Research Article

Cytotoxicity of Magnesium Oxide-Zinc Oxide-Titanium Nanocomposite ($MgZnTiO_4$) on LS 174T Colorectal Adenocarcinoma Cell Line

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ABSTRACT

Many efforts had been focused on the therapeutic approach of cancer for a long time by studying cytotoxic nanoparticles' activities. In this study, a mix of PVA 60% and PVP 35 %, with 5% reinforced bioceramic triple (ZnO, MgO, Tio2) was applied against LS 174T colorectal adenocarcinoma cell line. This study was planned to evaluate MZT cytotoxicity by thiazolyl blue tetrazolium bromide (MTT) cytotoxicity test on LS 174T cell line in compared with chemotherapeutic drugs that were included Doxorubicin (DOX) and 5-flourouracil (5FU). These nanoparticles were purchased from Skyspring Nanomaterials, Inc. USA, with 10-30 nm in diameter and 99.8%.purity. The size was further confirmed with X-ray Diffraction (XRD), Energy Dispersive X-ray Spectroscopy (EDS) and Scanning Electron Microscopy (SEM). The results showed that MZT nanocomposite was effectively and significantly inhibited the cell proliferation ($p<0.0005$) by decreasing the viability of LS 174T cells at different concentrations involved 1, 10, 100, 1000 and 2000 µg/ml, with 5.698 µg/ml half maximal inhibitory concentration (IC_{50}) of. DOX and 5FU IC_{50} values were 27.409 µg/ml and 62.84 µg/ml, respectively. It was concluded that MZT nanocomposite have an anticancer effect against LS 174T colorectal carcinoma and this type of cell line was good enough for applying MTT cytotoxicity assay.

Keywords:LS 174T colorectal adenocarcinoma, Cell culture, MTT Assay, Nanoparticles.

INTRODUCTION

Cancer is one of the leading diseases throughout the world in which a group of cells display uncontrolled growth, invasion, and sometimes metastasis [25]. Cancer is a disease that is characterized by the unchecked division and abnormal cells survival. Abnormal growth that occurs in the colon or rectum is called Colorectal Cancer (CRC). It is estimated that in 2017, the number of new cases of colon cancer will be 95,520 and the number of rectal cancer diagnosed in the US will be 39,910. . While the numbers of colon cancer are fairly equal in men (47,700) and women (47,820), a larger number of men (23,720) than women (16,190) will be diagnosed with rectal cancer. Due to estimation, 27,150 men and 23,110 women will die from CRC in 2017[26]. Bioactive ceramics are used in a number of different applications in implants, repair, and reconstruction of diseased or damaged body parts. Most bioactive ceramics medical applications relate to the skeletal system repair and hard tissue. They include several major groups such as calcium phosphate ceramics. The nanoparticles with their size in compare to of the biological structures are very smart materials for the sensing, and biological systems detection, recent progress in utilizing inorganic nanoparticles for biomedical applications has received more attention due to their applications as potential antibacterial agents, drug, gene delivery vehicles, in molecular diagnostics, and cancer therapy. Therapeutic Nano reactors have been proposed to treat cancers through low-toxicity prodrugs transformation to toxic therapeutics in the body. In this study, the effect of MgZnTiO4 (MZT) nanocomposite against LS 174T colorectal adenocarcinoma cell line in compared to doxorubicin and 5- fluorouracil is considered.

Materials And Methods

Pure and polymer mixed MZT nanocomposite Preparation

The polymer containing nanocomposite was prepared by adding 0.6gm of PVA(Shanghai Kaidu Industrial Development Co., Ltd China), 0.35 gm of PVP (Anhui LeafchemCo., Ltd, China), and 0.05 gm of MZT nanocomposite (total 1gm) to 10 ml of distilled water. The mixture was agitated by continuous string to get homogenous suspension. The obtained homogeneous suspension was kept for 1-2 h under constant stirring at the temperature of 60 0C to get white homogeneous solution. Pure MZT nanocomposite phase confirmation is verified by XRD and EDX analysis. The shape and morphology of particles were also studied by scan electron microscopy SEM. The study was planned to use selected dilutions of MZT nanocomposite as follows (1, 10, 100, 1000, 2000) µg/ml.

MZT nanocomposite characterizations

To investigate various properties of MZT nanocomposite, it has to go under a number of characterization techniques. The results which give the information about the different optical and structural properties of MZT nanocomposite prepared. The following characterization

techniques are applicable to get exact information about the crystal structure, surface morphology, and particle size.

X-Ray Diffraction (XRD)

The specific morphologies were analyzed because the properties of nano - and micro – structures are strongly depend on their shapes and sizes. The structural properties of MZT nanocomposite were analyzed by x-ray diffraction patterns and by specific technique [1]. The most popular method for the crystallite size estimation in nanomaterials that has been extensively used in this study is the X-ray diffraction analysis, the evaluation of crystallite sizes in the nanometer range warrants careful analytical skills. The broadening of the Bragg peaks is ascribed to the development of the crystallite refinement and internal stain. The full width at half maximum (FWHM) of the Brag peaks as a function of the diffraction angle is analyzed to size broadening and stain broadening. Crystallite size of the deposits is calculated by the X-ray diffraction (XRD) peak broadening. After subtracting the instrumental line broadening, that was estimated by quartz and silicon standards. All crystal structures of the preparation polymeric composites have been studied by the XRD (6000) diffraction device that was supplied by the Japanese company ((Ministry of Science and Technology). The XRD 6000 X-Ray diffraction device has been used to examine the mode of X-Ray diffraction of composite as follows:

Target: Cu

Wavelength: 1.5406 Å.

Voltage: 40 Kv.

Current: 30 mA.

Rank: 10.000 - 60.000 (deg) current Time: 0.60 (s).

The grain size (D) of the crystalline nanocomposte which plays an important role in the material properties could be estimated easily from the X-ray spectrum by means of Full Width at Half Maximum (FWHM) method that is often calculated by Scherrer relation [2].

$$D = \frac{0.9\lambda}{B\cos\theta} D = \frac{0.9\lambda}{B\cos\theta} \quad \ldots (3\text{-}1)$$

Where λ is the wave length of incident X-ray radiation (λ = 0.15406 nm), B is the intrinsic full width at Half Maximum of the peak, and θ is the Bragg's diffraction angle of the respective XRD peak. It is used to estimate the size of very small crystals from measured width of their diffraction curves.

Field emission Scanning Electron Microscopy (SEM)

The ultra-high SEM resolution was achieved for MZT nanocomposite, this technique offers new views into the dynamical interactions processes and morphological appearance of nanocomposite. SEM images of the MZT nanocomposite powder were taken by Jeol JSM-6335F scanning electron microscope that was equipped with magnification power of 250000 energy dispersive X-ray.

Energy Dispersive X-ray Spectroscopy (EDX)

The EDX of the MZT nanocomposite was done by the **SEM (HITACHI S-3000N/ State)** machine. The EDX demonstrates the required phase. All components including MgO, ZnO, and TiO2 were presented in the sample. The elemental compositions of the MZT nanocomposite were observed by energy dispersive X-ray spectroscopy Fig. (3)

MTT Cytotoxicity assay

The cytotoxicity of $MgZnTiO_4$ nanocomposite, $MgZnTiO_4$-Polymer nanocomposite, doxorubicin and 5- Fluorouracil was evaluated by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cells of LS 174T colorectal adenocarcinoma cell line were seeded onto 96 well plates with a concentration of l04cells/ml and incubated at the temperature of 37°C for 48-72 h, when the confluent monolayer of LS-174 cells was completely formed (80%-100%), different concentrations including (1,10,100,1000 and 2000μg/ml) of microtitered compounds were added to cultured wells except cells control in the triplicate at 100 μl volume. After 24 h incubation at the temperature of 37°C in 5% CO2, all media were discarded from microtiter plate's wells [10]. To remove any residual amount of composites or standard anticancer drugs due to avoid interaction with the final results, the LS-174 cell monolayers were cleaned with PBS solution (pH=7.2). Then remaining materials (100 μl) was added to all wells included drugs/composites treated cells, drugs/composites untreated cells, and blank wells. Then, 20 μl of MTT reagent was added to each well. After 3-4 h of incubation at the temperature of 37°C, 5% CO2, the formazan granules were formed as a mitochondrial enzymatic process of proportional correlation with LS 174T cells viability. The formazan was solubilized by adding diluted dimethylsulfoxide DMSO : isopropanol (1:1) on each wells included blank wells, then absorbance was read at 490 *nm* / reference wavelength of *630 nm* by ELISA reader, this MTT protocol assay was adapted by many reports [6] [7]. Mean blank absorption read was subscribed from other samples and controls absorptions. The inhibitory concentration was fitted by blotting of inhibition percentage versus log of concentration of other used compound. % inhibition was calculated by the following formula: % Inhibition (GI %) =⟦ (A−A1) ÷A⟧×100 [9]. Where A is the absorbance of untreated samples, and A1 is the absorbance of the treated test/standard. While the 50% of maximum inhibition (=Y in the formula of IC50 calculation) was calculated according as follows: 50% of maximum inhibition=Max GI % - 50% × (Max GI% - min GI %).[8]

Results and Discussion

MZT nanocomposite XRD analysis

The XRD was used to investigate the crystal phase structure of MZT nanocomposite. Fig. (1) shows the diffraction pattern of the sample. the obvious characteristic peaks are at the temperature of 36.28°, 31.79°, 25.31° and 43.28° and the phase identification of the prepared MZT nanocomposite was performed by XRD. Also, no diffraction peaks was not corresponded to MZT nanocomposite or other impurities that were observed in these patterns. The (100) plane had the strongest line for all investigated annealing temperatures. The average particle size of MZT nanocomposite was estimated to be around 55.65- 66.37 nm. These data demonstrate that the MZT nanocomposite's size increased by annealing temperature increasing. MZT nanocomposite showed stronger diffraction peaks at (1300^{0}C), and it indicates that the crystallinity of the MZT nanocomposite will be improved by annealing temperature increasing. [16, 18, and 20]. However, when the annealing temperature was higher, the diffraction the MZT nanocomposite peaks became weaker and this indicates that the crystalline quality of MZT nanocomposite decreased as a result of agglomeration. As shown in Fig. (1) and Table (1), upon the annealing temperature, the diffraction peaks sharpness improved in respect to the annealing temperature, and the full-width at half-maximum (FWHM) values were decreased by annealing temperature increasing. In addition, (18) plane has the strongest line for all investigated annealing temperatures. These results indicated that there is an improvement in the crystallinity of the MZT nanocomposite by annealing temperature increasing. [18 and 20].

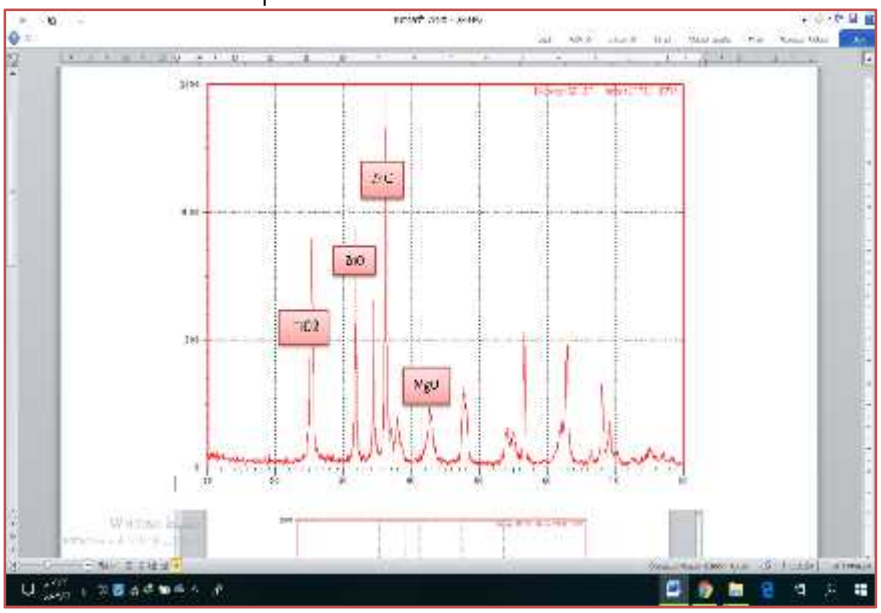

Fig. 1) :(XRDof MZT nanocomposite

Table (1): XRD MZT nanocomposite analysis presented by 2Θ, Intensity, FWHM, and diffraction values.

Elements	2Θ	I	FWHM	D(A)
ZnO	36.28	100	0.2679	2.47387
ZnO	31.79	60	0.2650	2.81198
TiO2	25.31	54	0.4984	3.51586
MgO	43.28	5	02666	2.08874

SEM

It's an ideal analytical technique for characterizing and visualizing the elemental composition of a specimen [11]. It has a spatial resolution of <1 nm and a number of other advantages, including much improved performance at low accelerating voltages [12]. It becomes more popular because of high magnification image that can be taken under high vacuum condition. This technique is widely used in metals, semiconductors, ceramics, medical, and biological field [13]. The SEM properties allow new approaches for science studies, and based on high quality, low voltage images are obtained from negligible electrical charging of samples, produces clearer, less electrostatically distorted images with spatial resolution down to 11/2 nm that's three to six times more than conventional SEM, reduced penetration of low kinetic energy electrons probes that is closer to the immediate material surface and smaller-area contamination spots that can be examined at electron accelerating voltages compatible with Energy Dispersive X-ray Spectroscopy [14]. In spite of the advanced capabilities of SEM, no study has yet described its use for the accurate ultrastructural nanoparticles localization inside cells. The results showed the newly topographical characteristics of the compounded nanocomposite with clear interaction of the three different component beads related to MgO, ZnO, and TiO2. Fig. (2) Illustrates the SEM images of MgO, ZnO, TiO2, and MZT nanocomposite.

EDX analysis of MZT nanocomposite

This method actually is an additional system to SEM and used for the purpose of estimating the chemical solid samples composition. This method uses returned X-ray analysis of the samples to evaluate the chemical composition and elemental samples surfaces analysis, also this method allows surface and spot analysis for samples. In addition, it is possible to obtain information about the chemical composition of the polished surfaces, fractured surfaces, powders, and thin films [19]. This type of analysis or X-ray energy distribution is called EDS. In general, this type of analysis can identify existing chemical elements in materials with atomic number that is more than five. Energy Dispersive X-ray Spectroscopy (EDX) is an analytical technique that is used to identify the elemental sample composition. It relies on the sample investigation through interactions between electromagnetic radiation and matter analyzing x-rays that is emitted by the matter in response to being hit with charged particles. During (EDX), a sample is exposed to an electron beam; these electrons collide with the electrons within the sample, causes some of them to be knocked out of their orbits. The vacated positions are filled by higher energy electrons which emit X-rays in the process. By analyzing the emitted X-rays, the elemental composition of the sample can be determined. This technique is a handy tool for performing the constitutional analysis of any kind of material. As presented in Fig. (3), the selected annealing temperature (1300 °C), which demonstrates the well-defined peaks origination related to the MZT nanocomposite It is clear that there is no other peak relates to any significant impurity in the EDX spectrum, up to the detection EDX limitation, confirming that the synthesized material is made of magnesium oxide, zinc oxide, and titanium oxide without any significant impurity, the same results also confirmed in the previous studies [15,16, 17]. The atomic percentages of MgO, ZnO and tio2 were generally close to the stoichiometric composition and had a 1:1:1 ratio, approximately. This result confirmed that the powder contained only MgO, ZnO, and tio2 elements and that MZT nanocomposite successfully formed the 83.82% comprise of the total weight with 81.79% atomic of. This test was also revealed the existence of other elements such as C, Ca, and Br with about 16.17% comprise of the total weight. These elements may be eradicated and removed by the subsequent events of annealing temperature to get the final pure nanocomposite. Table (2) shows the quantitative atomic and weight percentage of the compositional elements such as MgO, ZnO, and tiO2 present in MZT nanocomposite..

Table (2): The EDX quantitative atomic analysis and weight percentage of the compositional elements present in MgZnTiO4 nanocomposite

Element	Weight %	Atomic%
O	35.01	48.95
Mg	6.61	6.09
Ti	18.49	8.64
Zn	23.71	8.11
(C, Ca and Br)Elements	16.18%	28.21
Totals	100%	100%

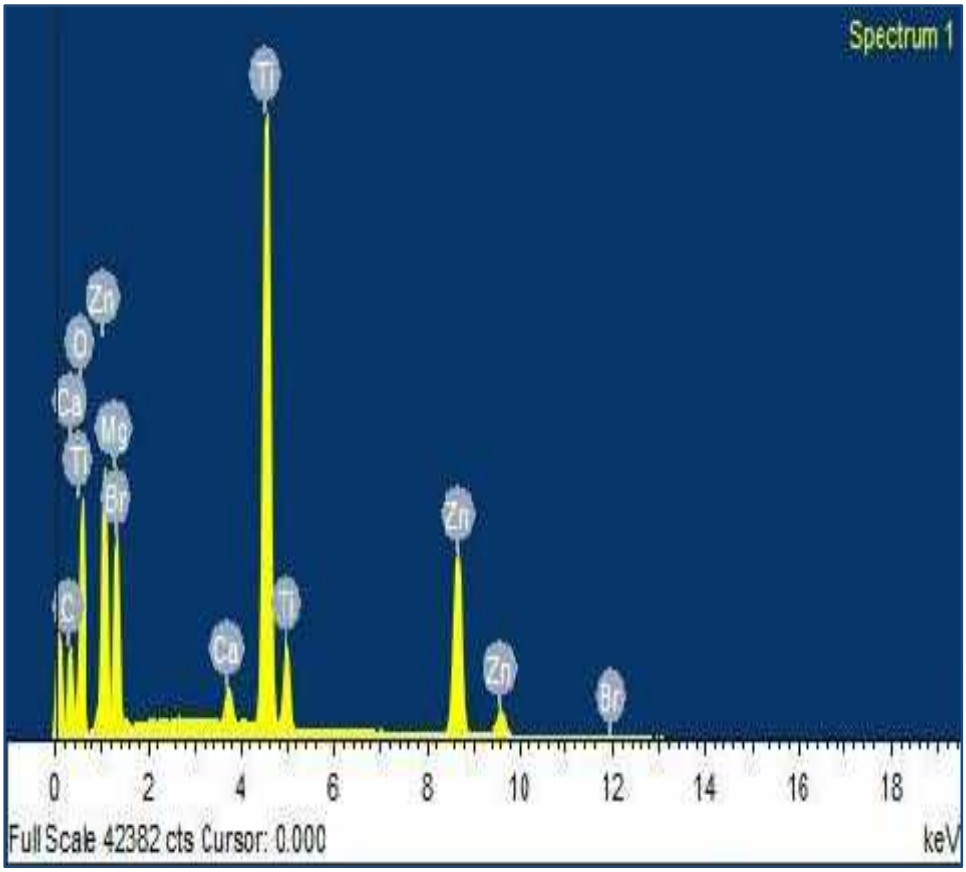

Fig. (2): EDX MZT nanocomposite analysis

Table (3): Cytotoxicity of MgZnTiO4 nanocomposite presented by IC50 and R^2 values

Drug/Compound	IC50 (µg/ml) by Log	R^{2*}
MZT-polymer nanocomposite	5.698	0.6968
DOX	27.409	0.4705
5FU	62.84	0.736

*DOX=Doxorubicin; 5FU=5-Flourouracil; IC50= Half maximal inhibitory concentration; R2 = correlation coefficient

Anti-cancer effect of MZT nanocomposite

Anticancer activity of MZT-polymer nanocomposite was performed by MTT assay on LS 174T colorectal adenocarcinoma Cell Line. The synthesized composite is selected and applied for anticancer activity. The compound showed significant anticancer activity against LS 174T colorectal adenocarcinoma Cell Line. Its value was equal to 5.698 µg/ml which is a significant value ($p<0.0005$) as shown in Fig. (6). This study was dealing with MZT-polymer nanocomposite for the assessment of cytotoxic activities against LS 174T colorectal adenocarcinoma Cell Line by DOX and 5-Flourouracil for comparison. (Fig. 7 & Fig. 8), In this study, MZT-polymer nanocomposite structure have offered a new medicinal agents with improved potency, good pharmacological actions, lesser toxicity, and hope to be proven as a useful

therapeutic compound, that may consequently become an interest in both industry and academia. Table (3) shows the results of MZT nanocomposite, DOX and 5- FU IC50 and R^2. The MTT tetrazolium reduction assay was the first corresponding homogeneous cell viability assay developed for any therapeutic agent potency measurement by a 96-well format that was valuable and suitable for high throughput screening [5]. Results showed that MTT assay was successful and valuable for the measurement of MZT-polymer nanocomposite, anticancer potency. Recently, MTT assay has been conducted to measure the cytotoxicity (IC50 values) of the combination drug therapy with DOX against the cancer Cell Lines [21]. Results showed that MZT-polymer nanocomposite may have the anticancer potency that exceeds DOX, or 5-Flourouracil, and the fact that gave us a hope to use MZT-polymer nanocomposite as an alternative anticancer in the future

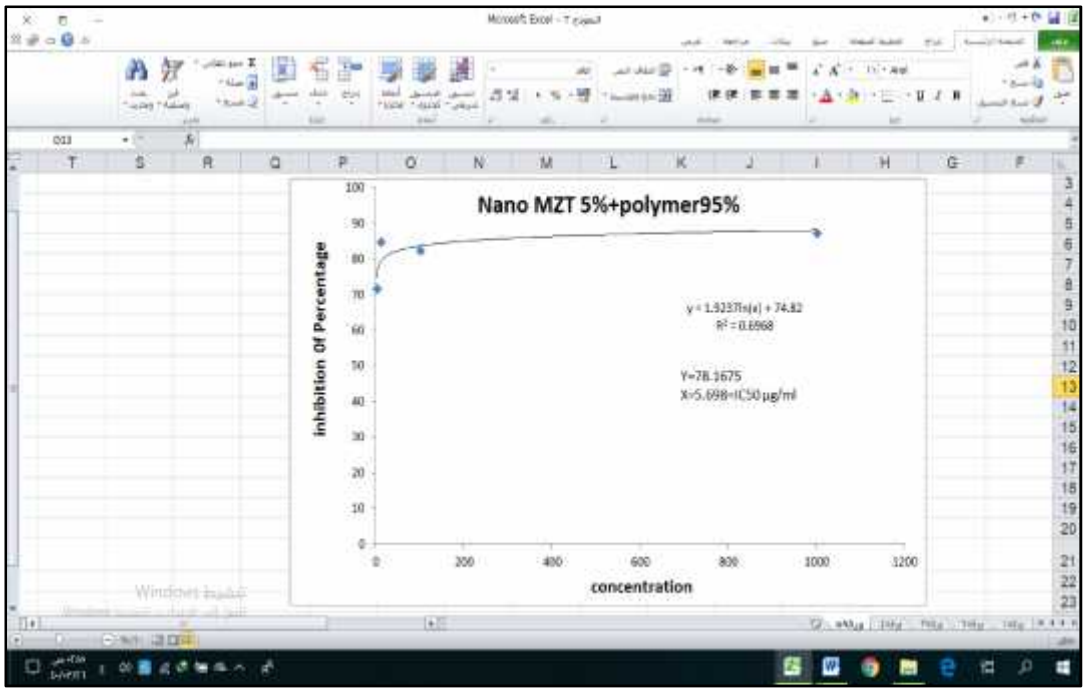

Fig. (3): Fig.(6): Anticancer activity of MZT-polymer nanocomposite presented by plotting of drug concentration versus inhibition% values

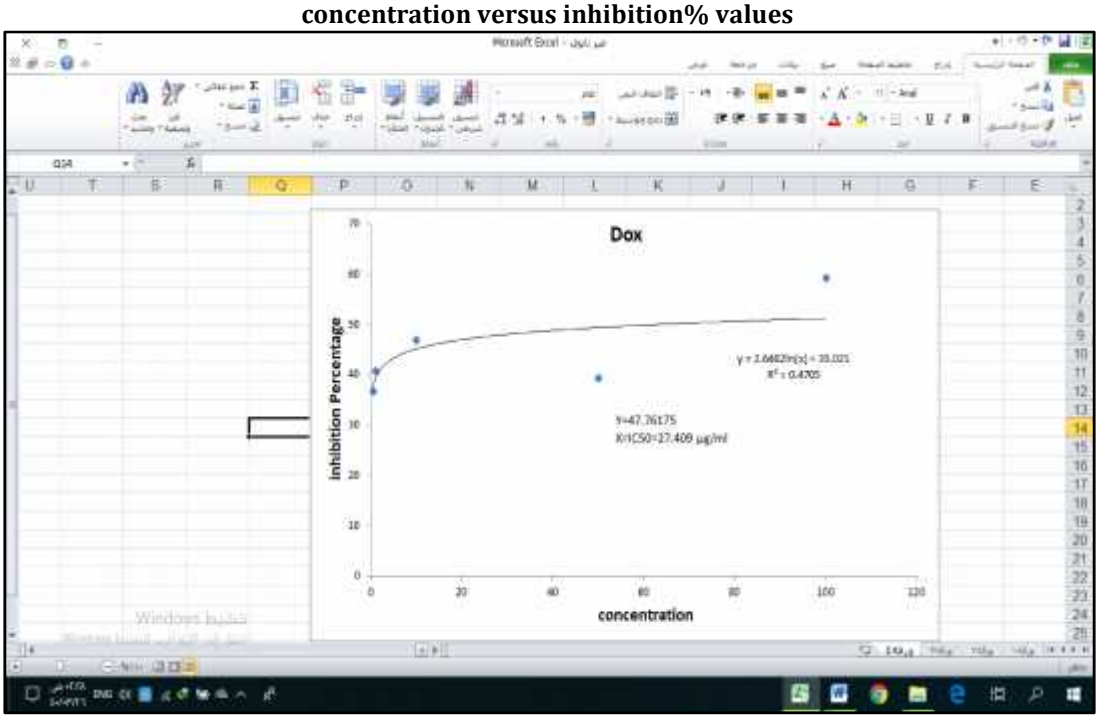

Fig.(4): Anticancer activity of Doxorubicin presented by plotting of drug concentration versus inhibition% values

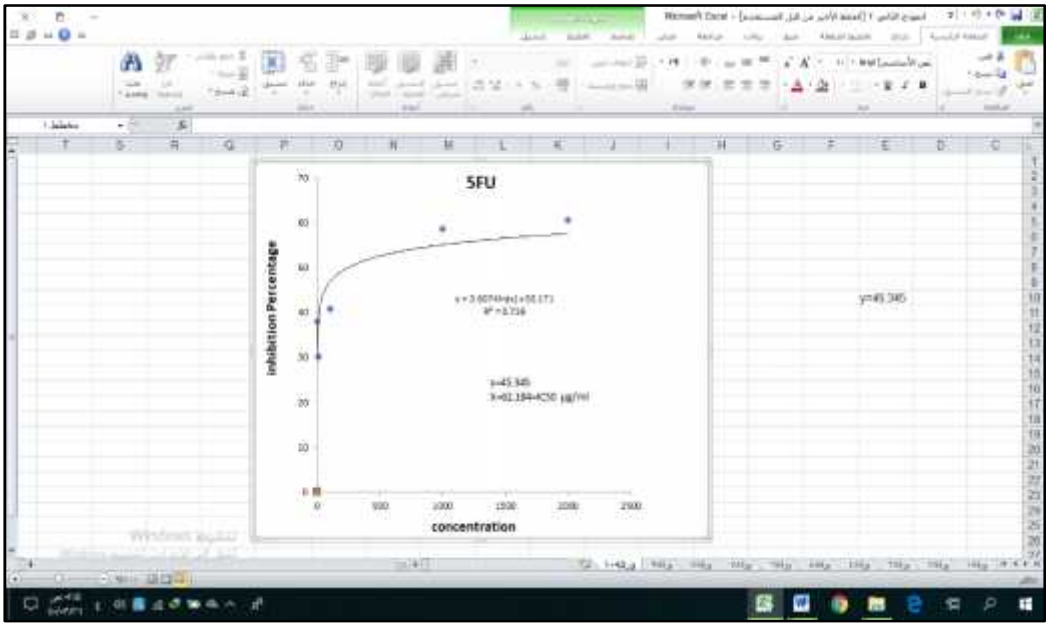

Fig.(5): Anticancer activity of 5-Flourouracil presented by plotting of drug concentration

In 2014 World Health Organization reported that 8.2 million people dead with colorectal cancers, lung, and breast cancer that identified as the most common worldwide problems in 2012 [32][33]. Current cancer treatments rely on chemotherapeutic agents and radiation that work in the body by killing rapidly dividing cells. The main drawback of conventional chemotherapy is the adverse effects on the body as it lacking selective and specific deliver action to the cancer cells, thus damage extending to the surrounding normal healthy tissues or rapidly dividing healthy cells such as the bone marrow cells, gastrointestinal tract, hair follicles, causing issues like cardiac, hepatic, pulmonary, gastrointestinal, and renal toxicities [34,35,36]. Drug delivery systems offer numerous advantages over chemotherapy such as targeted delivery at disease site, sustained release leading to prolonged bioavailability, lower required dosage and improved drug solubility among others [35,36,37]. Significant effect has been made by the nanotechnology application in medicine for the nanotech agent's development which can diagnose and cure the diseases, simultaneously [38]. Nanocarriers variety have been designed and successfully applied for the therapeutic agents' delivery such as GALLIC Acid formulation (Fe_3O_4-PEG-GA), graphene oxide, polymers-based delivery systems, layered double hydroxides, and gold nanoparticles [35-42]. Cytotoxic drugs can be efficiently delivered to tumor tissues using Nan carriers like nanoparticles which depend on difficult concepts of pharmacology. Multiple drugs can also be developed at the cancer site by nanomedicine which presents better cytotoxic effects. Nanomedicine also presents a targeted chemotherapeutic method which is still a developing field. This field enables the selective drugs delivery at the cancer site, due to increased permeability of the blood vessels at the tumor sites. The anticancer nanomedicine scope [27] is considered in this study. The 5-FU drug uses for cancer treatment by inhibiting essential biological processes. Recently understanding of the treating mechanism of 5-FU has led to the new ways development that raises its activity, Incorporating ZnO into 5-FU surface increased the killing rate of cells with great extent. Radical scavenging study shows that antioxidant ZnO-5-FU activity is more than pure 5-FU and inhibition% that increased by ZnO increasing that is incorporated until ratio reached to 10% where the surface of 5-FU fully coated with ZnO nano particles[28]. Recently, several ZnONPs are investigated for bacterial treatment and fungal diseases [12]. It also increases apoptosis in aged cells and also cancerous tissues [24]. They also are used for cosmetic formulation, ingredients, and food additives. However, most of the ZnO NPs pharmacological potentials are not still utilized [23, 12]. This came in agreement which was mentioned by other researchers that nanopartcles may exhibit an interesting profile of anticancer activity against cancerous Cell Line. ZnONPs significantly reduced the elevated serum levels of HCC-that are related to tumor markers alphafetoprotein, alpha-l-fucosidase, and the apoptotic marker caspase-3 in compared to the untreated HCC rats. In addition, treatment with ZnONPs decreased the elevated levels of hepatocyte integrity and oxidative stress markers as compared with the untreated HCC control group. Furthermore, the histopathological study reveales anaplasia and fibrous degenerations which were significantly corrected by ZnONPs treatment. [24, 3, 4]. The applying efficient synthetic methods based on scaffold methodology and monitoring about the role of MZT-polymer nanocomposite as a

fluorophores for live-cell imaging is recommended. PVA is one of the best known polymers. It is a polymer with several interesting physical properties, which are very useful in technical applications. It has been used in various applications, and is used extensively in semiconductors applications, medical purposes, making paper, and textile industries in the manufacture of membranes resistant to oxygen in the coating photographic film [29]. The advantage of poly vinyl alcohol is the ability to blend into the water and is resistant to do solvents and oils [30]. It is produced commercially by the hydrolysis of poly (vinyl acetate). Because of its unique properties of solubility and biodegradability as well as biocompatibility it finds several applications and its utilization is growing steadily in recent years [9]. Polyvinyl pyrrolidone (PVP) is also one of the hydrophilic, biocompatible polymers and it is used in many biomedical applications [31].PVP polymers are available in several viscosity grades, ranging from low to high molecular weight that couples with solubility in aqueous and organic solvent systems and combines with its non-toxic character. Its industrial applications include, for example, adhesives to improve strength, detergents, and soaps, the textile, ceramic, electrical, metallurgical industries, and as a polymerization additive. Polymer products are used in the pharmaceutical industry [31]. As shown in Fig.(5-J), the polymer containing media seems to be not hazardous to cells and the confluent monolayer still with the same viability and morphological characteristics of untreated control cells (Fig.4-A). This result has an advantage to the polymer for being used as a structural complementary molecule favoring the nanocomposite with polymerization. This came in agreement with that mentioned by other publications. [31]. Molecular docking is very important that should be used in future studies in order to explore suitable substitution patterns on the MZT-polymer nanocomposite scaffold to obtain selective and highly active inhibitors or modulators against specific receptors of CRC cells. This study describes the usefulness of MZT-polymer nanocomposite as valuable agents for various applications in the cancer therapy field. It was concluded that MZT-polymer nanocomposite have an anticancer effect on LS 174T colorectal adenocarcinoma Cell Line.

Conclusion

In conclusions, from the sequencing results, all three selected isolates were identified as LAB. However, according to the results obtained in probiotic tests analysis, these LABs do not have high probiotic potentials as compared to the positive probiotic strains, Lactobacillus acidophilus. Therefore, more goat's milk samples from different goats species as well as different localities should be screen to find potential probiotic strains from local goat's milk.

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