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Zinc-oxide Nanoparticles Biosynthesis from *RHODOTORULA GLUTINIS* and test its Anticancer cytotoxicity

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Abstract

This study focuses on the extracellular green synthesis of zinc oxide nanoparticles (ZnO NPs) using cell filtrate from yeast Rhodotorula glutinis and investigate the anticancer activity of these nanoparticles and compare it with the cytotoxicity effect of its biomass extraction on a breast cancer cell line. The optical properties of the nanoparticles were analyzed using UV—vis spectroscopy which showed a distinct absorption peak at (375.50) nm, while Fourier transform infrared spectroscopy (FT-IR) was proofed presence of characteristic functional groups belongs to ZnO, C=C, C=O, C-H and OH. The data obtained from the MTT assay indicate that the ZnO NPs exhibit high toxicity and significantly decrease cell viability in the breast cancer cell line compared to the biomass extraction and untreated cell line.

Key word: *MTT assay, Nanoparticles, anticancer, Zinc oxide, fungi*

Introduction

Nanotechnology has gained popularity in recent years because to its significant influence on several industries such as electronics, energy, space, medicines, biomedical, environment, and agriculture. This technique depends on synthesis and manipulation of micron-sized particles, which are minuscule particles measuring between 0.1 and 100 nm. These particles have several advantageous characteristics, including similar strength (such as resistance to being crushed), unique catalytic properties on their surfaces, a high ratio of surface area to volume, distinct energy levels, and the ability to modify their electronic properties (Iravani, 2014).

Nanoparticles may be observed throughout various biological organisms, including plants, microbes, beetle parts, and bones, they may also manifest in artificial forms, including grout, cement, gemstones, and several other things (Balakumaran, 2016). They are now utilized in several domains, including medical imaging, cancer therapy, genetic therapy, antibacterial drugs, biosensors, and pharmacological drivers for specific administration, Nano pesticides, and delivering DNA in agricultural products. (Moghaddam et al., 2015). Medical nanotechnology

seeks to amalgamate modern nanotechnologies with biotech as well as various biological instruments to fight diseases and repair tissue damage, therefore enhancing human health. (Calabi et al., 2010).

Fungi provide a crucial biological resource for nanoparticle formation owing to their beneficial capacity to generate extracellular enzymes that decrease metal ions and promote nano production. (Muhsin & Hachim, 2014), Furthermore, fungi secrete proteins and secondary metabolites extracellularly, leading to a reduction in metal ions and the rapid synthesis of nanoparticles, extracellular myco synthesis is an efficient technique for the production of nanoparticles. (Gade et al., 2008).

Yeasts are considered a good reservoir of nanoparticles because they are inexpensive, exhibit rapid development, and may be easily prepared and handled (Salvadori et al., 2019). *Rhodotorula glutinis* is a lipid-rich red yeast that synthesizes a range of valuable substances, such as microbial lipids, pigments, and enzymes. *R. glutinis* has a lipid content nearly 70% in its D. W. biomass. No toxicity included and can be easily cultivated and harvested. Due to its ability to accumulate polyunsaturated fatty acid triacylglycerol, similar to vegetable oils, it considered a suitable host for the biodiesel manufacturing. *R. glutinis* synthesizes β-carotene, a valuable compound known for its anti-carcinogenic and antioxidant properties (Pi et al., 2018).

These yeasts have the ability to generate a diverse array of commercially useful substances, such as lipids, carotenoids, and enzymes. An obvious advantage of these organisms is their capacity to thrive and generate metabolites when exposed to substrates that consist of diverse industrial waste products. This greatly enhances the economic feasibility of biotechnological procedures. *Rhodotorula* yeasts are quite prevalent in the environment. They are isolated from the surrounding environment, including air, soil, grass, lakes, oceans, food (such as milk and fruit juices), human skin, and excrement (Kot et al., 2016).

Chemotherapy, surgery, and radiation are effective treatments for several types of cancers. Chemotherapy, while effective in targeting cancer cells, also has a significant harmful effect on normal cells. Therefore, there is a pressing need to develop a controlled drug release system that can selectively destroy cancer cells without harming normal cells (Gu et al., 2002). The field of nanomedicine has great promise for the cure and early diagnosis of cancer (Maeng et al., 2010).

Zinc oxide (ZnO) is a significant nanoparticle material classified within the multifunctional inorganic nanoparticles category and offers a diverse range of uses. Nanoparticles are extensively utilized across several fields, notably physics, chemistry, and biology (Dulta et al., 2021). ZnO nanoparticles are extensively utilized for the purpose of eradicating cancer cells through their extraordinary physical and chemical features. In addition, ZnO exhibit a wide-ranging antibacterial activity against microorganisms that are often seen in clinical settings. Moreover, they demonstrate a significant antibacterial impact when applied to wound dressings of individuals with diabetes (Budime & Poklar, 2018).

Medication delivery and cancer therapy are the primary and most impactful applications of nanoparticles in the field of medicine. Nanoparticles are employed for targeted medicine delivery. Administering the recommended therapeutic dosage decreases the occurrence of adverse effects,

hence leading to a reduction in costs and suffering (Drbohlavova et al., 2013). Nanoparticles possess unique characteristics as a result of their very large surface area in proportion to their size (Tolaymat et al., 2010).

Current study aimed to investigate the cytotoxicity of yeast *Rhodotorula glutinis* ZnO Nanoparticles in cell line breast cancer and compare it with biomass extraction of the same microorganism.

Material and methods

1- Collection of specimens

Various clinical specimens were procured from individuals afflicted with diverse ailments, including diabetes and tooth disease, at the dentistry facility in Diwaniya Hospital (Iraq). Specimens were obtained from the oral cavity. Yeast Identification According to the physical traits of colonies and microscopic analysis mentioned in the study by (Hasan et al., 2023). In addition, we employed a biochemical test called HI Candida Identification to confirm the identified species (Sari et al., 2015).

2- Zinc oxide nanoparticles (ZnONPs) synthesized using *R.glutinis*

Zinc nanoparticles were synthesized using Yeast Peptone Dextrose (YPD) broth. Media consists of peptone 20.0 gr, dextrose 20 gr, yeast extract 10.0 gr, also a maximum of one liter of clean water. A concentration of 1 × 107 spores ml−1 of *Rhodotorula* sp. was introduced into a 250 mL Erlenmeyer flask comprising 50 mL of broth (YPD). The infected flask was incubated for 5 days at 28 ◦C, with continuous shaking at a speed of 150 rpm. The yeast cultures were isolated by passing them through a sterile Whatman Filter paper No. (1) and there after rinsing it many times with sterile bidistilled water. We suspended 10g of moist biomass in (50) milliliters of sterile bidistilled water and lefts in 30°C and a speed of (150) round per minute for 72 hours. Suspension of cells was obtained via filtering it using paper No. (1), and the deposits without any fungal presence were utilized for the biosynthesis of ZNPs. To produce ZnNPs, mix a $ZnNO_3$ 5 mM solution with an equivalent amount filtrate of fungi in water and let it for four days at 30°C. The bio-synthesizing parameters adjusted to optimize the releasing of NPs within a limited timeframe. The most effective form of $ZnNO_3$ were combined by the liquid produced thr the fungus and kept at a temperature 25°C for a duration of (24) hours. The formation of nanoparticles is evidenced by the presence of a white precipitate that settles at the bottom of the flask (Ammar et al., 2021).

3- Characterization of ZnONPs

Technique of Ultra violet (UV)– vis spectroscopy was employed to evaluate the optical characteristics of the nanoparticles. The UV-vis spectrum was obtained using a UV-Vis spectrophotometer with a wavelength range of (200-1100) nm. Determination of functional groups contained in the produced nanoparticles conducted by analysis. The FTIR spectrophotometer was utilized to analyze the interaction between ZNPs and biomolecules Subsequently, the material was dried and subjected to FTIR analysis. The band widths are measured within the range of 450 to 4000 cm -1as summarized by Umoren et al., (2014).

4- Cultivate cell lines for cytotoxicity assays

In order to carry out the experiment according to the original concept, Rawafed Scientific Research Company in iraq developed the MCF7 cell line, specifically designed for studying breast cancer. The process of preservation using liquid nitrogen was developed, the culture medium and (10%) fetal bovine serum (FBS) were incubated in a (5%) carbon dioxide incubator at (37) °C for (24) hours, as described by (Castro-Garza et al., 2007). The Hella breast cancer cell line (MCF7) was subjected to 3- (4, 5- dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to assess the cytotoxic effects of *R. glutinis* ZnONPs and extraction. Photo (1) each well of the 96-well plates contains 200µl of full culture media to nourish the cancer cells. The cells are then cultivated for (24) hours and seen under an inverted microscope to confirm their development and the formation of a cell layer that covers 85-90% of the well. The wells were exposed to a series of diluted solutions containing *R. glutinis* ZnONPs and extraction at concentrations (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL). Following a one-day period, the Elisa reader assessed the data by measuring the optical density using the crystal violet cytotoxicity assay. Data were going compared with an untreated cell line of (MCF7) also a negative control of dimethyl sulphoxide (DMSO) (Chaing et al., 2003).

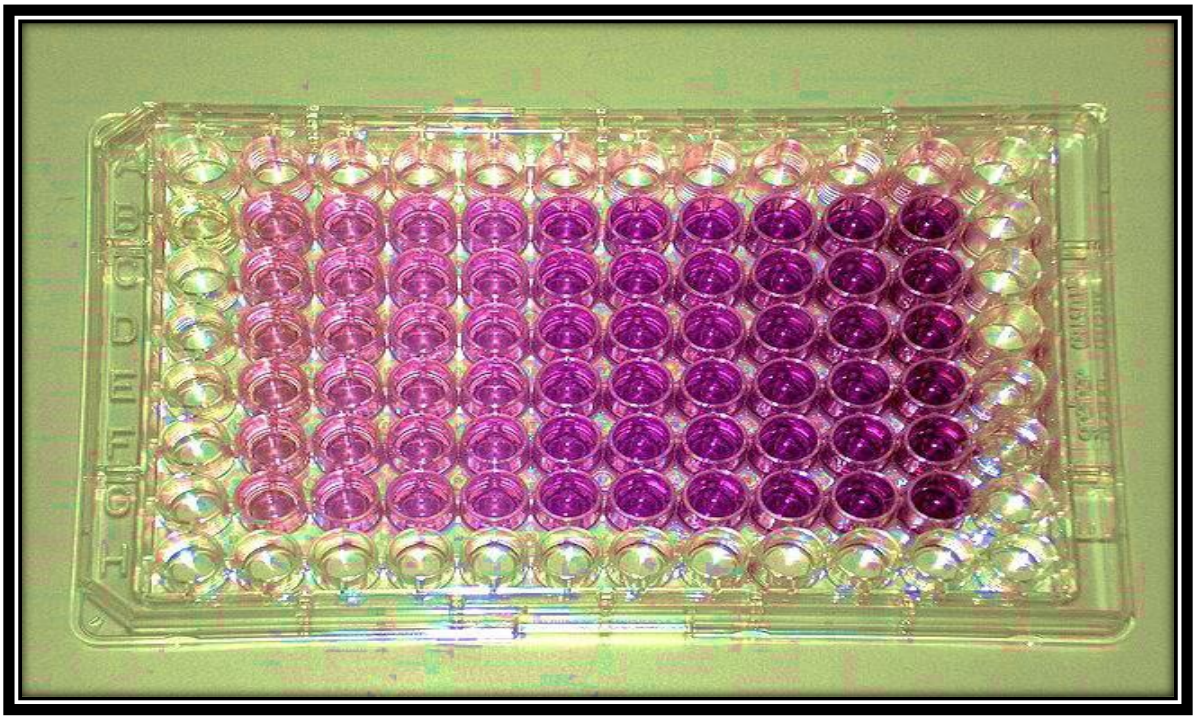
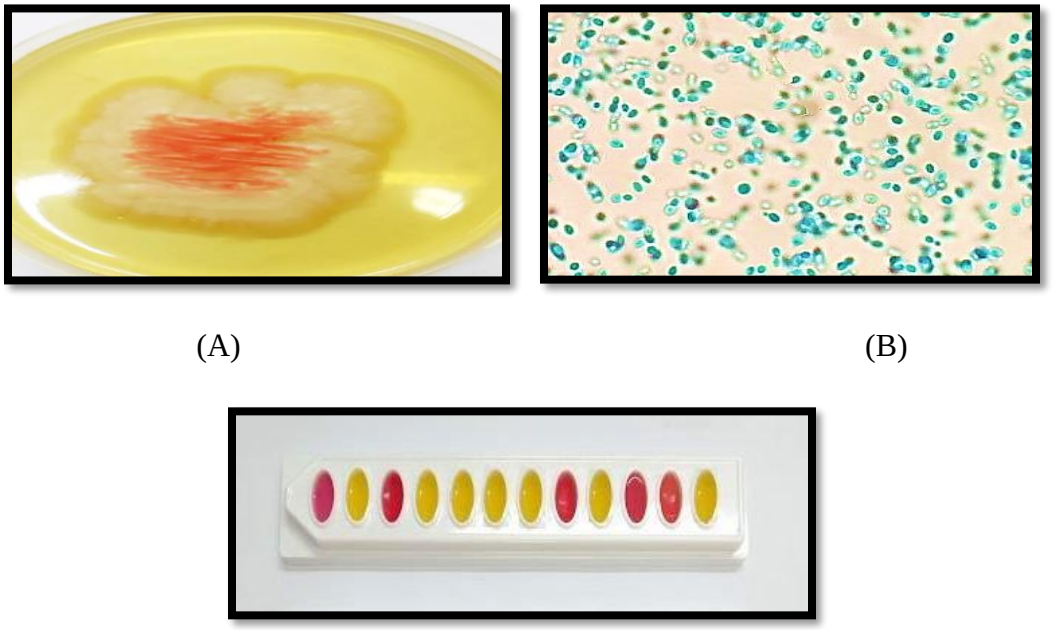


Photo (1): 96 well plate of MTT assay.

Results and Discussions

Fungal identification

R. glutinis colonies on SDA were coral pink, smooth, and moist to mucoid yeast. The colonies are often pink, crimson, or reddish-orange. Because capsules are present, there is a risk that colonies will become mucoid. Microscopy revealed spherical to elongated budding yeast-like cells called blastoconidia (Hoog et al., 2005). Based on its description and biochemical tests, this isolate was identified as *Rhodotorula glutinis*. The results of this test demonstrated the ability of *Rhodotorula* species to secret Urease enzyme and utilize carbohydrate fermentation, the urease activity of *Rhodotorula glutinis* was verified to be positive, as indicated by the shift in color to pink/red in the corresponding well, demonstrating the breakdown of urea by hydrolysis. Furthermore, demonstrated good assimilation of maltose, sucrose, galactose, cellobiose, xylose, and trehalose among the studied carbohydrates. This assimilation was accompanied by a color shift to yellow, indicating the generation of acid. Nevertheless, the yeast failed to metabolize lactose or raffinose, as evidenced by the absence of a color change (still red). The direct assessment of melibiose, inositol, and dulcitol findings was not conducted, nevertheless, it is generally observed that these results are negative for *Rhodotorula glutinis*. as indicated in the table (1) and Figure (1) below.



(A) (B)

(C)

Fig. 1_ (A) *Rhodotorula glutinis* growth on SDA at 37°C for 4 days and (B) microscopic characteristic with rounded blastoconidia observed in a 40x magnification.
(C) HiCandida identification kit

Table 1. Tests of HiCandida identification kit for biochemical characterization of yeast identification

No.	Substrate	Fermentation Reaction	Color Change
1	**Urease**	Positive	Pink/Red
2	**Melibiose**	Not mentioned (Likely Negative)	No Change/Red
3	**Lactose**	Negative	No Change/Red
4	**Maltose**	Positive	Yellow
5	**Sucrose**	Positive	Yellow
6	**Galactose**	Positive	Yellow
7	**Cellobiose**	Positive	Yellow
8	**Inositol**	Not mentioned (Likely Negative)	No Change/Red
9	**Xylose**	Positive	Yellow
10	**Dulcitol**	Not mentioned (Likely Negative)	No Change/Red
11	**Raffinose**	Negative	No Change/Red
12	**Trehalose**	Positive	Yellow

The findings of this test align with the results reported by Hedayati et al., (2015), demonstrating that the HiCandida identification kit accurately identifies *Rhodotorula* species. This is consistent with the findings of who also confirmed the effectiveness of this kit for diagnostic purposes (Singh et al., 2007).

Utilizing *R. glutinis* for the Eco-friendly production of ZnONPs through green synthesis.

The use of optical measuring techniques detected color changes in fungal metabolites, indicating the presence of ZnNPs. The stimulation of surface plasmon resonance zinc ions is responsible for this phenomenon (Raj et al., 2015). The optical parameters of these nanoparticles were determined by analyzing them using a UV-VIS spectrophotometer. The presence of a distinct absorption peak at (261.50) nm is unique to ZnO nanoparticles, whereas the wide peak observed at (375.50) nm indicates absorption spectra of ZnONPs. This result in line with ZnONPs, as mentioned in a recent study by (Al-Dhabi & Arasu, 2018) who found the absorbtion in (274)nm peak.**(Figure 2).**

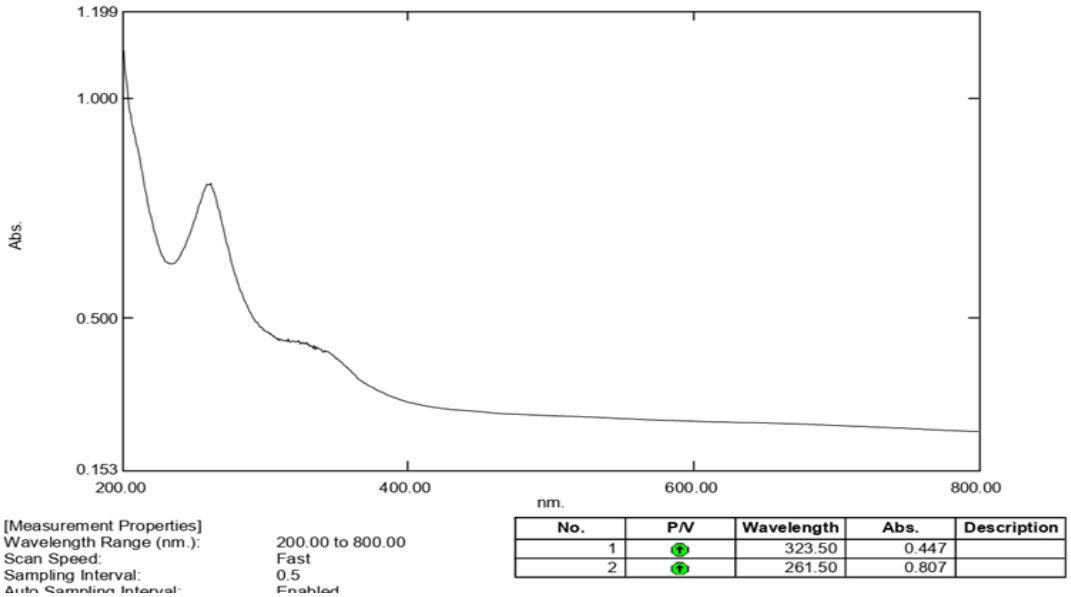


Fig_ 2. Analysis of zinc oxide nanoparticles generated by mycosynthesis using a UV-Vis spectrophotometer.

A separate investigation has verified the decrease in metal ions and the creation of nanoparticles, which have a peak at (350) nm (Sangeetha G, 2012). An benefit of utilizing yeast cells as NPs carriers is that a straightforward encapsulation procedure may be accomplished by employing simply yeast cells, water, and chemicals, hence reducing the necessity for stabilizers (Klis et al., 2006).

FT -IR Spectroscopy was employed to quantify the engagement of IR light, clearly seen in Figure (3). The zinc nanoparticles that were created were examined using FT-IR in order to detect the several unique functional groups that are linked to them. The peaks correspond to the unique functional groups contained in the synthesized zinc oxide nanoparticles. Figure (3) displays the absorption peaks of the samples, which are seen at wavelengths of (3411.91, 1651.74, 1614.91 ,1505.91, 1349.18, 1360.21,1143.82, 987.33, 789.09, 532.13, 439.92 cm-1). The absorption peak gotten at a wavenumber of (532.13) cm-1 can be attributed to the vibrational mode of metal-oxygen (ZnO stretching vibrations). The peak observed at (1651.74) cm-1 is credited to the stretching vibration of the N-H bond in a primary amine, as well as the C=C bonds stretch in an alkyl group and the amino group in an open chain. The peak located at 1614.91cm-1 is qualified to the extending of the C=O carbonyl bond, the stretch of C=C alkyl bond, also open chain amino group presence. The peak (1143.82) cm-1 is allied to stretch quivering the bond of C-N in aliphatic amine and in-plane aromatic bending of C-H. (3411.91) cm -1 is ascribed to the stretching vibration of the OH bond in alcohols, phenols, and aromatic primary amines. The inclusion of these functional components enhances the efficacy of produced zinc oxide nanoparticles as antibacterial agents (Rajan et al., 2016).

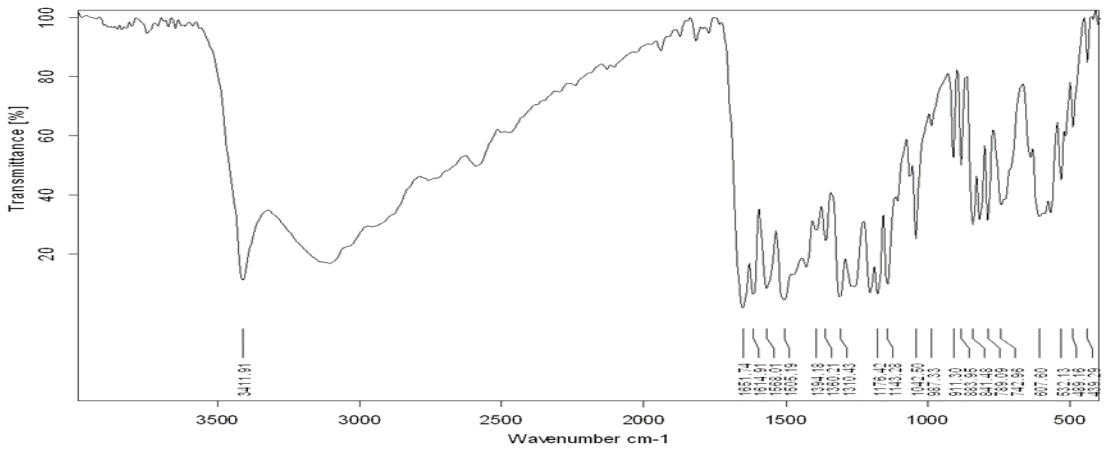


Fig_3. Displays the FTIR spectrum of ZnONPs, which were produced by *Rhodotulla glutiniis.* The spectrum exhibits clear and distinguishable peaks.

Cytotoxicity study

Zinc oxide nanoparticles (ZnONPs) produced by green synthesis have distinctive physiochemical characteristics, including biocompatibility, exceptional selectivity, significant cytotoxicity, and convenient production. Consequently, they hold great potential as a promising option for anticancer treatment. However, anti-cancer activity of green synthesized ZnONPs from *R.glutinis* was appraised in both cancer and normal cell line by MTT assay and the bachwash were illustrated in figure (4) there is high toxicity effect when we used ZnONps mediated by *Rhodotorula glutinis* with all different concentration that we used from (6.25 µg/mL) with cell viability (75.26%) compared with untreated MCF7 cancer cell line (99.5%). The cell viability decreased in other concentrations (12.5 µg/mL, 25 µg/mL, 50µg/mL, 100µg/mL)corresponding to cell viability (68.63%, 52.41%, 49.33%, 39.88%) respectively. *R. glutinis* extraction of (6.25 µg/mL) and compared with untreated (MCF7) cell line with cell viability (62.31%) and then the cell viability begins decrease with use other concentrations (12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL) while cell viability (52.63%, 38.67%, 31.05%, 29.53%) respectively. Our findings align with the research conducted by (Gao et al., 2019), who found that zinc oxide nanoparticles had a significant impact on cancer cells.

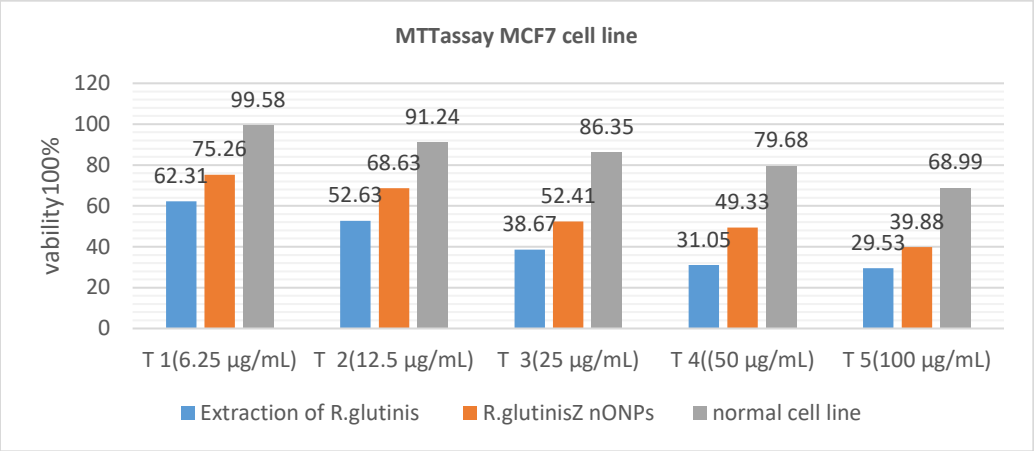


Fig_4. Cell viability percentage of MCF7 cell line with different concentration after incubation for 24 h.

Our results are consistent with the findings of (G et al., 2015), data evident strong anti-cancer activity of ZnO, when applied alone and combined with a nano-bio-composite, contra the cell line MCF- 7.

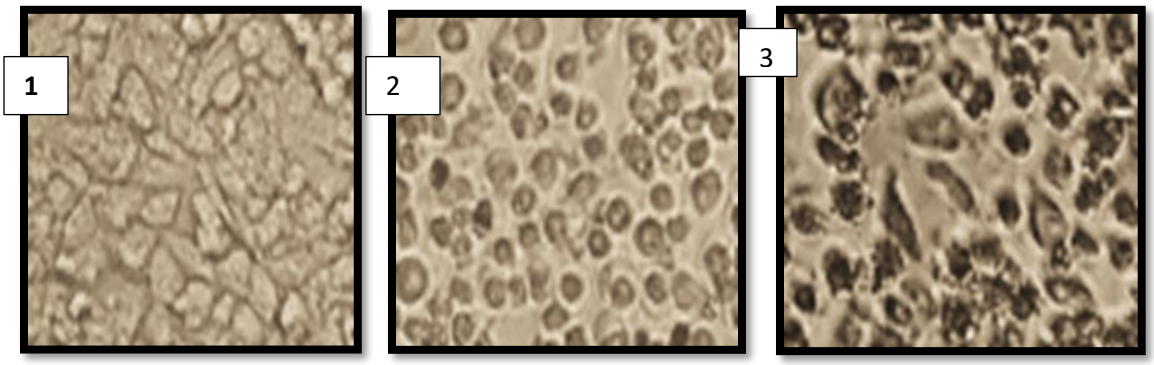
Present-day study supports the facts of (Hammad et al., 2023), that the toxic effective of bi-osynthesized ZnONPs on $CACO_2$ cells upsurges in a dosage-dependent manner. The doses used were (0.0, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50,100% µg/mL) of ZnONPs. The cell viability proportions decreased as follows: (100, 100, 100,100, 96.03, 87.24, 70.81, 54.12, 32.39, 21.4, 10.81, 6.53, and 2.74%) correspondingly.

Consistent with other studies, an initial observation suggested a notable decrease in cell viability,ZnONPs have farther efficacy towards HCT- 116 cell line of colon cancer compared to Au nanoparticles. This is attributed to ZnO NPs having a lower IC50 value than Au NPs. This finding has been corroborated by previous investigations (Low DYS et al, 2021). The enhanced anticancer of Au and ZnO nanoparticles efficacy can be qualified for ions liberation of Au^{+3} & Zn^{2+}. This, cogitate, facilitates the infiltration of reactive oxygen species (ROS) by cell membrane, ultimately leading to cells demise. Releasing of reactive oxygen species (ROS) have crucial pathway for the anticancer exertion of NPs (Martínez-Torres AC et al, 2018).

Rhodotorula sp contains chemical compounds that have the ability to inhibit cell growth, and some natural chemicals with antioxidant properties have demonstrated potential in treating cancer. These compounds are now being studied as potential candidates for anticancer therapy (Kaur et al., 2021). In vitro, the carotenoid extracts effectively suppressed the growth of human breast cancer cells. The IC50 values for MCF7 and MDAMB-231 cells were 29.11 and 7.82 µg/ml, respectively (Samuel et al., 2021).

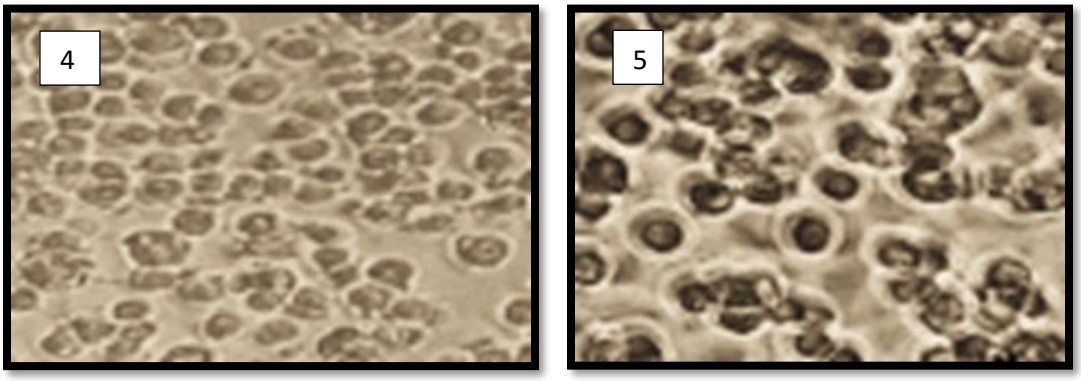
Figure (5) displays a significant change in the physical structure of the Mcf7 cell line. There was a higher number of apoptotic cells seen in cells treated with ZnONPs compared to control cells. After (24) hours of treatment, the cells showed strong signs of apoptotic body formation and

nuclear fragmentation, which is consistent with our study indicating that fungal-derived ZnO NPs induce apoptosis in a way that depends on the doses. ZnO NPs have the ability to specifically target and eliminate cancerous cells, which makes them a very auspicious treatment for treating cancer. Extensive research has demonstrated that fungi-derived ZnO NPs effectively control oxidative stress, regulate DNA replication and repair processes, influence cell cycle progression, and induce apoptosis in several cancer cell lines (Arakha et al., 2017; Siddiqi et al., 2018).



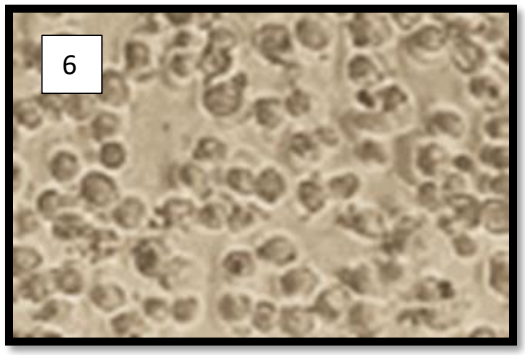
0µg/ml **6.25 µg/mL** **6.25 µg/mL**

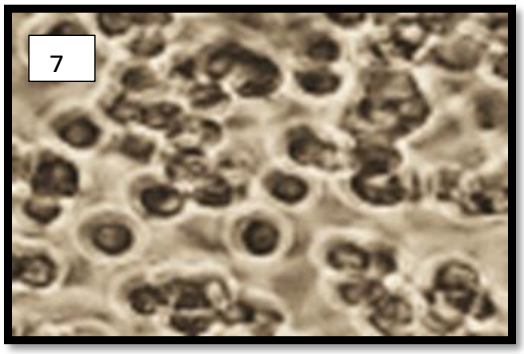
1-normal mcf7 cell line
2 treatment of mcf- 7 with biomass extraction *R.glutinis*
3- treatment of mcf-7 with *R.glutinis*ZNoNps



12.5 µg/mL 12.5 µg/mL

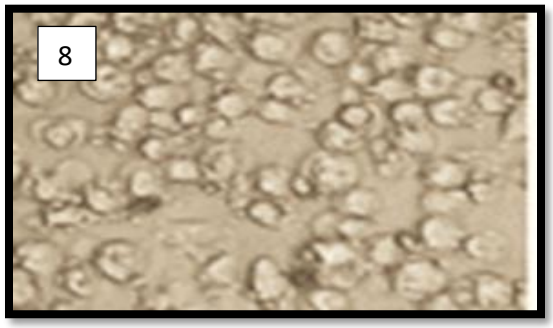
4 – treatment of normal mcf-7 with biomass extraction *R.glutinis*
5 – treatment of mcf-7 with *R.glutinis* ZNoNps

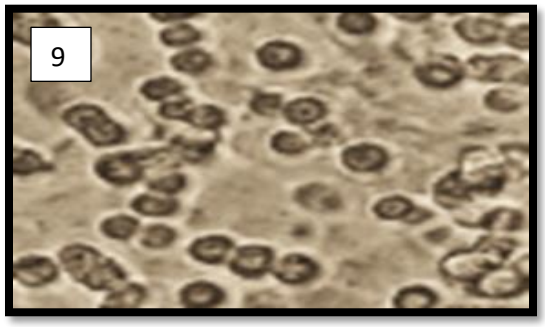




25 µg/mL 25 µg/mL

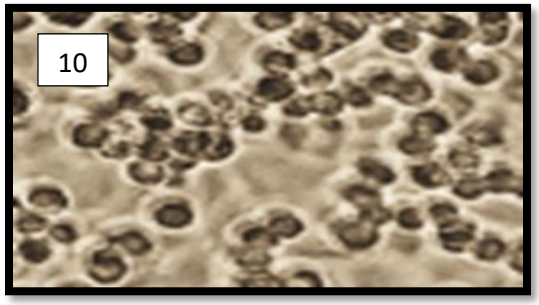
6 – treatment of mcf-7 with biomass extraction *R.glutinis*
7 – treatment of mcf-7 with *R.glutinis*ZNoNps

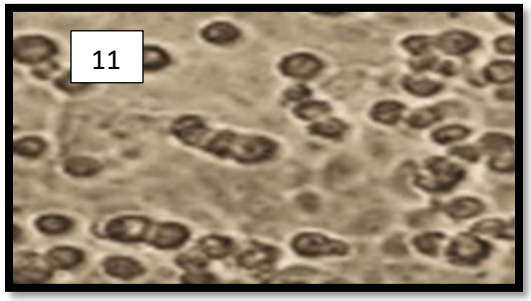




50 µg/mL 50 µg/mL

8 – treatment of mcf-7 with biomass extraction *R.glutinis*
9 – treatment of mcf-7 with *R.glutinis*ZNoNps





100 µg/mL 100 µg/mL

10 – treatment of mcf-7 with biomass extraction *R.glutinis*
11 – treatment of mcf-7 with *R.glutinis*ZNoNps

Fig_5. Inverted microscope of Mcf7 cell line

Conclusions

Fungi have great potential for synthesizing metal nanoparticles extracellularly due to their ability to secrete high concentrations of metabolites into the culture media. In this study, yeast *R.glutinis* was used to mediate the synthesis of ZnO NPs. These nanoparticles were found to efficiently suppress the viability and cell proliferation of MCF7 cell line. This makes them a promising candidate for anticancer therapy. Yet, withal delving is required to investigate their prospect genotoxicity as well as explore their usage in various biomedical inquires.

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