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Zinc Oxide Nanoparticles-Glutaminase Conjugates As Anti- A-549 Cells (Lung Carcinoma)

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Abstract

Zinc oxide nanoparticles were produced using green synthesis method by *Aspegillus niger*, F1a. The nanoparticles were purified and their characteristics were analyzed using different techniques *viz*. UV–visible spectrophotometry, FTIR and TEM. The morphology, structure and the dimensions of zinc nanoparticles were recorded as spherical shaped particles ranging in size from 6.35-10.5nm. Glutamines enzyme was produced biologically by the same strain used for nanoparticles production. The enzyme was purified up to 1.33 fold and 7.31% recovery. Mixture of gltaminase and zinc nanoparticles (protein corona PC) was prepared and tested on A-549 cells (Lung carcinoma). Cytotoxicity measurements have shown increasing percentage of cancer cells inhibition from 73.17 % and 88.04% by zinc nanoparticles and glutaminase enzyme, respectively independently up to 97.06% by mixture of both zinc nanoparticles and glutaminase was approved. The interaction of this protein corona was studied using EDX, NMR and TEM.

Key words: Glutaminase enzyme; zinc oxide; nanoparticles; protein corona and lung carcinoma.

.Introduction

The potential of nanoparticles, specially, zinc oxide nanoparticles as cures for cancer has been established in much research due to their unique physical and chemical properties that introduced them as efficient tool in different applications [1-8]. Gutaminase enzyme was approved individually as promising drug for cancer cells growth retardation by a hydrolysis reaction involving breakdown of addicted cancer cells component (glutamine) into glutamic acid and ammonia [9-12]. Enzymes are famous with their potential therapeutic activity, accordingly; maintenance and protection of the enzymes with the aid of nanoparticles considered a point of great interest [13&14]. There are several methods for establishing enzyme-nano association either by enzyme immobilization onto nano-based support as this method prevents contamination with other compounds, increasing the stability and half life time of the enzymes but also, may led to the interaction of nano-enzyme with other existing enzymes [15&16); or by covalent/non-covalent attachment on modified matrices. The later method may cause half-life increasing of the enzymes used, less degradability during the specific reactions and changing their diffusing and kinetic parameters [17]. Also, protein may interact with nanoparticle through its free amine groups or cysteine residues or by the electrostatic attraction of negatively charged carboxylate groups, specifically in enzymes with retaining their biocatalytic activity in the bioconjugate material [18-20].

Using microorganisms as small biological machinery facilitated the production process for nanoparticles and glutaminase enzyme as well. Maximizing the productivity and avoiding high temperature and pressure needed by most other chemical and physical methods were started signals

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[21]. Lung cancer has been placed at the top degree for causing mortality in humans. According to the newest cancer statics of world health organization (WHO) in 2020, the most common worldwide causes of cancer death in 2020 was attributed to lung cancer of about 1.80 million deaths. The trend of producing protein interacting with nanoparticles (protein corona) whether if they were bind to each other or just exhibiting adsorption reaction gave new spots on new therapy. The possible medical applications using protein corona as biological cure need to be extensively studied. It is clearly that, this research aimed to the production of microbial vital cure for lung cancer with simple and effective strategy. The nano-glutaminase represented a promising therapy for such purpose.

Materials and Methods

Preparation of zinc nanoparticles

In 500ml Erlenmeyer flask, the following medium was prepared as (g/l) K2HPO4, 2.0; (NH4)2SO4, 1.0; Yeast extract, 0.6; MgSO4.7H2O, 0.1; KH2PO4, 7.0; and glucose, 10.0. The pH was adjusted at 7. Inoculation with 2.48x106spores/ml of Aspergillus niger, F1a (where; F corresponded to fungi, a to acido) was done with subsequent incubation at 150 rpm and 33°C for 7 days. Cell free filtrate was gained followed by addition of 5 mM Zinc nitrate while keeping in a shaking incubator at 150 rpm and 33 °C for 2 days. The filtrate was centrifuged at 14,000 rpm for 10 min at 4oC. Washing with isopropanol was done followed by recentrifugation at the same conditions. White precipitate of zinc nanoparticles was gained [22].

Characterization of zinc nanoparticles

The presence of zinc nanoparticles was monitored across wavelength range 200-700nm using PerkinElmer Lambda 45 UV/Vis Spectrophotometers. The examination of morphology and the dimensions of zinc nanoparticles was observed using XRD, FTIR and transmission electron microscopy technique ((JEOL JEM 2100 (200 kev – 0.143 resolution)).

Preparation of glutaminase enzyme

The medium used for the production was composed of (g/l) as NaCl 10; K2HPO4, 10; MgSO4.7H2O, 10; L-glutamine, 20 with the addition of MnSO4.H2O, 100 ppm and 3.59% isoleucine. The pH was kept at 1.8 followed by inoculation with 2.48x106spores/ml of Aspergillus niger, F1a. The previous component concentrations and the strain were obtained from previous studies on glutaminase production. Incubation was at 35oC for 7days [23].

Purification of glutaminase enzyme

Preparation of cell free filtrate was prepared followed by ammonium sulphate precipitation at 80% at which the enzyme exhibited the highest specific activity. The precipitated protein was undergo dialysis against phosphate buffer pH8 0.1M. The buffer was changed every 2-3hours for 24 hours then, the crude glutaminase was concentrated against sugar crystals. The concentrates were re-suspended in minimal volume of the same buffer to be applied on the sephadex G-200 column of 1.8X30 cm.

Preparation and examination of glutaminase enzyme- zinc nano mixture

A total volume of 2ml mixture was prepared. At 4oC, weight of 12mg zinc nanoparticles were resuspended in 1ml of distilled H2O. Then, 1ml of purified glutaminase enzyme with concentration 4.76mg/ml was added and the mixture was kept at the same temperature for analysis. The nano-glutaminse mixture was examined using EDX and ((JEOL JEM 2100 (200 kev – 0.143 resolution)) transmission electron microscopy technique (TEM).

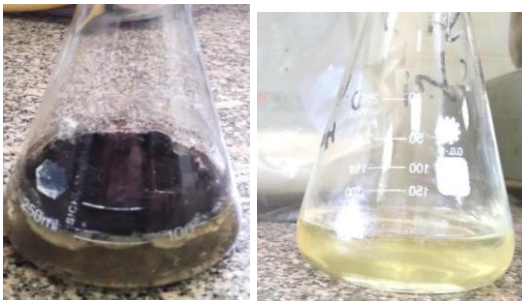
Application of nano-glutaminase mixture as anticancer agent

The test was carried out in the Regional Center for Mycology & Biotechnology; Al-Azhar University. Cell line propagation and cytotoxicity evaluation were carried out according to [24 & 25]. For cytotoxicity

assay, the cells were seeded in 96-well plate at a cell concentration of 1×104 cells per well in 100µl of growth medium. Fresh medium containing Different concentrations of the mixture were made using two-fold serial dilutions method. Each dilution was added after 24 h of seeding, separately. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells at 37°C, for 24 h, the viable cells yield was determined by a colorimetric method. The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The previous mentioned protocol was made also for two samples represented the zinc nanoparticles and purified glutaminase, separately as lines for comparison and determination of their effect on A-459 lung cell line.

Results and discussions

The first sign for zinc nanoparticles production by Aspergillus niger, F1a was changing the color of the specific growth medium into pale yellow (Fig.1) and appearance of white precipitate after the purification steps. The examination of produced zinc nanoparticles was primarily confirmed using PerkinElmer Lambda 45 UV/Vis Spectrophotometers across the studied range from 200-700nm, a peak was noticed clearly at 328 nm (fig. 2) which was in agreement with Umamaheswari et al. [26] as a characteristic absorption peak of ZNO nanoparticles from leaf extracts of Raphanus sativus var. Longipinnatus was observed at 369 nm. Also, Kalpana et al. [22] recorded zinc oxide nanoparticles production from A. niger with observed clear absorption peak at 320nm, similar to [27], the examined UV spectrum range of ZnO from flower extract of Nyctanthes arbor-tristis was 320–390 nm showing an absorption peak at 369 nm. moreover, Santhoshkumar et al. [28] approved absorption peak of ZnO nanoparticles from Passiflora caerulea fresh leaf extract at 380 nm across the examined UV spectrum) wavelength range of 300–500 nm.



(a) (b)

Fig.1. Zinc nanoparticles production showing dark color of the specific growth medium for Aspegillus niger, F1a at (a), while in (b) the color become pale yellow following addition of Zinc nitrate and incubation due to zinc nanoparticles production.

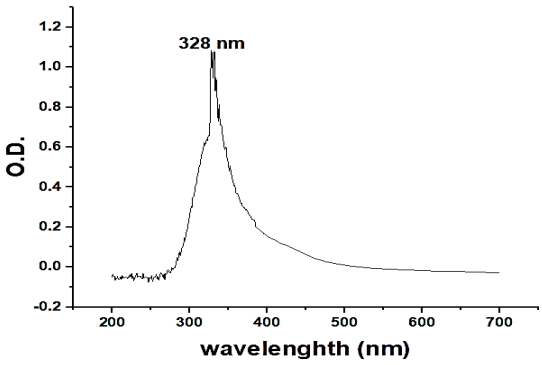


Fig.2. UV spectrum of zinc nanoparticles from Aspegillus niger, F1a

FT-IR spectroscopy spectrum resulted in various peaks at 3442.81, 2083.55, 1634.88, 1080.66, 1044.65, 545.76 and 486.73. The broad peak at 3442.81 correspond to H bonded OH stretch. Peak at 1634.88 results from the stretching bands of C=O functional groups (Fig.3).

In this study, FTIR spectrum showed similar

pattern as that by Kalpana et al. [22] study which revealed that, the strong aromatic ring and carboxylic acid appearance in FTIR band is responsible for ZnO NPs synthesis. Moreover, absorption peak was appeared at 3199 corresponding to O–H stretch and 1587 correspond to C-C stretch. While, Kavitha et al. [29] reported the O–H stretching vibration at the peak of 3334 cm−1and –O axial stretching band appears at 1656 cm−1. Another study has showed the peak at 1637 cm−1 is due to C=C aromatic stretching [30]. Also, The FT-IR spectrum of CA-ZnONPs produced from Cassia auriculata flowers showed broad OH hydrogen bond at 3441along with Zn-O stretching band monitored at 472 cm-1 [31].

Glutaminase enzyme was produced by Aspegillus niger, F1a and purified up to 1.33 fold and 7.31% recovery. The purification protocol led to the appearance of two fractions with the highest specific activities (Fig.4 & Table 1). In previous studies, purification pattern of Aspergillus niger, F1a glutaminase using G-100 sephadex column led to increasing glutaminase purity with 3.88 fold and recovery 6.28% [23]. In another study, El-Gendy et al. [32] reported purification of the glutaminase from crude extract of Aspergillus sp. ALAA-2000 with purification fold 36.72 with yields 37.42 %. Moreover, an intracellular L-glutaminase from Penicillium brevicompactum NRC 829 was purified with heat treatment at 50oC for 20 min followed by gel filtration on sephadex G-100 and G-200 columns to homogeneity 162.75 fold [33].

Different concentrations of purified glutaminase enzyme (0-4.8 mg//ml) was tested on A-549 cells. The results indicated remarkable anticancer activity of glutaminase enzyme with IC50=1.57mg/ml. this was compatible with several studies in which gutaminase enzyme has been tested on different cancer cell lines and showed remarkable signs [11&34].

In this concern, Dutt et al. [35] studied the effect of different concentrations of glutaminase produced from Aspergillus oryzae S2 using MTT assay and showed that, glutaminase had toxic effect on MCF7 cells with IC50 was 283.288 µg/ml. Also, the anticancer activity of purified glutamianse from Streptomyces canarius FR (KC460654) was determined against five types of human cancer cell lines Hep-G2 cell, HeLa cells, HCT-116 cell, RAW 264.7 (macrophage-like, Abelson leukemia virus transformed) and MCF-7 cells using MTT assay with inhibition expressed as IC50 (6.8, 8.3, 64.7 and 59.3 µg/ml); the MCF-7 growth was not affected [36].

In addition, different concentrations of the purified glumainases from Aspergillus niger, F1a was tested on three different cells lines (human hepatocellular carcinoma (HepG-2), human breast cancer cell line (MCF-7) and colon carcinoma (HCT-116)) with IC50of 3.9, 6.15 and 4.08µg/ml, respectively [23].

Conformational studies of zinc oxide nanoparticles-glutaminase mixture were performed using NMR 1H analysis (Fig.5).

The spectrum showed peaks at 1.0266, 1.0342, 1.0418, 1.0517 and 1.0692 corresponding to H of methyl group –CH3 and methylene groups -CH2. Peaks at 3.4129, 3.4304, 3.4479 and 3.4654 were corresponding to different H of–CH. Peak at 4.5436 corresponded to H2 of amino group –NH2.

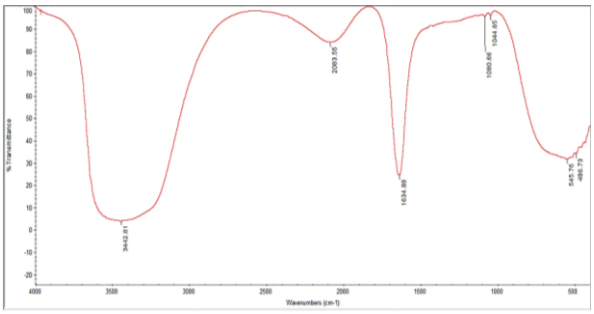


Fig.3. FTIR spectrum of purified zinc oxide nanoparticles

Table (1): purification patteren of glutaminase enzyme from Aspegillus niger, F1a

Amm. Sulfate concen. (%)	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Fold	Recovery (%)
Crude extract	3227	86.51	37.30	1	100
$(NH_4)_2SO_4$ precipitation	2775	65.22	42.55	1.14	85.99
Sphadex G-200	235.50	4.755	49.53	1.33	7.31

In addition, the EDAX analysis of synthesized protein corona, revealed that, the following chemical elements were found including C, O, N, P, K, S and Zn with percentages 29.76%, 41.39%, and 24.57, 1.33, 2.75, 0.07 and 0.13% respectively (Fig.6).

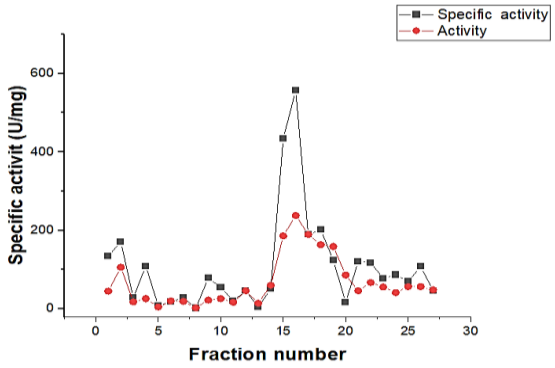


Fig.4. Purification pattern of glutaminase enzyme by Aspegillus niger, F1a using Sephadex G-200 and elution with phosohate buffer 0.1M, pH

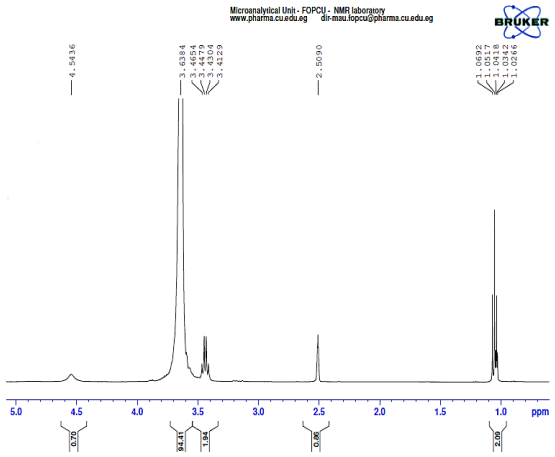


Fig.5. 1H NMR spectroscopy spectrum of protein corona from Aspegillus niger, F1a

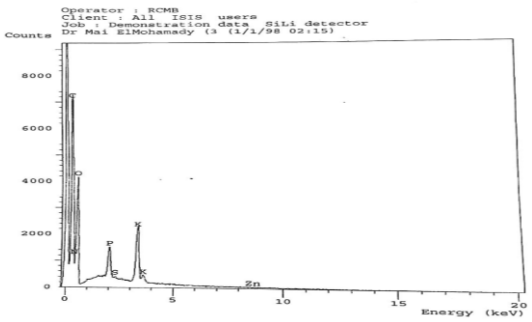
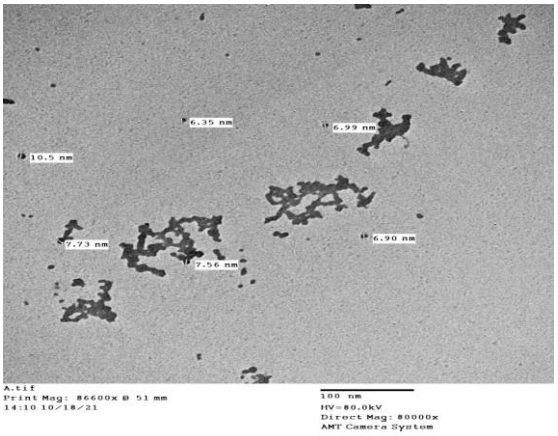
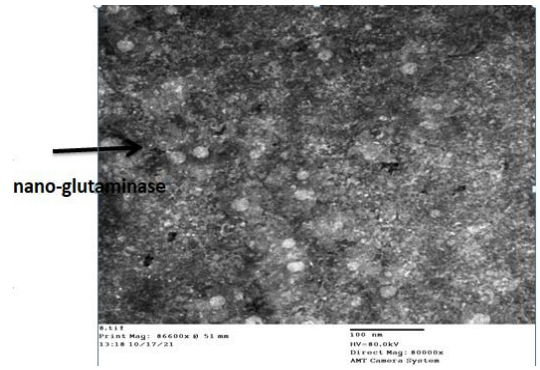


Fig. 6. EDX analysis of zinc oxide nanoparticles-glutaminase mixture



(a)



(b)

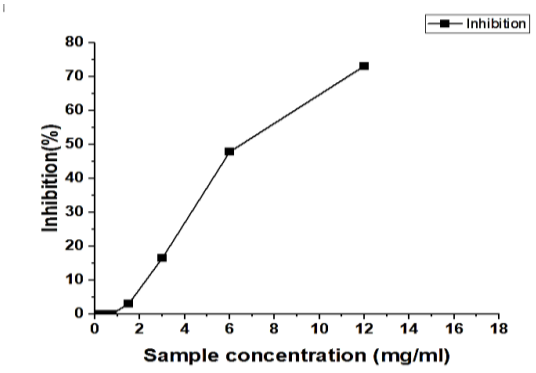
Fig.7. TEM images of a) zinc nanoparticles indicated the size and their spherical shape; b) protein coronan of zinc nanoparticles binded to glutaminase of Aspegillus niger, F1a

The produced zinc nanoparticles form *Aspegillus niger*, F1a appeared as spheres with size range from 6.35-10.5nm (Fig. 7) which may be the reason of its ability to interact with glutaminase enzyme molecules efficiently as the nanoparticles possesses unique properties owing to their small size and large surface to volume ratio which facilated their interaction with biological molecules such as proteins with specific interest enzymes [37]. In addition, protein–nanoparticle interactions are produced due to the presence of free amine groups or cysteine residues. Also, it may be initiated through the electrostatic attraction of negatively charged carboxylate groups, specifically in enzymes [18].

zinc oxide nanoparticles (ZnO NPs) were selected because it have unique physical and chemical properties including the ability of radiation adsorption, large excitation binding energy, higher stability, wide band gap (3.37 eV), electrochemical coupling coefficient, n-type semiconductivity, high rate of chemical reaction in presence of catalyst, inexpensive luminescent material and non-toxic [38]. The possible mechanisms for nanoparticles being act as cancer cells inhibitors may be due causing DNA damage pathways, paraptosis, autophagy, radiosensitising, overcoming chemoresist-ance, inhibition of carcinogen-activation of P450 enzymes (phase Imetabolism) or induction of carcinogen-detoxifying enzymes(phase II metabolism) or oxidative stress modulation or induction of apoptosis or arresting of the cancer cell cycle [39 & 40].

The nanoprotein interaction or as it named protein corona (PC) led to enhancement of the bioreactivity of both nanoparticles and glutaminase enzyme against the tested A-549 cancer cell line (Fig8&9) indicated by increasing percentage of cancer cells inhibition from 73.17 % and 88.04% by zinc nanoparticles and glutaminase enzyme, respectively independently up to 97.06% by mixture of both nano and glutaminase with IC50 of 6.3mg/ml, 1.57mg/ml and 0.91+ 0.36 (mg/ml) of mixture of zinc nanoparticles & glutaminase, respectively.

In comparison to other studies, A549 Cell lines were treated with different concentrations of ZnO NPs from leaf extracts of Raphanus sativus var. Longipinnatus (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg/ ml) for 48 h and compared with DMEM as control by the MTT assay. The IC50 was found to be 40µg/ ml [9], While IC50 was 116.9 µg/ ml on A-549 cells from leaf extract of Annona muricata as reported by [41]. In addition, Nilavukkarasi et al. [42] synthesized ZnO NPs using the leaf extract of Capparis zeylanic, their cytotoxic results indicated that, higher concentrations (500 and 250mg/ml) of ZnO nanoparticles has led to decreases in cell viability of A549 cancer cells to 22% and 38% respectively.



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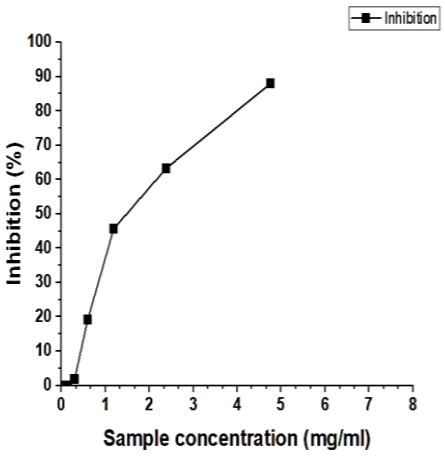

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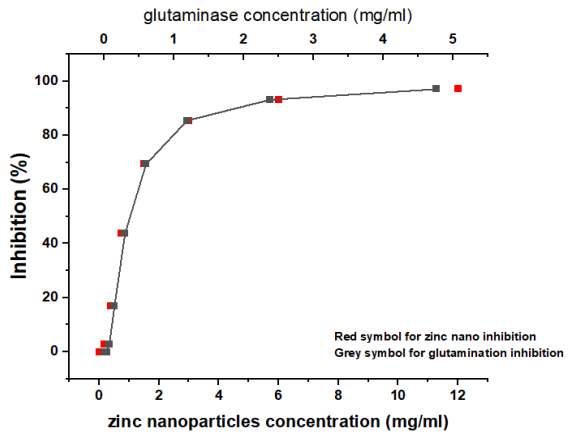
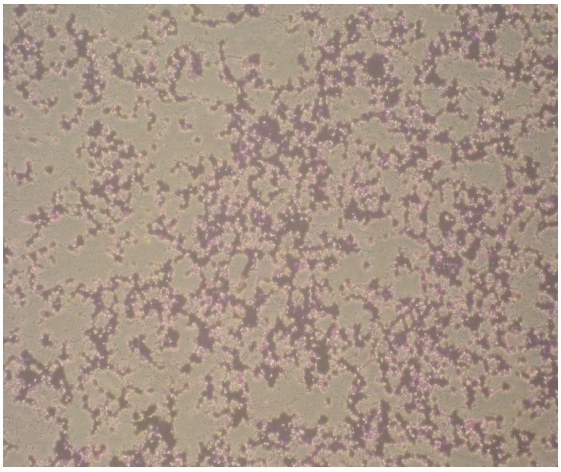


(c)

Fig.8. Cytotoxicity of different concentration of Aspergillus niger, F1a purified a) zinc nanoparticles; b)glutaminase enzyme; c) zinc nanoparticles-glutaminase mixture against A-549 lung carcinoma.



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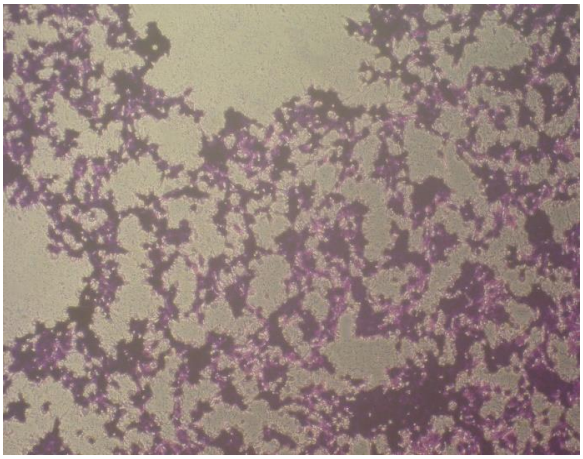

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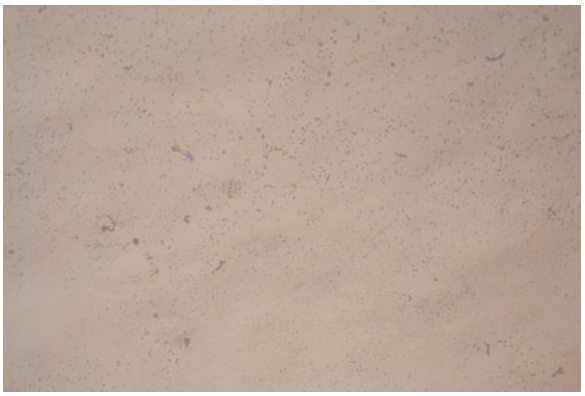

(c)

Fig.9. Cytotoxicity of different concentration of Aspergillus niger, F1a purified a) zinc nanoparticles; b)glutaminase enzyme; c) zinc nanoparticles-glutaminase mixture against A-549 lung carcinoma.

Conclusion

In this present investigation, ZnO nanoparticles were synthesized biologically using acido stable Aspegillus niger, F1a strain efficiently along with glutaminase production. A mixture of both zinc oxide nanoparticles and glutaminase enzyme represented suitable enhancing suppression tool against lung carcinoma.

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