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Original Research

Estimation of the Antibacterial and Anti-Tumor Impacts of Soy Milk and Ecofriendly Myco-Manufactured Zinc Oxide Nanomaterials. *In vitro* Appraisal

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Abstract

The necessity for the creation of safe, dependable, biocompatible, and efficient methods to make nanoparticles drives an increasing number of researchers to consider using biological systems as potential eco-friendly nanofactories. Fungi have an innate ability to reduce and oxidize metal ions into metal oxide nanoparticles, thus behaving as nanofactories. In the present study, *Trichoderma harzianum* was used to prepare ZnO nanoparticles (ZnO NPS). ZnO NPS showed a peak at 370 nm upon testing using UV spectrophotometer and percentages of zinc(72.0±0.2 %) and oxygen(28.0 ±0.3) upon analysis using Edx with an irregular shape, 16.5 nm size and characterized XRD pattern. Soymilk as well as the prepared ZnONPs were investigated either separately or in their mixture (1:1) for their antibacterial and anti-tumor effects. The mixture showed a promising antibacterial impact on *Enterococcus faecalis* (ATCC29212) and *Escherichia coli* (ATCC25922) which was confirmed using a transmission electron microscope. ZnONPs mixed with soymilk showed a promising anti-tumor action towards human colorectal adenocarcinoma cell line Caco-2 through boosting apoptotic rate, which was detected by flow cytometry. The current report highlights the possibility of mixing eco-friendly biosynthesized nanoparticles with natural products to enhance their biomedical impact with minimal toxicity as possible innovative pharmaceutical applications.

Keywords: Soymilk, *Trichoderma harzianum,* nanotechnology, antibacterial, electron micropscope antitumor, apoptosis

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Introduction

One of the major legume crops in the world is soybean, which contains a variety of physiologically active compounds such as protein, phospholipid, oligosaccharides, and others [1, 2]. Since it can reduce lactose intolerance-related signs of intestinal discomfort, including diarrhea and pain, soy milk is frequently recommended as a substitute for milk [3, 4]. Due to its sensory qualities like those of raw beans and the bloating brought on by the intestinal microbiota's metabolization, soybean milk may not be preferred by everyone [5]. According to Paulo et al. [6], the market for cow's milk substitutes has grown as a result of rising lactose intolerance, hypercholesterolemia prevalence, and flexitarian dietary preferences. Non-dairy plant-based drinks are a good substitute for dairy products because they include bioactive substances that have positive effects on health and draw customers who care about their well-being [7, 8].

Nanotechnology offers a good platform for modifying and enhancing the fundamental properties of metal as nanoparticles with potential uses in diagnosis, indicators, distinguish operators for biological imaging, antimicrobials, tranquilizer delivery platforms, and nano drugs for the treatment of various diseases [9, 10]. There are several ways to obtain nanomaterials, but the production of nanoparticles requires greater attention because of the growing demand to develop safe, cost-effective, and environmentally friendly improvements. The chemical and physical synthesis has many drawbacks which urge a demand for creating a new route for the nanomaterial green protocol [11, 12]. Zinc dioxide (ZnO_2) nanoparticles were used with minimal cytotoxicity and several antimicrobial activities, which varied according to the prepared particle size [13, 14].

Trichoderma sp. is fungal species applied to the production of NPs which could be applied to enhance output that enhances crop through combating phytopathogens. ZnO NPs are useful products for plants and microbes in soil. Various research groups reported that ZnONPs can eradicate microbes and act as a substitute to chemical fertilizers, as an ecofriendly alterantive material for this purpose [15-17]. This paper examines the antibacterial and anti-tumor properties of soybean milk, both alone and in combination with ZnO nanoparticles.

Experimental Procedure

Microbial Culture & Soymilk

Trichoderma harzianum (RCMB 017 009) was used for the synthesis of zinc nanomaterials. All other bacterial pathogens and common intestinal flora were kindly provided by the culture collection unit of the Regional Center for Mycology & Biotechnology, (RCMB), Al-Azhar University, Cairo, Egypt. Individual fungal colonies were sub-cultured on Potato Dextrose Agar (PDA) media. Stock fungal cultures were maintained by sub-culturing the fungi on slants of Malt Extract Agar medium and preserving the agar at −20°C.

Soymilk was obtained from Agriculture Research Center (ARC, Egypt).

ZnONPs Bioproduction

T. harzianum culture was cultivated in a 250 mL Erlenmeyer flask containing 100 mL of broth medium comprising/L; Yeast extract; 3.0 g, Malt extract; 3.0 g, Peptone; 5.0 g, Dextrose (Glucose); 10.0 g, and the final pH was adjusted to 6.2±0.2 using 1N HCl. The fungal culture was grown with continuous shaking during incubation on a rotary shaker (Eppendorff, USA) (150 rpm) at 28°C for 72 hrs. After incubation, fungal pellets were obtained from the culture broth by centrifugation (4000 rpm – Eppendrorff, USA) at 4°C for 10 minutes and then washed twice with sterile distilled water. The harvested fungal biomass (Approx.15g wet weight) was resuspended in 100 mL sterile deionized distilled water and incubated while shaking (150 rpm) at 28°C for another 72 hrs. After incubation, the water cell-free filtrate was obtained by filtration and then added to 0.01 M zinc acetate dihydrate solution. The entire mixture was put into a shaker (150 rpm) at 28°C for a period of 48 hrs. After 48 hrs the bio-transformed product of Zinc oxide nanoparticles (ZnONPs) were collected for further purification [18, 19].

Purification of ZnONPs

The solution containing the zinc oxide nanoparticles was centrifuged for 15 minutes at 10,000 rpm, and then the pellet was re-scattered in sterile deionized water to remove any unwieldy organic particles. To ensure greater separation of the free elements from the metal nanoparticles, the centrifugation and re-scattering steps were repeated three times in sterile deionized water. The sanitized pellets were then subjected to a 30-minute sonication procedure using an ultrasonicator (Jeveriy Instrument Supplies, Italy) to achieve increased dissimilarity. The Lyophilizer (Thermo Electron Corporation, Micro Modulyo 230 stop drier, USA) was used to solidify and dry the specimens. The collected, purified Zinc oxide nanoparticles (ZnONPs) were then submitted for further characterization [20, 21].

ZnONPs Characterization

The biotransformation process of ZnO metal ion salt into Zinc oxide nanoparticles was assessed using different protocols. Samples of bio-transformed products of zinc oxide nanoparticles (ZnONPs) were characterized by globally accepted nano structure characterizations techniques using UV-visible spectrophotometer (Spectronic Milton Roy 1201, USA),

particle size and shape analyzing system of transmission Electron Microscopy (TEM) (TEM JEOL 1010, Japan), Energy Dispersive Spectroscopy (EDX) connected to Scanning Electron Microscope (SEM/EDX, JSM-5500 LV JEOL, SEM, Japan), and X-ray Diffraction (XRD) analysis (IRPrestige-21®, German) techniques [22, 23].

Antibacterial Activity

Staphylococcus aureus (ATCC25923) and *Staphylococcus epidermidis* (ATCC12228) were grown in tryptic soya agar for 24 h at 37°C. *Enterococcus faecalis* (ATCC29212), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella typhimurium* (ATCC14028), and *Escherichia coli* (ATCC25922) were grown on macconkey agar media for 24 h at 37°C [22-28]. To obtain the turbidity of 0.5McFarland standards, the tested bacteria were cultivated in nutrient broth and incubated overnight at 37°C, yielding 1.5 $X10^8$ CFU/mL. Agar well diffusion was used to test the antibacterial activity of zinc acetate salt, soy milk, Zinc oxide nanoparticles, and combination of soymilk and zinc oxide nanoparticles (1:1). Bacterial suspensions were grown on Mueller Hinton agar plates. Using a sterile cork borer, wells (6 mm) were drilled into the inoculation medium. Separately, 100 µL of specimens were added to each well. The plates were then placed in the refrigerator for 30 minutes to improve the samples ability to diffuse into the agar. Negative and positive controls were DMSO (10%) and Gentamycin (10 µg/ml). The plates were incubated for 24 hours at 37°C [29, 30].

For MIC detection, the sample that showed the greatest inhibition of bacterial growth using the agar well diffusion method was further examined using the micro-dilution method The stock solution was diluted in two-fold serial dilutions using broth as the diluent to reach concentrations between 1000 and 1.9 µg/mL. Lastly, 10 µL of the bacterial solution (10^5 CFU/mL) was obtained and added to each well. Wells containing uninoculated medium with and without samples were used as a control to make sure the medium was sterile and pure. A third control well was utilized that included infected media but no extract to make sure the organism could flourish in the medium. After being incubated for 24 hours at 37°C, the turbidity was evaluated as a gauge of microbial growth. The MIC value is the lowest sample dilution that prevented any discernible growth of the tested bacterium [31, 32].

Antitumor and Cytotoxic Activity

African green monkey cells (VERO) and human colorectal adenocarcinoma cells (Caco-2) were used to examine the samples for cytotoxic effects. Between 500 and 15.63 ug/mL of soybean or soybean plus ZnONP were added to the cells and allowed to adhere for 24 hours until confluence. Cells were then incubated at 37°C for 24 hours. After that, the new medium was added, and after 4 hours at 37°C, 100 µL of the MTT solution (5 mg/mL) was used. The absorbance at 570 nm was discovered using a microplate reader (SUNRISE, USA) [33, 34].

Flow Cytometric Analysis

For the flow cytometry analysis, Caco-2 cells were treated with ZnO nanoparticles, soybean, and ZnO nanoparticle mixture, or left untreated. The Caco-2 cells were separated with trypsin in 0.25% pancreatin and washed with phosphate-buffered saline. The death rate was calculated using an Annexin V-FITC and propidium iodide staining kit (B.D. Bioscience, USA). Cells were incubated for ten minutes at room temperature in a buffer containing Annexin V-FITC and/or P.I. stock solution. Analysis was done using flow cytometry (BD bioscience, USA) [35, 36].

Transmission Electron Microscope

Changes in ultrastructure were examined in *Enterococcus faecalis* and *Escherichia coli* after treatment with soybean milk, ZnO nanoparticles mixed with soybean milk, or a standard drug (Gentamycin). Cells were fixed with 2.5% glutaraldehyde for two hours. The blocks were then colored with 1% uranyl acetate and treated for two hours with 2% osmium tetroxide before being dried with a progressive ethanol series. The specimens were then inserted using resin. The materials were cut into slices using an ultra-microtome (Leica, Wetzkar, Germany). Then, a transmission electron microscope (JOEL, Tokyo, Japan) was used to analyze the slices [37].

Statistical Variations

The analytical variation was calculated using the GraphPad Prism 5 program (San Diego, CA, USA). To find differences between groups where $P \leq 0.05$ is considered significant, one-way analysis (ANOVA) was applied, followed by Tukey's post-hoc test [38-40].

Results

Characterization of Prepared ZnONPs

UV Visible Spectrophotometer

A UV-Vis spectrophotometer's spectrum analysis was used to ascertain how the ZnO NPs were made. To find out more about the optical characteristics of the synthesized zinc oxide nanoparticles, a UV/Visible investigation was also conducted. Analysis of the UV spectra was done between 200 and 800 nm. The production of zinc oxide nanoparticles is indicated by the ZnO nanoparticles' predominant absorption peaks at wavelengths of 370 nm, as shown (Fig. 1a).

TEM Results

The shape of the synthesized zinc oxide nanoparticles was investigated using TEM microscopy. Due to the presence of some debris of fungal metabolites on the surface of the ZnO NPs, which serves as a capping and stabilizing agent, the nanoparticles were irregular, agglomerated, with a mean particle size of 16.5 nm, as can be seen in the TEM picture (Fig. 1b).

XRD Analysis

ZnO nanoparticles with their intensity profile and XRD pattern that represent their structural characteristics have been tested. The hexagonal crystalline structure of ZnO NPs was shown to exhibit different diffraction in the spectra, which corresponded to peak values of (100), (101), (102), (110), (103), (200), (201), and (202), respectively as demonstrated in Fig. 1 (c, d)

Edx Results

EDX analysis, showed the reduction of a zinc ion into a zinc element in the reaction mixture. The elemental composition analysis of the zinc nanoparticles validated the excellent purity of the synthesized zinc oxide nanoparticles and demonstrated the existence of Zn and O in the sample. The relative proportions of zinc and oxygen in the synthesized zinc oxide nanoparticles (72.0±0.2% zinc and 28.0±0.3% oxygen) as shown in Fig. 1e) and Table 1.

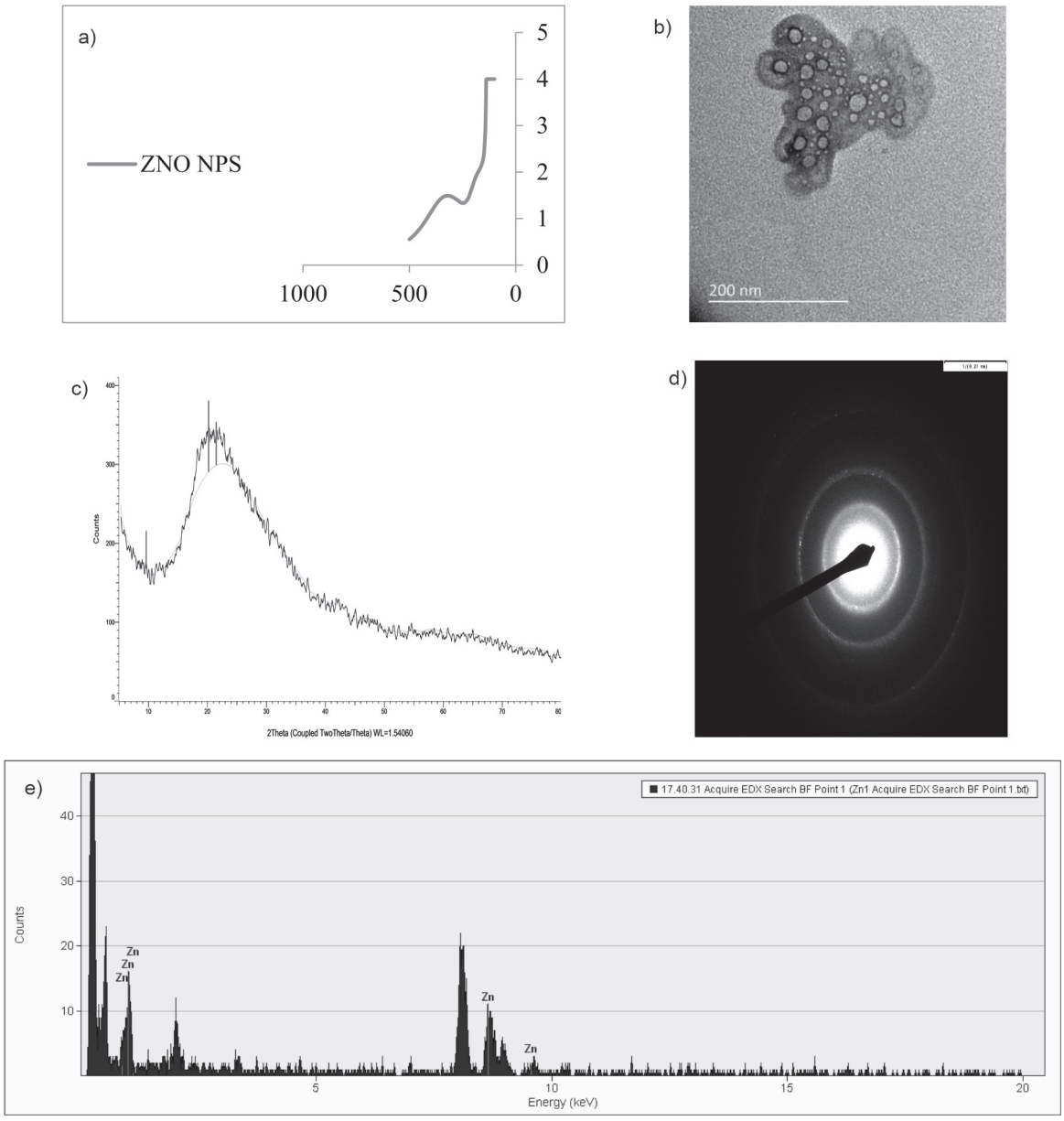

Fig. 1. Characterization of ZnONPS: a) UV spectrum of ZnO NPS; b) TEM of ZnO NPS; (c, d) XRD and intententsity pattern of ZnO NPS; e) Edx pattern of ZnO NPS.

Table 1. Different perecntages of elements in ZnO NPs by EDX testing.

Element	Atomic %± Standard devaiation of mean
Zn	72.0±0.2
O	28.0 ±0.3

Antibacterial Assay

The antibacterial activity of soy milk, the prepared ZnO NPs made using the *T. harzianum* as well as combination of ZnO NPs + Soy milk was tested against three strains of Gram-positive bacteria, including, *Staphylococcus aureus* (ATCC25923), *Staphylococcus epidermidis* (ATCC12228) and *Enterococcus faecalis* (ATCC29212) three strains of Gram-negative bacteria including *Pseudomonas aeruginosa* (ATCC27853), *Salmonella typhimurium*(ATCC14028), *Escherichia coli* (ATCC25922). The mycosynthesized ZnO NPs showed the highest value of inhibition versus *Enterococcus faecalis* than any tested Gram-positive bacteria (12.1±0.6 mm) and slightly increased upon mixing with soymilk reaching (14.3±0.1 mm) with MIC values of the ZnONPs and combination treatments of 125.0±1.2 µg/ml and 31.25±0.8 µg/ml respectively. Moreover, the myco-synthesized ZnO NPs showed the highest value of inhibition versus *E.coli* among tested Gram- negative bacteria for ZnO NPs and a combination of ZnO NPs with soy milk of 11.6±0.6 and 13.5±0.2 respectively and MIC of 250 µg/ml for the ZnO NPs and combination treatments as depicted in (Tables 2, 3).

Ultrastructure Examination

Electron microscopy investigation of *E. faecalis* (Gram-positive bacteria) verified the antibacterial effects of several successful treatments (Fig. 2). Untreated *E. faecalis* were well-organized cells with flat surface layers and visible interior organelles (Fig. 2a). In contrast, treated *E. faecalis* with ZnO NPS led to the formation of holes in the outermost layer of the bacteria and the lysis of cellular organelles as shown in (Fig. 2b). Treating *E. faecalis* with soymilk enhanced the bactericidal impact and led to the lysis of internal organelles as shown in (Fig. 4c), with a similar effect when compared to standard medication as shown in (Fig. 2d).

The effectiveness of several antibacterial treatments against *E. coli* (Gram-negative bacteria) is shown in (Fig. 3). Untreated *E. coli* cells were well-organized with uniformly smooth surface layers and discernible interior organelles (Fig. 3a). Besides, in (Fig. 3b), ZnO NPs treatment of *E. coli* caused cellular organelle lysis and the development of pores in the surface of the bacterium, treatment using a mixture of Zn ONPs and soymilk enhanced bactericidal effects with lysis of internal organelles as shown in (Fig. 3c), with an outcome comparable to that generated by standard drug as shown in (Fig. 3d).

Table 2. Antibacterial impact of different treatments (Data are represented as means±SD).

Sample code / Tested microorganisms	Zinc actate	Soy milk	ZnONPs	ZnONPs + Soy milk	Control
Bacteria:					Gentamycin
Gram Positive Bacteria					
Staphylococcus aureus (ATCC25923)	ND	ND	10.0±1.1	12.2±0.4	24.0±1.6
Staphylococcus epidermidis (ATCC12228)	ND	ND	10.2±0.8	11.1±0.3	200.0±1.8
Enterococcus faecalis (ATCC29212)	ND	ND	12.1±0.6	14.3±0.1	27.0±1.5
Gram Negative Bacteria					
Pseudomonas aeruginosa (ATCC27853)	ND	ND	9.3±0.3	13.5±0.9	25.0±1.7
Salmonella typhimurium (ATCC14028)	NA	ND	8.5±0.4	10.2±0.6	22.0±1.4
Escherichia coli (ATCC25922)	NA	ND	11.6±0.6	13.5±0.2	30.0±1.6

ND: Not detected

Table 3. Minimal inhibitory concnertations (µg/ml) for ZnO NPS & ZnONPs mixed with Soy bean milk (1:1) Versus highly affected bacteria (Data are represented as means±S.D).

Tested microrgnaism	ZnONPs	ZnONPs + Soy milk	Control
Enterococcus faecalis	125.0±1.2	31.25±0.8	5.59±0.2
Escherichia coli	250.0±0.6	250.0±0.5	4.25±0.3

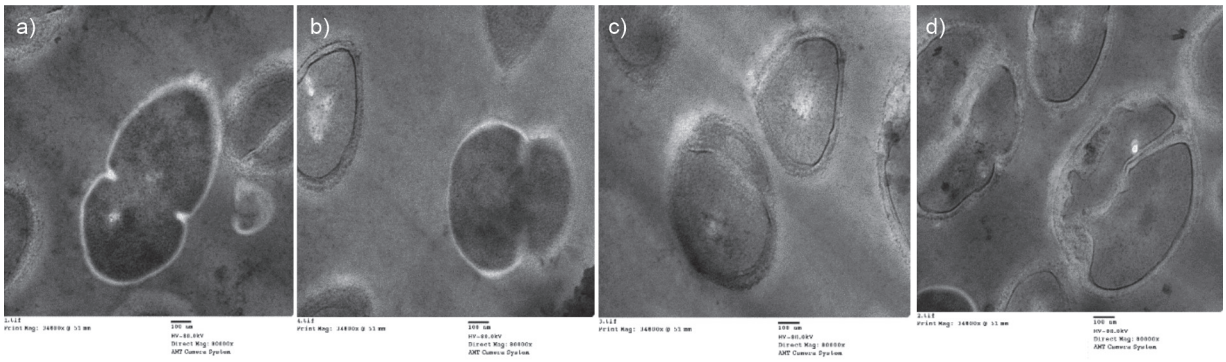

Fig. 2. TEM micrographs of *E. faecalis* a) Control; b) Upon treatment with ZnO NPs; c) Upon treamtnet with ZnO NPs mixed with soymilk; d) treated with 10 µg/ml Genatmaycin; (Magnification 80,000 X).

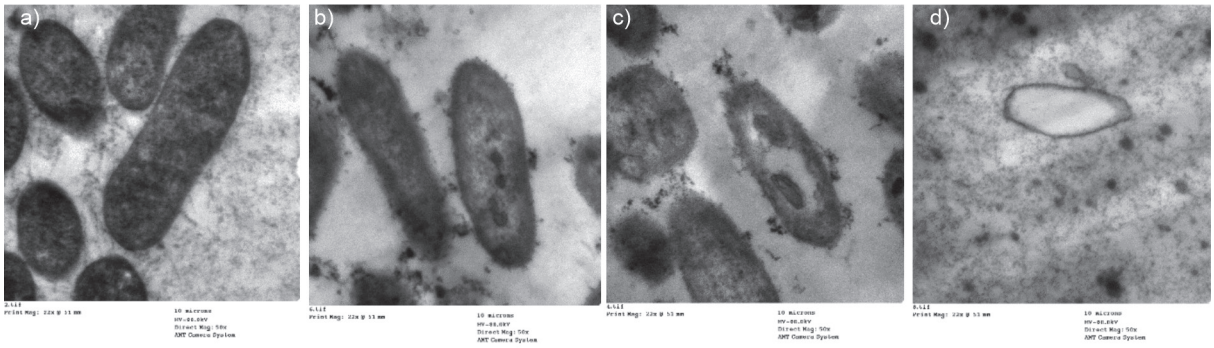

Fig. 3. TEM micrographs of *E. coli* a) Control; b) Upon treatment with ZnO NPs; c) Upon treamtnet with ZnONPs mixed with soymilk; d) treated with 10µg/ml Genatmaycin (Magnification 50,000 X).

Anti-Tumor and Cytotoxicity Testing

Soymilk exhibited good anti-tumor effects in Caco-2 cells (IC_{50}= 105.0±2.3 µg/ml. Besides, Zn ONPs have promising antitumor effect versus Caco-2 cells with an IC_{50} = 82.6±1.4 µg/ml as depicted in (Fig. 4). A combination of ZnO NPs + Soy milk showed antitumor action with IC_{50} = 70.6±/1.9µg/ml. Furthermore, testing soymilk, ZnO NPs, and their combination on Vero cells revealed to assure their minimal toxicity with CC_{50} = 45.9±0.6, 97±1.1 and 111.2±1.4 µg/ml respectively assured its efficacy and potential for usage in various applications as shown in (Table 4).

Apoptosis Testing

To confirm the role of ZnONPS and its combination with soymilk versus Caco-2 cells through flow cytometric testing, there was a significant boosting (P<0.05) of the apoptotic rate of Caco-2 cells treated by ZnONPS and its combination with soymilk, as shown in (Fig. 5).

Discussion

There is a recent demand for vegans and lactose intolerant people to use soy milk in place of dairy milk. Regarding water consumption, potential for global warming, and land use, soy milk has excellent environmental performance [41]. Besides, to shield bacterial cells from harmful environments including the low pH of stomach acid, bile salt, and different enzymes for digestion in the gastrointestinal system, soymilk might be employed as a dietary carrier for probiotics [42].

The use of zinc in buildings and architecture is ecologically favorable [43]. ZnO nanoparticles make excellent choices for uses in biology since they are straightforward to create, nontoxic, and accessible [44, 45].

In the present investigation zinc oxide nanoparticles were prepared using *T. harzianum*, with a peak at 370 nm. In accordance with Huang et al., [46] who prepared zinc oxide with the same UV spectrum as

Table 4. Cytotoxicity assay of Soymilk, ZnONPs and ZnONPs + Soy milk (1:1) versus VERO cells. (Data are repreaented as means±SD).

Cytotoxicity	Soymilk	ZnONPs	ZnONPs + Soy milk
CC_{50}	45.9±0.6	97±1.1	111.2±1.4

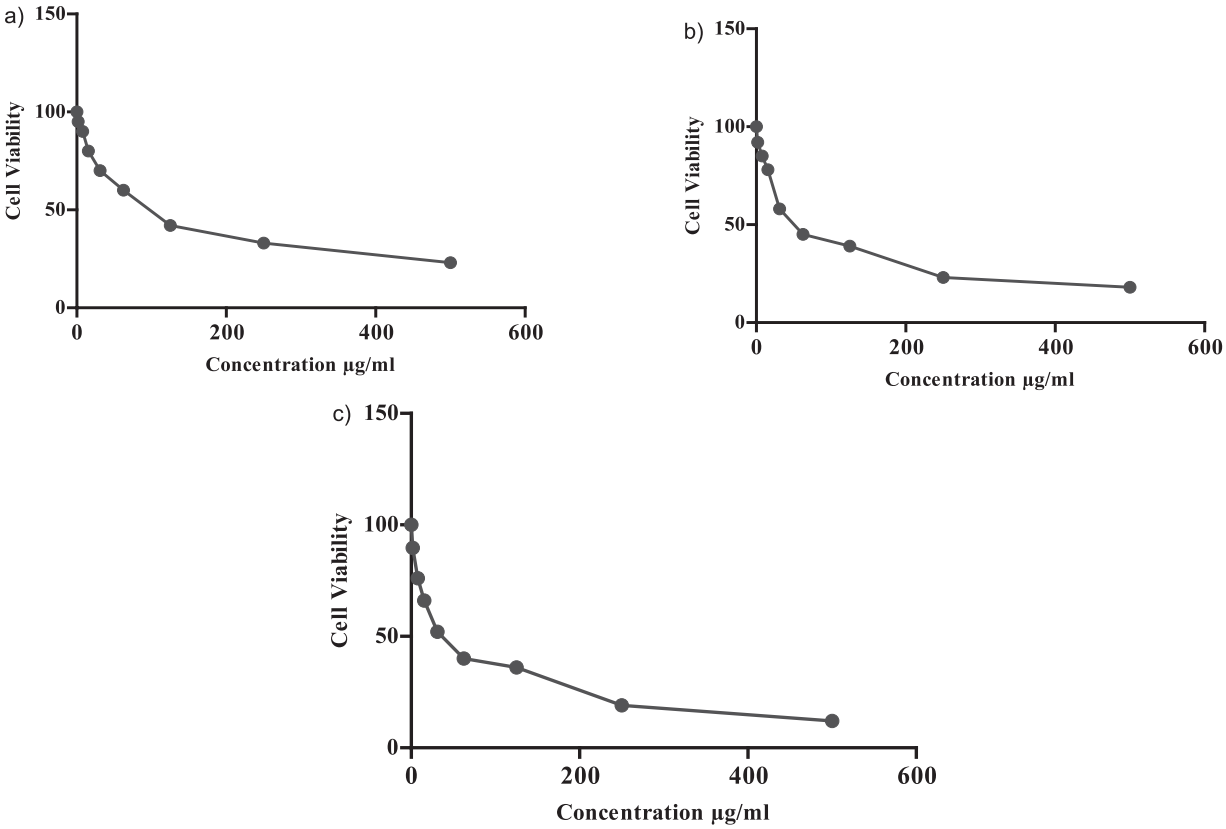

Fig. 4. Inhibitory activity of different treatements versus human colorectal adenocarcinoma cell line Caco-2 was detected under these experimental conditions a) Soy milk with IC_{50} = 105±2.3 µg/ml; b) ZnO NPs with IC_{50} = 82.6±1.4 µg/ml and c) mixture of ZnO NPs and soymilk (1:1) IC_{50} = 70.6±/1.9 µg/ml**(**Data are represented as means±S.D**)**

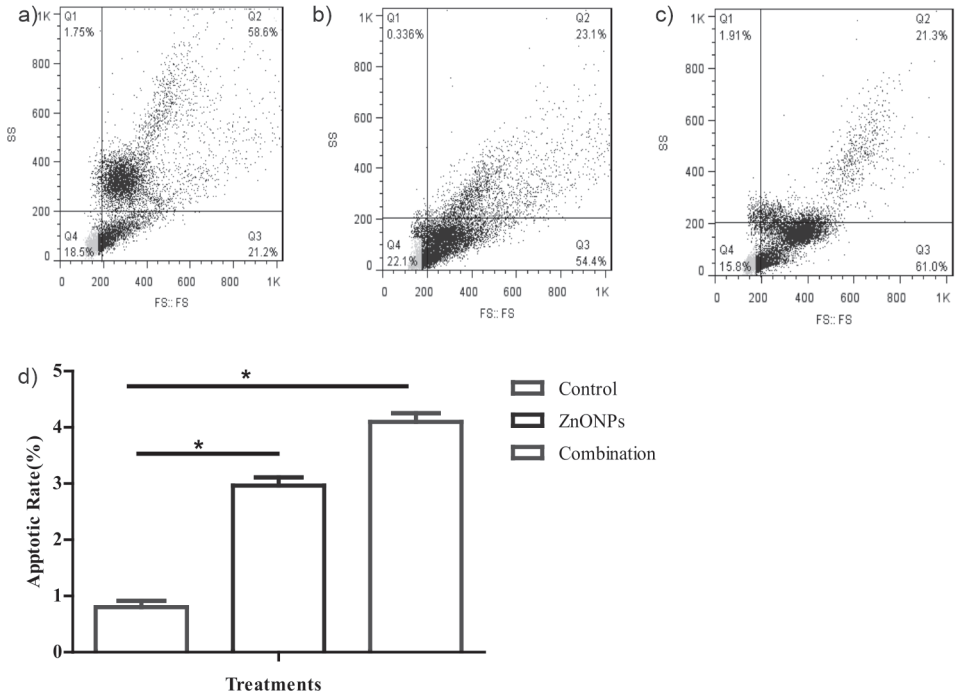

Fig. 5. Flow cytometric analysis of different treatements versus against human colorectal adenocarcinoma cell line Caco-2 upon treatment with a) Untreated Caco-2cells; b) treated cells by ZnO NPs c) Treated cells by mixture of ZnO NPs and soymilk (1:1); d) Sataistical analysis revealing variation in apoptotic rate upn using ZnONPs and mixture of ZnO NPs and soymilk (1:1). (Data are represented as means±SD * $P \leq 0.05$).

a nanocarrier to enhance drug roles. Furthermore, EDX analysis revealed the existence of zinc and oxygen with notable percentages and predominance of zinc as reported by Wojnarowicz et al., [47]. The prepared ZnO nanoparticles have a similar XRD pattern as those prepared by Kamal et al., [48] who used mushrooms to synthesize ZnO nanoparticles.

It is generally known that fungi release significant amounts of proteins that are essential to their life cycle. According to Jain et al. [49] the majority of these proteins contain hydrolytic enzymes such as amylases, cellulases, and proteases. These proteins may be responsible for both the nano-biosynthesis and the monodisperse nanoparticles' sustainability towards coagulation and oxidation.

Several research groups reported the roles of soymilk towards tumor bearing animals' fecal microbiota [50]. In the present investigation ZnO nanoparticles, soymilk, and a mixture of soymilk combined with ZnO NPs were tested against wide range of Gram-positive and Gram-negative bacteria The present results revealed that the combination of ZnO nanoparticles with soymilk have synergestic effect towards *E. faecalis* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria) through enhancing destruction of cellular structure.

This current approach is a new approach for mixing natural products, such as soymilk, with ZnO nanoparticles to enhance antibacterial effects. Several research groups reported the synergistic effect of ZnO nanoparticles with various antibiotics on Pseudomonas aeruginosa and Staphylococcus aureus [51, 52]. The exopolysaccharide formation process, which is essential for the formation of complex biofilms and interferes with biofilm integrity, may be the mechanism of ZnO NPs that underlies this action [53]. In addition, a recent clinical trial reported the possible application of soymilk to combat gut and urinary microbiota [54].

The combination of ZnO NPs with soymilk has an effective anti-tumor impact towards *Caco-2*cells through acceleration of apoptotic rate to a level higher than apoptotic rate relative to ZnO Nanoparticles alone. Furthermore, the safety of various treatments for Vero cells has been detected. Nano-ZnO's tiny particle size makes it easier for the body to absorb zinc. Nano-ZnO is therefore commonly used in food. Additionally, ZnO has been designated as a generally recognized as a safe product by the US Food and Drug Administration (FDA). ZnO NPs have a variety of medical applications, including diabetic therapy, wound healing, and bioimaging. They are also relatively inexpensive and less toxic than other metal oxide NPs [55-57]. Many investigators reported the development of antimicrobial resistance [58-61]. Thus the using of natural products combined with nanoparticles is an excellent approach for overcoming the developed resitance.

Conclusion

The combination of soymilk with myco-synthesized ZnO nanoparticles has a synergisticimpact which highlights the possibility of using this combination for antibacterial and antitumor applications for future *in vivo* studies.

Conflict of Interest

The author confirms that they have no conflict of interest.

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