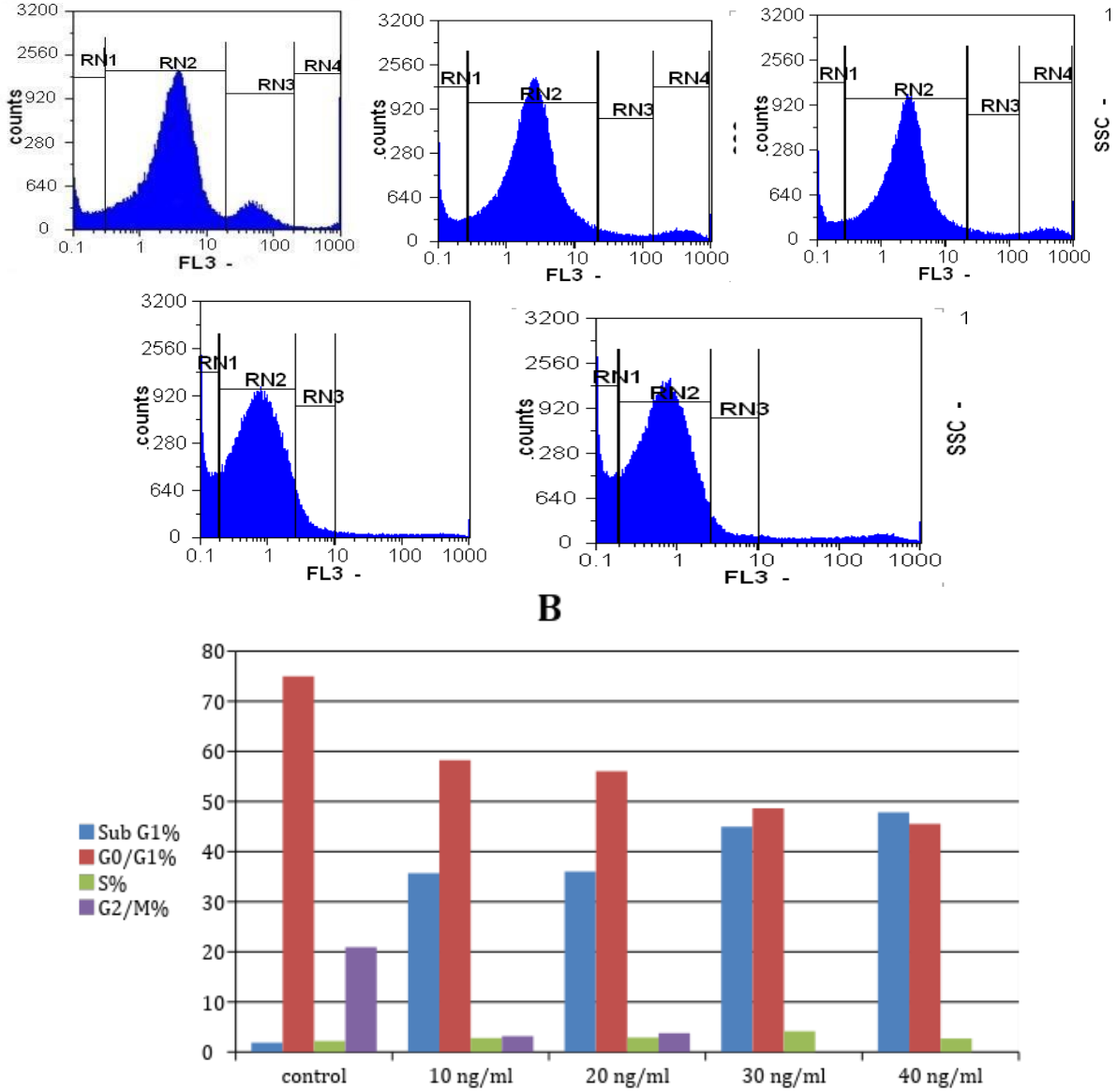

Figure 14: Cell cycle distribution of HepG2 cells treated with 10 to 40 ng/ml of LOX (A) original cell cycle diagrams (B) quantitative distribution of percentage of cells in different phases of cell cycle

Fragmentation of DNA

The apoptotic DNA fragmentation caused by different concentrations of L-lysine alpha-oxidase (10, 20, 30, and 40 ng/ml) on HepG2 cell line was analyzed. It was observed that oligonucleosomal fragments increased by

increasing enzyme concentration, while untreated cells showed complete genomic DNA without any fragmentation in1.8% agarose gel (Fig. 15).

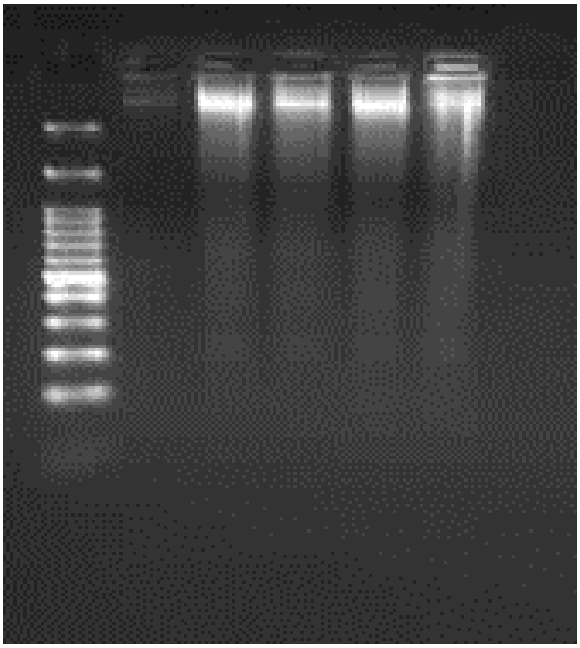

Figure 15: The apoptotic DNA fragmentation caused by different concentrations of LOX. (A) (B) control (untreated) cells, (C) cells exposed to 10 ng/ml, (D) cells exposed to 20 ng/ml (E) cells exposed to 30 ng/ml (F) cells exposed to 40 ng/ml

L-lysine alpha-oxidase was purified by successive steps including ammonium sulphate fractionation, ion exchange QFF and gel filtration (Sephacryl S-200) chromatography. Ammonium sulphate precipitation at a concentration of 60% of L-lysine alpha-oxidase produced by *Trichoderma viride* Y244-2 was recommended by [2], [28]reported ammonium sulphate precipitation at a concentration of 25% of L-lysine alpha-oxidase from *Trichoderma* cf. *aureoviride* RIFAI VKM F-4268D whereas purification based on copper sulfate precipitation of L-lysine alpha-oxidase produced by *Trichoderma sp*. 6 was described by [29]. Arinbasarova *et al* [2] purified L-lysine alpha-oxidase from *Trichoderma viride* Y244-2 by DEAE-Sephadex A-50 and reported total activity of 13,400 U/ml, 1.2-fold increase in purity with specific activity of 31.30 U/mg. They revealed that Sephadex G-200 gel filtration exhibited total activity of 4,780 U/ml and 1-fold increase in purity with 66.15 U/mg specific activity. Makrushin *et al.* [29] reported a new improved technique for L-lysine alpha-oxidase purification from *Trichoderma* sp. 6 based on ammonium sulfate precipitation and octyl-sepharose and DEAE-Toyopearl chromatography. This approach made it possible to obtain homogeneous enzyme with high specific activity (99 U/mg) and good yield (66%).

In this study, L-lysine alpha-oxidase produced by *T. harzianam* Rifai AUMC No.848 was analyzed using SDS-PAGE and found to be two sub units of 58 KDa molecular weight whereas native gel electrophoresis showed 118 kDa band representing the monomeric nature of enzyme. Similarly to our study, according to SDS electrophoresis data, L-lysine alpha-oxidase is molecule with identical subunits of 57-58 kDa; Arinbasarova *et al.* [28] reported the molecular mass of the enzyme subunit to be 57– 58 kDa on SDS-PAGE. In contrast, the corresponding values for L-lysine alpha-oxidase from *Trichoderma viride* Y 244-2 were about 116 and 56 kDa [2], whereas and Kalra *et al.* [30] determined the molecular weight of L-lysine alpha-oxidase to be between 25-100 kDa.

The activity of purified L-lysine alpha-oxidase produced by *Trichoderma harzianam* increased with increasing of incubation time with a peak after 25 min. While Kusakabe *et al.* [2] reported the best incubation time for activity of L-lysine alpha-oxidase produced by *Trichoderma viride* Y244-2 to be 30 min, L-lysine alpha-oxidase produced by *Trichoderma viride* Y-244-2-90 recorded 20 min as the best incubation time for activity [15] and Lukasheva and Berezov [16] observed decrease in the activity of L-lysine alpha-oxidase during 10-20 min, and then the activity remained virtually constant.

Kusakab *et al.* [2] reported that L-lysine alpha-oxidase produced by *Trichoderma viride* Y-244-2 showed maximum reactivity in the pH range 7.4 to 9.2. The maximum enzyme activity was observed at pH 7.8–8.2. Investigation of the pH dependence of the activity of L-lysine alpha-oxidase from *Trichoderma harzianam* Rifai using the polarographic method revealed a wide plateau in the range of pH from 4.5 to 10.5.

We demonstrated that optimal temperature of L-lysine alpha-oxidase activity ranged from 30 to 50 °C. These results were similar to those described by Makrushin *et al.* [29] who showed that the enzyme retained its activity at temperature up to 50 °C. The L-lysine alpha-oxidase produced by *Trichoderma viride* Y244-2 was assayed at various temperatures [16]. L-lysine alpha-oxidase from *Trichoderma harzianam* Rifai exhibited a relatively high stability as reported by [2]; after 3 h of incubation at 60 °C, the enzyme retained more than 60% of its activity.

Furthermore, effect of different substrates on enzyme activity was tested. We observed the maximum activity using lysine. L-lysine alpha-oxidase activity increased with increasing lysine (inducer substrate for this enzyme) concentration; 160 mM of L-lysine and then the activity remained virtually constant at 180 and 200 mM of L-lysine with Km and *Vmax* values 3.33 mM and 277.5 IU, respectively. [15] showed a variety of substrates subjected to the action of the purified enzyme produced by *Trichoderma* Y244-2-90. Makrushin *et al*. [29] reported that L-lysine is the main substrate of L-lysine alpha-oxidase with 100% activity.

Studying the effect of different metallic salts and various compounds compared to the control revealed that nickel and potassium achieved increase in the L-lysine alpha-oxidase. Kusakabe *et al*. [15] found that the enzyme was inhibited by copper ions, mercuric chloride. The activator has not been found. The enzyme was stabilized by sodium chloride, potassium chloride, and phosphates. Lukasheva and Berezov [16]reported that Mn^{2+} and Co^{2+} increased the rate of L-lysine oxidation. L-lysine alpha-oxidase obtained in this thesis from *T. harzianum* Rifai AUMC No.848 was not belonging to the metalloenzymes as EDTA at different concentration. These results were similar to those obtained by [2] demonstrated that EDTA did not significantly change L-lysine alpha-oxidase activity.

The spectrum of L-lysine alpha-oxidase produced by *T. harzianum* Rifai AUMC No.848 was typical having maximum at 278, 390, and 465 nm. These results agree with results of [28] and [29]. While [2] observed the maximums spectrum at 278, 390, and 465nm.

The cytotoxic effect of L-lysine alpha-oxidase on human colon carcinoma cell line (Caco-2), human epithelial type 2 (HEp-2) cells, and hepatoma cell line HepG2 was evaluated by MTT colorimetric assay. The enzyme was more toxic and selective to cancer cells than normal cells; thus considered a potential anticancer drug. These results match those of [16] who showed that the viability of leukemia L5178Y cells was decreased when treated by L-lysine alpha-oxidase. Also, the enzyme suppressed effectively the cell growth of MM1 cell line [31]. Cytotoxicity of L-lysine alpha-oxidase on HepG2 by inhibiting cell viability in a dose 38.82 µg/ml after 24 hours of incubation was reported by [32].

The apoptotic effect of L-lysine alpha oxidase was confirmed by detecting morphological changes in Caco-2, HEp-2, HepG2, and fibroblasts, nuclear staining by propidium iodide, ethidium bromide and acridine orange dye, assaying cell-cycle phase by flow cytometry and DNA fragmentation. Guo *et al*. [32] reported that LOX exhibited anti-tumor activity without interrupting HepG2 cell cycle. They showed that L-lysine alpha-oxidase caused the cytoplasmic vacuolation, shrinkage, detachment from culture matrix and death of HepG2. HepG2 cells were incubated for 1 h, treated with LOX for 24 h and stained Hoechst 33258. Nuclear condensation and/or fragmentation confirmed cell apoptosis.

Conclusion

L-lysine alpha-oxidase obtained in this thesis from *T. harzianum* Rifai AUMC No.848 was not belonging to the metalloenzymes. The enzyme was more toxic and selective to cancer cells than normal cells.

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