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In vitro assessment of cytotoxic and genotoxic activities of the anticancer drug doxorubicin

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Abstract
An antibiotic doxorubicin is generated from the bacterium *Streptomyces paucities*. Since-1960s, it has been used extensively as a chemotherapeutic drug. Doxorubicin belongs to the class of chemotherapy drugs known as anthracyclines. Doxorubicin (Dox) is an effective anticancer medication for solid and hematologic tumors. However, it can poison multiple organs in a variety of people. In this work, the effects of different doxorubicin doses (100,50,25,12.500,6.250 and 3.125 µg/ml) on cell viability were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method in the cell lines HCT116 (colon), PC3 (prostate), Hep-G2 (human hepatocellular carcinoma), and 293T (human embryonic kidney as the control). PI staining and Annexin V/PI staining were used in flow cytometry to measure cell cycle arrest and apoptosis, respectively. The findings demonstrated that cytotoxicity was caused by doxorubicin in HCT116, PC3, Hep-G2, and 293T. IC_{50} was 24.30, 2.640, 14.72, and 13.43 µg/ml, respectively, using various concentrations. These findings confirmed that doxorubicin has cytotoxic effects on both cancerous and non-cancerous cells by reducing cell viability. Using flow cytometry to measure the cell cycle, and apoptosis, it was discovered that doxorubicin damaged PC3 cells with a considerable increase in apoptosis and cell cycle arrest in the G2/M phase. In addition, when PC3 cell lines were treated with a high dosage of doxorubicin, the mRNA expression levels of *p53* and *Casp3* dose genes whereas *Bcl-2* fell off. At the same time, the effects of doxorubicin on various yeast knockout strains (YKO) were investigated. To determine the three various doxorubicin concentrations, the Comet assay was performed because doxorubicin might lead to DNA damage. The genotypes of YKO were chosen using the Clustal Omega Multiple Sequence Alignment (EMBL-EBI) of saccharomyces and human gene sequence homology. The comet assay showed improved yeast cell sensitivity, which was unquestionably confirmed.
Keywords: Doxorubicin, Comet assay, RT-PCR, flow cytometry, apoptosis, and cell lines.

Introduction

Different drugs have different mechanisms of action and effects on cancer cells. For example, methionine-coated magnetite nanoparticles can deliver letrozole, an aromatase
inhibitor, to breast cancer cells and induce cell death [1]. Other drugs, such as cisplatin, can damage DNA and trigger immune responses that enhance the efficacy of immunotherapy [2]**.**

Doxorubicin is an anthracycline antibiotic that can cause genetic damage by intercalating with DNA and generating free radicals [2-3]**.** It can bind to both nuclear and mitochondrial DNA, leading to DNA strand breaks and apoptosis of cancer cells [3]**.** However, doxorubicin also has toxic effects on normal tissues, especially the heart [2-3]**.**

The choice of evaluated method depends on the type of drug and the cancer model used [5]. Cytotoxicity of a drug can be assessed by various methods, such as cell viability, apoptosis, or necrosis assays. Cytologically, oxidative DNA damage (DAPI labeling) and mitotic disruption were assessed, as well as genotoxicity using the comet assay test and micronuclei generation. Cellular viability is unaffected by Bisphenol A (BPA) exposure in and of itself, according to a cytotoxicity investigation. But the genotoxic research revealed that BPA caused oxidative damage to cell lines in the Medical Research Council cell strain 5 (MRC-5) and increased DNA damage in the human epithelial cell tumor (Hep-2) cell lines. Following exposure to BPA, more micronuclei were seen in both cell lines [6]**.**

Cytotoxicity is a measure of how a drug is toxic to cancer cells *in vitro*. Genotoxicity test is a technique that evaluates the potential of a chemical substance to cause DNA damage or mutations, which may lead to cancer or other genetic defects. *In vitro*, genotoxicity tests use bacterial or mammalian cells to assess the effects of a chemical on DNA or chromosomes. Some of these tests require metabolic activation systems, such as S9 mix or human *CYP* genes, to simulate the biotransformation of the chemical in the body. Anticancer drugs are a class of

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chemicals that often show positive genotoxic findings in the standard battery of *in vitro* tests but may not pose a risk to human cells at therapeutic doses. Therefore, comprehensive genetic toxicology screening and healthy volunteer studies are needed to demonstrate the safety of anticancer drugs with genotoxic findings [7,8-9]**.**

Chromosomal abnormalities, mitotic index, and micronuclei production were used as toxicological endpoints to assess the genotoxic effects of doxorubicin on Swiss mice bone marrow cells. These findings demonstrated that doxorubicin affects mouse bone marrow cells in a genotoxic as well as cytotoxic manner [10]**.**

According to [11]**,** the primary regulatory mechanism of cell growth is the control of cell division; therefore, cell cycle analysis is a novel and effective strategy for controlling and eliminating cancer. Apoptosis and the cell cycle are related, and together they influence how sensitive cancer cells are to chemotherapy; on the other hand, apoptosis was indicated by the concentration of cells in the subG1 phase [12].

A fifteen-year-old Caucasian male with a diagnosis of hepatocellular carcinoma donated his liver tissue to create the human liver cancer cell line Hep-G2. Over 350,000 people received a liver cancer diagnosis in 2013, while more than 160,000 of these individuals passed away as a result of the disease [13].

Confirmed that doxorubicin has a genotoxic effect on Hep-G2 cell lines. Melatonin and its nanoparticles considerably reduced the doxorubicin-induced genotoxicity in both types of comet assay experiment [14]. Furthermore, Hep-G2 cell lines treated with both forms of melatonin produced fewer intracellular reactive oxygen species and had higher intracellular glutathione concentrations. Nano melatonin, however, was more successful in reducing oxidative stress, while DNA damage brought by doxorubicin was recorded.

Examined the development, genotoxicity, and apoptosis effects of some food addictive of human cell lines *in vitro.* In the human cell lines, genes linked to apoptosis and cell cycle arrest were also investigated [15]. Moreover, [16], [17] confirmed the lethal effect of the compounds and showed that *Saccharomyces cerevisiae* cells were more susceptible, both cancerous and non-cancerous cells were stopped from proliferating by those specific compounds found in food. The comet assay was used to study the effects of chemicals on various yeast knockout strains to determine the optimal doses at which this combination of dietary chemicals could cause DNA damage.

Determined the optimal extraction solvent, they evaluated the potential anticancer activity of *T. nilotica* crude extract using ethyl acetate towards human lung cancer (A549) cell lines [18]. The plant's extract proved to be highly effective against human lung cancer (A549), according to the results. The cytotoxic activity of ethyl acetate crude extract on A549 and Wi38 cell lines, as determined by the Neutral Red assay, showed no effect on normal cell lines (Wi38). On the other hand, a more marked decline in the viability of cells on human cancer cell lines indicated that it caused cytotoxicity on cancer cell lines (A549).

Used the MTT test to investigate the potential cytotoxicity of two species, *Z. album* and *S. palaestina* treatment of Hep-G2 cells resulted in a significant augmentation of G2/M phase cell cycle arrest, as shown by IC50 flow cytometric analysis. The mRNA was measured using real-time PCR. Hep-G2 cells exhibited a down-regulation of Cyclin B1 and CDK1 levels of expression in comparison to the cells not treated [19].

Determined how different doses of cadmium chloride affected the viability of hepatocellular carcinoma (Hep-G2), lung cancer (A549), and healthy lung cells (Wi38) (control) cell lines. Improved yeast cell sensitivity was obtained in the comet experiment, and this has been unequivocally confirmed [20].

This study aimed at evaluating the cytotoxic and genotoxic properties of the anticancer medication doxorubicin *in vitro*.

Materials and Methods

Materials

1. **Doxorubicin drug**
   - 1.1. **Properties**
     Physical properties: Doxorubicin hydrochloride is an orange-red lyophilized powder that is stable for 2 years when stored at room temperature and protected from light, its molecular weight is 543.522 Da [21].
   - 1.2. **Concentrations:** Doxorubicin concentrations used were 100, 50, 25, 12.500, 6.250 and 3.125 µg/ml.
2. **Cell lines**
   - 2.1. **Mammalian cell lines:** The American Type Culture Collection (ATCC, Rockville, MD) provided the tumor cell lines HCT116 (Colon cell lines), PC3 (Prostate cell lines), Hep-G2 (Human hepatocellular carcinoma cell lines), and 293T (normal human embryonic kidney cell lines).
   - 2.2. **Lonza:** (Belgium) provided the fetal bovine serum, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin, and 0.25% Trypsin-EDTA as a medium for cell lines.
   - 2.3. **Cell lines propagation:** On RPMI-1640 medium treated with 10% inactivated fetal calf serum and 50 g/ml gentamycin, the cell lines were cultured. The cell lines were sub-cultured two to three times per week.

2. Methods

2.1. Cytotoxicity evaluation

Cytotoxicity evaluation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was applied. The tumor cell lines were placed in Corning 96-well cell culture plates at a concentration of $5x10^4$ cells/well for antitumor tests, and they were then incubated for 24 hours. The three replicates of the doxorubicin concentrations were then applied in 96-well plates. Dimethyl sulfoxide (DMSO) 0.5% was used as the control. The MTT test was used to assess the number of viable cell lines after 24 hours of incubation according to[22].

2.2. Cell cycle analysis by propidium iodide (PI) assay using flow cytometry

Cell lines were digested for 10 minutes at 37°C using warm trypsin-EDTA and warm phosphate buffer saline (PBS), ethylene diamine tetra acetate (EDTA) (0.25%) (500 µl + 500 µl), Then all steps were applied according to[15]. In attune flow cytometry, the stained cell lines were read (Applied Bio-system, USA).

2.3. Apoptosis analysis by Annexin V-FITC assay using flow cytometry

After centrifuging at 1-5 ×10^5 cells were collected, and the supernatant was discarded. Then, the collected cells were washed twice in warm PBS buffer and re-suspended in 500 µl of 1X binding buffer. Propidium iodide (PI) 5 µl and 5 µl of Annexin V-FITC should be added, and the mixture was then incubated at room temperature for 5 min in the dark [23]. Utilize flow cytometry to examine Annexin V-FITC binding (Applied Bio-system, USA).

2.4. Assessment of genotoxicity induction of some related genes; *Casp3*, *Bcl-2* and *p53* in HepG3 cell lines by doxorubicin using RT-PCR (Quantitative RT-PCR

Using the Gene JET RNA Purified Kit (Thermo Scientific, # K0731, USA), total RNA was extracted from PC3 in accordance with the manufacturer's instructions. To create cDNA, total RNA (5 µg) had been reverse-transcribed utilizing Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) according to [24]. Using the Step One Plus real-time PCR system (Applied Biosystems, USA), the relative expression of the genes associated with apoptosis was assessed using the cDNA as a template. Primer 5.0 software program was used to design the primers. Used forward and reverse primers for *Casp3*, *Bcl-2*, *p53* genes, and *GAPDH* as house-keeping gene, and their sequences are shown in Table (1).

Table (1): Forward and reverse primer sequences for *Casp3, Bcl-2, p53,* and *GAPDH* genes.

Gene	Forward primer (5′→ 3′)	Reverse primer (3′→ 5′)
Casp3	TTCATTATTCAGGCCTGCCGAGG	TTCTGACAGGCCATGTCATCCTC
Bcl2	CATGCAAGAGGGAAACACCAGA	GTGCTTTGCATTCTTGATGAGGG
p53	AGAGTCTATAGG CCACCCC	GCTCGACGCTAGGATCTG AC
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Target genes and the housekeeping gene's cycle threshold (Ct) values were obtained, and the $2^{-\Delta\Delta Ct}$ method was used to quantify relative gene expression [25].

5. Yeast comet assay (YCA)

In vitro, Comet assay was conducted according to [17,25]. Using doxorubicin on yeast culture media at doses of 25, 50, and 75 µg/ml additionally, a medium devoid of chemical components was used as an untreated control.

5.1. Knockout yeast strains of choice

In this study, the sequences of each strain have been selected and linked with human sequence data stored in NCBI (The National Centre for Biotechnology Information) which used as knockout yeast strains with completely different genotypes. To match the used yeast genes, four genes that are linked with human genes related to cancer were selected (Table 2).

5.2. Selection of yeast strains deficient in genes like human cancer genes

Based on Clustal Omega Multiple Sequence Alignment (EMBL-EBI alignment) between human and yeast sequence similarity (Table 2), the genotypes of yeast (knockout) strains were selected.

Table (2): Selected yeast genes (from different strains) which matched with cancer-related human genes.

Selected yeast strain	Selected genes of yeast strain	Homologous genes in human
YMR039C	*SUppressor of TFIIB mutations (SUB1)*	*SUppressor of TFIIB mutations (SUB1)*
YMR156C	*Three Prime Phosphatase (TPP1)*	*polynucleotide kinase3' phosphatase (PNKP)*
YMR166C	*Mitochondrial Magnesium Exporter (MME1)*	*Membrane metalloendopeptidase (MME)*
YMR040W	*Yeast Endoplasmic reticulum Transmembrane protein (YET2)*	*B Cell Receptor Associated Protein 31 (BCAP31)*

5.3. Protein-protein interaction prediction

The interaction network was used in line with the order. MANIA (http://www.genemania.org) is a flexible and user-friendly web interface for analyzing sequence lists, prioritizing genes for particular studies, and assessing gene function hypothesis.

6. Statistical analysis

All data were presented as means with standard errors. One-way analysis of variance (ANOVA using SPSS 18.0 software, 2011) was used to determine the statistical significance, Half maximal inhibitory concentration (IC_{50}) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function, IC_{50}= (0.5 - b)/a. while b= the intercept b for fitting your data, a = slope factor.

Results and Discussion

1. Cytotoxicity evaluation

Cytotoxicity evaluation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was applied. In respect to the concentration of doxorubicin doses, cell viability generally declined gradually as seen in Table (3). Doxorubicin's cytotoxicity and cell toxicity increased, as the concentration of the tested doxorubicin increased, while the viability of all cell lines decreased. Table (3) and Figure (1) showed the dose-response curves for cell viability and the dose eliciting 50% cell growth inhibition (IC_{50}) for HCT116, PC3, Hep-G2, and 293T cell lines.

Tetrazolium, which was utilized as a positive control in the current investigation, had its IC_{50} levels tested against each dose of doxorubicin. The results demonstrated that doxorubicin concentration produced slight cytotoxicity in all cancer cells (Table 3), including human colon cancer (HCT116) which gave IC_{50} = 24.30 µg/ml, human hepatocellular carcinoma (Hep-G2); IC_{50} = 14.72 µg/ml. prostate cell line (PC3) showed IC_{50} = 2.64 µg/ml. Doxorubicin also demonstrated a cytotoxic effect against human embryonic kidney normal cell lines (293T) as the control where IC_{50} = 13.43 µg/ml (Table 3 and Fig. 1). These results revealed that doxorubicin had a cytotoxic impact and reduced cell viability in both malignant and non-malignant cells.

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Table (3) Effect of different doxorubicin concentrations on carcinoma cells and normal cells (control).

Cell line	Dox. Conc. µg/ml	Viability %	Sd	Toxicity % cytotoxicity	IC50 µg/ml
HCT116 [Colon carcinoma cell line]	100.000	7.666	2.616	92.333	**24.30**
	50.000	28.633	3.201	71.367	
	25.000	42.933	5.259	57.067	
	12.500	77.633	1.801	22.367	
	6.250	91.767	1.514	8.233	
	3.125	96.633	0.907	3.367	
PC3 [Prostate cancer cell line]	100.000	0	0	100	**2.640**
	50.000	0	0	100	
	25.000	0	0	100	
	12.500	16.033	3.656	83.967	
	6.250	23.833	2.136	76.167	
	3.125	44.667	2.301	55.333	
HepG 2 [Human hepatocellular carcinoma cell line]	100.000	0	0	100	**14.72**
	50.000	11.633	2.572	88.367	
	25.000	14.867	0.010	85.133	
	12.500	63.567	2.747	36.433	
	6.250	88.567	0.902	11.433	
	3.125	96.567	0.586	3.433	
293T [Human embryonic kidney] (control)	100.000	0	0	100	**13.43**
	50.000	11.633	2.572	88.367	
	25.000	36.033	4.038	63.967	
	12.500	52.067	3.001	47.933	
	6.250	70.633	3.907	29.367	
	3.125	89.233	1.343	10.767	

Sd= standard deviation **IC_{50}=**The half-maximal inhibitory concentration

The results showed worth accumulation of the different doxorubicin concentrations in human colon cancer (HCT116), prostate cell line (PC3), hepatocellular carcinoma (Hep-G2) and human embryonic kidney as the control. Doxorubicin was the most effective on the viability reduction of HCT116 cell line among the four cell lines where the other concentrations had marked effect, while PC3 was the lowest without considerable change. These results are in agreement with [16] who treated four distinct human cells included colon carcinoma (Caco-3), breast carcinoma (MCF7), lung carcinoma (A549), and normal lung cell line (Wi38) with some food additives and confirmed the presence of substantial difference in the ability to survive and morphology of the cell lines among the control and treated groups supported the idea that these substances have a carcinogenic effect. At the same time, [24] confirmed the effect of heavy metal cytotoxic activity on proliferation of Hep-G2, A549, and Wi38 carcinoma cells using the MTT cytotoxic in comparison with the positive control.

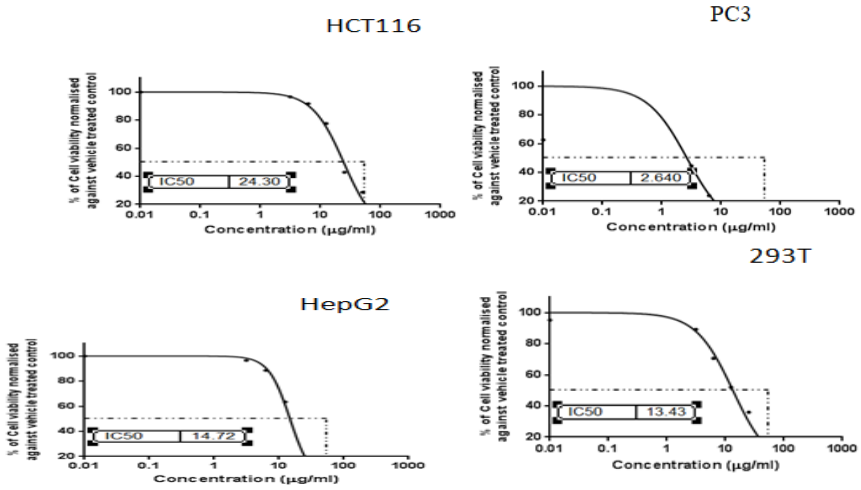

Figure (1): Inhibition of growth caused by doxorubicin concentration, Malignant human cells are HCT116, PC3, HepG2, and non-malignant human lung cells 293T (control). MTT assay was used to measure viability. Results are expressed as the mean (n = 3); *p= 0.05 or less when compared with the control. IC_{50} was measured for the four cell lines.

2. Cell cycle analysis by PI assay using flow cytometry

The DNA content average of PC3 cell line was affected by doxorubicin at dosage 50 µg/ml. The G0/G1 phase showed a decline in DNA content average from 51.03 (control) to 46.23% (treatment) (Table 4). The S phase showed the same results for decline in DNA content average from 44.13 (control) to 38.61 (treatment). As shown in Table (4), the DNA content average of PC3 cell line treated with doxorubicin (15.16) increased when compared with the control (4.84) during the G2/M phase and pre-G1 phase from 2.03 (control) to 11.38 (treatment). Additionally, doxorubicin at 50 µg/ml had an impact on PC3 cell line during cell cycle distribution. In comparison to the control, the G0/G1 and S phases exhibited a modest decline. However, there was a sharp increase in the proportion of PC3 cell lines in the G2/M phase. These findings verified that doxorubicin has a notable lethal impact by causing the cell cycle's G2/M phase arrest as seen in Figure (2) and revealed a considerable DNA concentration of PC3 cell line in the G2/M phase.

Table (4): Average of DNA content% in each cell cycle phase using PC3 cell line alone as the control and PC3 cell line with 50 µg/ml doxorubicin as treatment.

Groups	Average of DNA content%, each in cell cycle phase			
	G0/G1 phase	S phase	G2/M phase	Pre-G1
PC3 -control	51.03	44.13	4.84	2.03
PC3-treated with doxorubicin	46.23	38.61	15.16	11.38

A. Untreated PC3 cell **B. Treated PC3 cell**

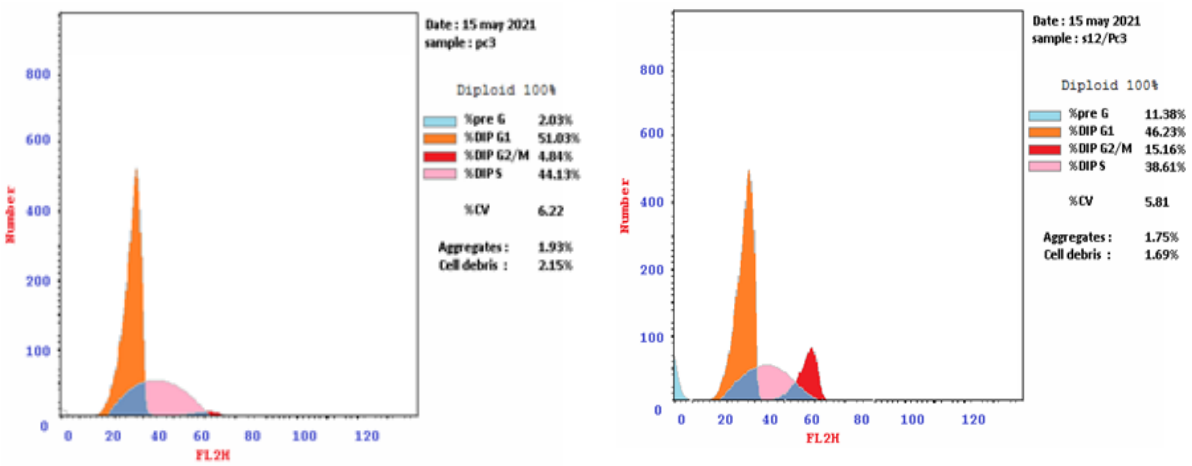

Figure (2): A. PC3 prostate cancer cell line – untreated (control), B. Prostate cancer cell line (PC3) after treated with doxorubicin at a concentration of 50 µg/ml and effect at G2/M cell cycle arrest.

The current work's results verified that doxorubicin has a cytotoxic impact by inducing G2/M phase cell cycle arrest in PC3 cell lines, starting at 3.125 µg/ml and increasing to 100 µg/ml doxorubicin concentrations. Moreover, flow cytometric analysis showed that doxorubicin treatment increased G2/M phase cell cycle arrest in human PC3 cell lines. The cytotoxicity of doxorubicin varies significantly according on the studied concentrations: 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml, which are equal to human dosages.

[24] demonstrated a considerable concentration of Hep-G2 cells in the G2/M phase as well as the significant inhibition of cell growth caused by ZnSo4 through G2/M phase cell cycle arrest. Human cell growth, cytotoxicity, and programmable cell death (apoptotic) were studied in vitro. Genes associated with cell cycle arrest as well as apoptosis have also been studied in the CoCa-3 cell lines [15]. Analysis using flow cytometry revealed suppression of the Hep-G2 liver cancer cell viability rate. The results of [27] demonstrated a notable buildup of Hep-G2 cells in the G2/M phase and verified that additives cause the cell cycle to arrest in the G2/M phase, which has a harmful effect. Additionally, the rate of carcinoma of the liver (Hep-G2) survival of cells was inhibited by flow cytometry analysis, indicating that DBP (di-butyl phthalate)'s lethal effect on cell cycle arrest-based cell cycle distribution needed to be evaluated. Furthermore, the findings of [28] demonstrated a notable build-up of Hep-G2 cell lines in the G2/M phase and validated that DBP exerts lethal effects by inducing cell cycle arrest in the G2/M phase.

3. Apoptosis analysis by Annexin V-FITC assay using flow cytometry

The mitochondrial pathway and the caspase cascade are just two of the signaling channels that regulate the closely regulated process of apoptosis. Cell culture was used to examine the effects of doxorubicin on PC3 cell line at a dosage of 50 µg/ml to identify cell necrosis and apoptosis. Using Annexin V-FITC/PI double-labeled flow cytometry, apoptosis and necrosis were assessed (Figure 3). The percentage of early and late apoptotic cells was used to compute the apoptotic rate. As shown in Table (5), doxorubicin treatment of PC3 cell line resulted in a change in the apoptosis rate, which was 0.61% and 0.23% for cells that are apoptotic early and late, respectively. While the PC3 cell lines-treated with doxorubicin concentration for early and late apoptotic cells were 0.74% and 3.87%, respectively. PC3-control showed 1.19% necrosis which reached 6.77% in PC3-treated with doxorubicin. These findings revealed that doxorubicin showed considerable

difference affected PC3 cell lines in terms of apoptotic and necrotic effects.

The findings of the present investigation are generally consistent with earlier research showing that cadmium chloride causes the Hep-G2 cell line to undergo apoptosis. In contrast to the control, cadmium chloride-treated cells gradually increase the number of annexin-positive cells. [20] demonstrated that cancer and normal cell lines responded differently to doxorubicin-induced genotoxic stress, as evidenced by apoptosis and damage to DNA in these cells. PC3 was one of the cell lines that was also sensitized to doxorubicin, while A549 and LNCaP were just slightly or not at all sensitized. According to [29] **,** sensitization was more noticeable at 1 µM doxorubicin given for 48 hours than at lower levels and shorter treatments.

Table (5): Apoptotic and necrotic effect on PC3 cell line as control and treatment with 50 µg/ml doxorubicin from 24:48 hours.

Cell line treatment	Apoptosis %		Necrosis %
	Early	Late	Necrosis
PC3 -control	0.61	0.23	1.19
PC3 –treated with doxorubicin	0.74	3.87	6.77

A. Untreated PC3 cancer cell lines **B. treated PC3 cancer cell lines**

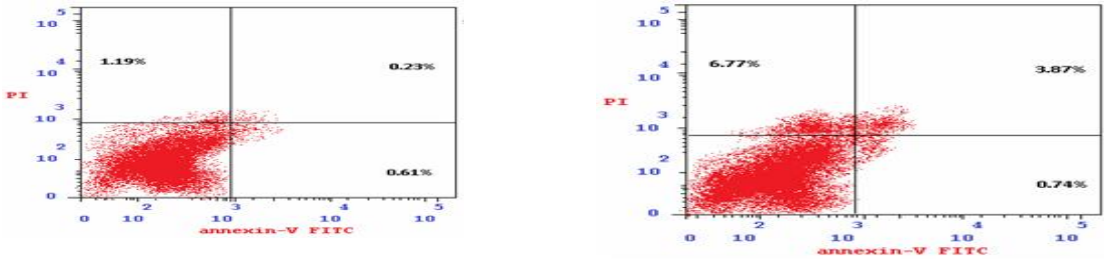

Figure (3): A. Untreated (control) prostate cancer cell line (PC3). B. treated prostate cancer cell lines (PC3) with doxorubicin concentration (50 µg/ml). Lower left (prostate cells) - upper left (necrotic cells) – lower right (early apoptosis) –upper right (late apoptosis).

4. Assessment of genotoxicity induction of some related genes; *casp3*, *Bcl-2* and *p53* in HepG3 cell lines by doxorubicin using RT-PCR (quantitative RT-PCR)

How doxorubicin-induced genotoxicity on PC3 prostate cancer cell lines was assessed. Real-time PCR (qPCR) was used to determine the amounts at which apoptosis-related genes including *casp3*, *p53*, and *Bcl-2* are expressed in PC3 cell lines. In comparison to the control, *Casp3* increased by 1.506, and *p53* by 1.219 (Table 6). The results of *Bcl-2* dropped by 0.390 compared to the control (Table 6) demonstrating that the expression levels of the *casp3* and *p53* genes were more in the treatment group in comparison to the untreated group (Figure 4). These results showed that doxorubicin killed PC3 cell lines primarily through upregulating the genes *casp3* and *p53* during apoptosis, while downregulating *Bcl-2*.

Doxorubicin may modify the RT-qPCR's reverse transcription and amplification reactions, raising the possibility of incorrectly interpreting the results of gene expression. Overall, the study's results are consistent with earlier research that demonstrated how differently doxorubicin-resistant and susceptible cells responded to genotoxic stress brought on by UV radiation. This was demonstrated by the cells' varying levels of ROS production, damaged DNA, and apoptosis [30].

Table (6): Effect of doxorubicin compound administration on the relative expression of *casp3*, *p53* and *Bcl-2* genes in PC3 cell lines using RT-PCR technique.

Sample data	Control cells			Test cells				FLD
Sample code (*Casp3*)	***GAPDH***	***casp3***	**ΔCTC**	***GAPDH***	***casp3***	**ΔCTE**	**ΔΔ CT**	$2^{-\Delta\Delta CT}$
	HC	**TC**	**TC-HC**	**HE**	**HE**	**TE-HE**	**ΔCTE-ΔCTC**	**Eamp=1.896**
PC3 treated	22.93	34.12	11.2	22.21	32.76	10.6	-0.64	1.506
PC3 control	22.93	34.12	11.2	22.93	34.12	11.2	0	1
Sample code (*p53*)	***GAPDH***	***p53***	**ΔCTC**	***GAPDH***	***p53***	**ΔCTE**	**ΔΔ CT**	$2^{-\Delta\Delta CT}$
	HC	**TC**	**TC-HC**	**HE**	**HE**	**TE-HE**	**ΔCTE-ΔCTC**	**Eamp=1.896**
PC3 treated cell line	22.93	33.42	10.5	22.21	32.39	10.2	-0.31	1.219
PC3 control cell line	22.93	33.42	10.5	22.93	33.42	10.5	0	1
Sample code (*Bcl-2)*	***GAPDH***	***Bcl-2***	**ΔCTC**	***GAPDH***	***Bcl-2***	**ΔCTE**	**ΔΔ CT**	$2^{-\Delta\Delta CT}$
	HC	**TC**	**TC-HC**	**HE**	**HE**	**TE-HE**	**ΔCTE-ΔCTC**	**Eamp=1.896**
PC3 treated cell	22.93	28.28	5.35	22.21	29.03	6.82	1.47	0.390
PC3 control cell	22.93	28.28	5.35	22.93	28.28	5.35	0	1

The values are gene being tested control (TC), housekeeping gene control (HC), housekeeping gene experimental (HE), Gene being tested experimental (TE).

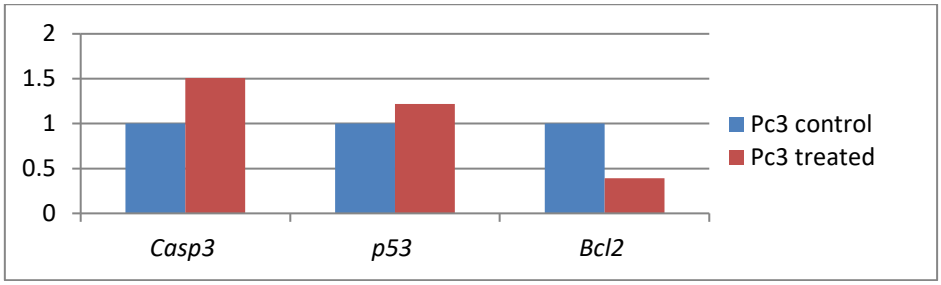

Figure (4): Effects of 50 µg/ml doxorubicin on apoptotic-related genes, mRNA expression of *casp3*, *Bcl-2* and *p53* was assessed by quantitative RT-PCR, *P < 0.05, compared to the control group.

5. Genotoxicity on yeast knockout strains (YKO) tested with doxorubicin by extra-terrestrial object assay

According to the alien object assay, doxorubicin has various degrees of yeast-specific genotoxic effects on YKO. The genotoxic impact of doxorubicin was demonstrated at doses of 25, 50, and 75 µg /ml. Genotoxicity was high for the *SUB1*, *YET2*, *TPP1* and *MME1* genes, but low for the *MMT1* gene sequence. Table (7) displays the breakdown of the share of identified comets for doxorubicin. It should be mentioned that the yeast produced significantly more comets than the different management for each of the four examined genes (Figure 5). demonstrating that the medication under test caused significant levels of deoxyribonucleic acid

[31] discovered that alone the highest dosage resulted in a noteworthy rise in oxidative DNA damage in the liver ($P < 0.05$). Eleven DNA damage and repair genes had their expression significantly altered, according to gene expression profiling of the cardiac tissues. These findings showed that doxorubicin is genotoxic to the heart and that reactive oxygen species generation may be the main mechanism causing DNA damage.

6. Selection of YKO devoid of genes similar to specific human cancer genes *in vitro*.

The genotypes of saccharomyces knockout strains were selected by means of genome comparisons between yeast and humans. Figure (6) displays the results of an destruction to be found. More precisely, since twice as many comets form in the controlled culture as in the treated culture, we typically use the three concentrations as a sensitivity measure instead of the methods. Each cell that had received micro gram linear units of doxorubicin had a pre-treatment with doxorubicin at the previous concentrations. It was evident that each of the four examined genes had been seriously harmed by doxorubicin therapy. These findings imply that doxorubicin is genetically toxic to Saccharomyces genes and that oxygen species that are reactive generation may be the main mechanism by which DNA damage is caused. alignment between the yeast and human SUB1 sequences. SUB1 is a transcriptional regulator that participates in nonhomologous

end-joining (NHEJ) of dissolves in plasmid DNA but not chromosomal DNA, facilitates elongation through factors that change RNAP II, contributes to peroxide resistance through Rad2p, recruit's polymerase at *RNAP II* and *RNAP III* genes to aid in the response to hyperosmotic stress, and negatively regulates sporulation. The results of these genes supported the findings of [17] and demonstrated the viability of a linkage between the human and yeast.

Table (7): Image analysis of comet assay parameters in cells of all groups after doxorubicin therapy.

Concentrations	Tail Length (px)	Tail DNA (%)	Tail Moment	Tail Olive Moment
Control *SUB1* (A)	**1.26**	**21.60208**	**0.342913**	**0.566601**
(25 µg/ml (A1)	4.12	27.89608	2.166781	1.806877
(50 µg/ml(A2)	4.54	25.28048	1.499524	1.862723
(75 µg/ml(A3)	12.64	30.48544	7.619874	5.460589
Control *TPP1* (B)	**3.14**	**16.5178**	**0.88856**	**1.338438**
(25 µg/ml (B1)	9.02	33.46624	5.191757	3.851707
(50 µg/ml (B2)	16.18	30.00267	9.87075	7.453799
(75 µg/ml (B3)	9.9	37.29237	5.384803	4.282819
Control *MME1* (C)	**2.14**	**24.61826**	**0.771117**	**0.964382**
(25 µg/ml (C1)	9.92	30.10626	5.568558	3.967005
(50 µg/ml (C2)	11.08	28.84174	6.010902	4.981345
(75 µg/ml (C3)	12.92	44.23602	7.966663	5.713019
Control *YET2* (D)	**6.6**	**32.13105**	**4.037921**	**3.378572**
(25 µg/ml (D1)	8.18	27.14059	3.79463	3.571177
(50 µg/ml (D2)	11.78	23.54841	6.559439	5.45859
(75 µg/ml (D3)	14.88	31.06162	8.034072	6.470201

Different letters (A, B, C and D) due to different groups as in figure showed considerable difference.

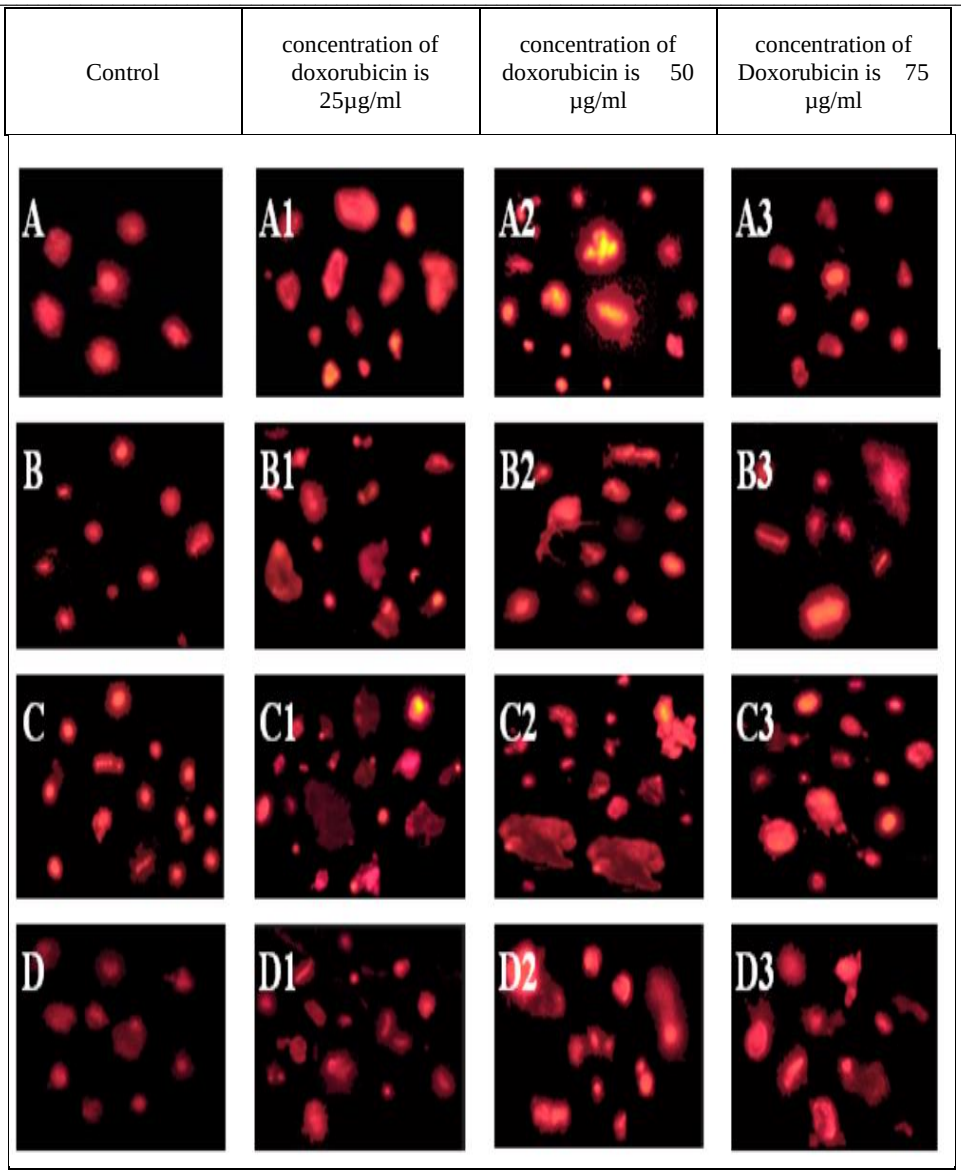

Figure (5) Using the Comet assay and doxorubicin at a dosage of 25, 50, 75 µg /ml, photomicrographs of DNA damage in yeast strains were taken. Cells that act as a check A represents the control of *SUB1* gene; A1, A2, A3 represent the treated *SUB1* gene; B represents the control of *TPP1* gene; B1, B2, B3 represent the treated *TPP1* gene; C represents the control of *MME1* gene; C1, C2, C3 represent the treated *MME1* gene; D represents the control of *YET2* gene; D1, D2, D3 represent the treated YET2 gene.

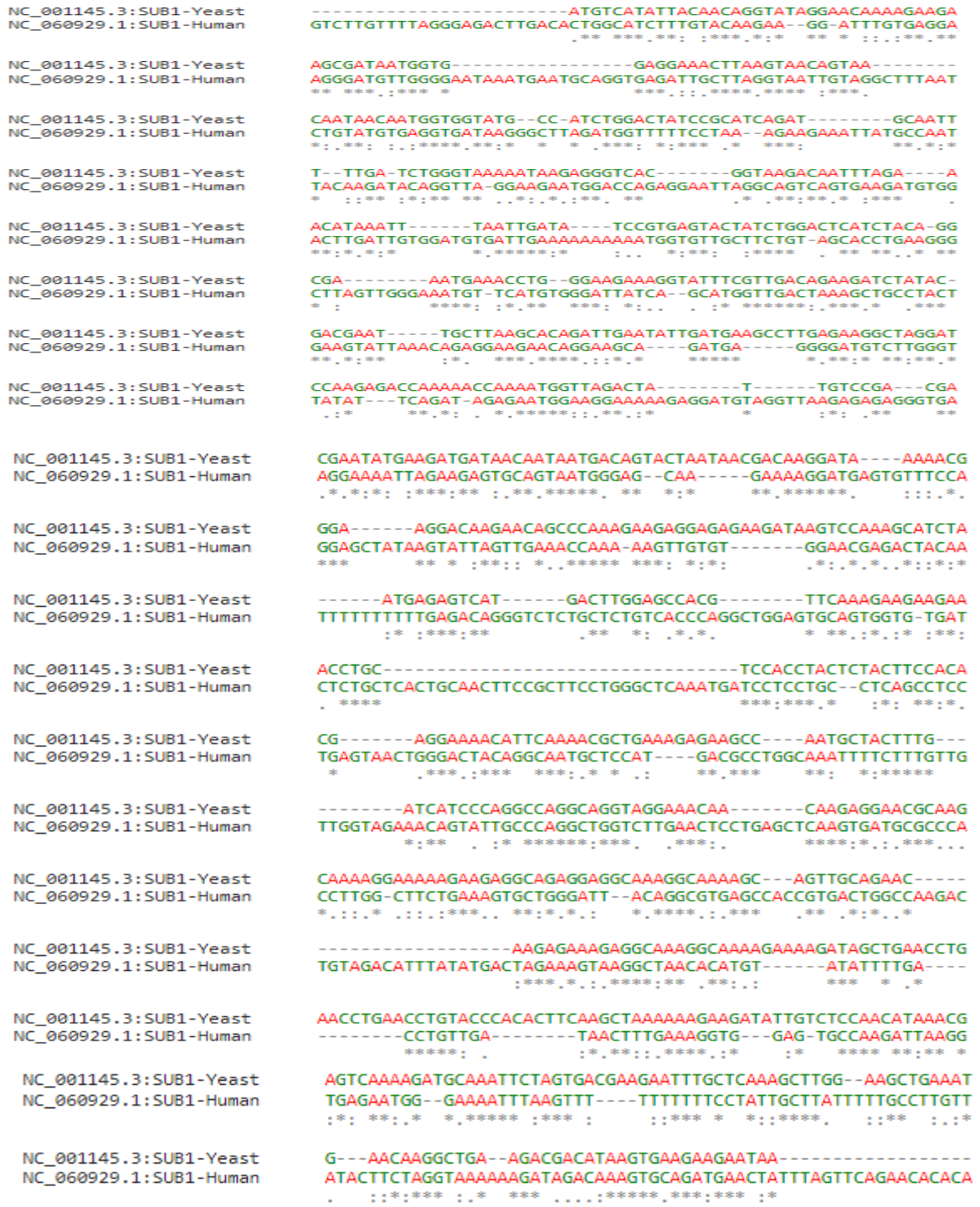

Figure (6) Gene alignment between human gene *SUB1* and the yeast *SUB1* in the Clustal Omega web site ('*' indicates identical between two aligned, '-' indicates gaps missing of one) and ('.' indicates low similarity, ':' indicates more similarity used to denote the level of similarity that are not identical) at position.

Furthermore, an alignment of the humans PNKP and the yeast TPP1 DNA 3'-phosphatase revealed that the latter is a homolog of human polynucleotide kinase/3'-phosphatase and repairs endogenous damage to double-stranded DNA. ts activity is specific to the removal of 3' phosphates at strand breaks and is comparable to the l-2-haloacid dehalogenase superfamily (Figure 7).

The alignment findings between the yeast MME1 and human MME sequences are displayed in Figure (8). Similar to human mitochondrial ATP-Mg/Pi carriers, this transporter of the inside membrane of the mitochondria transfers magnesium and is crucial in maintaining the balance of Mg2+ within the mitochondria**.**

The results of co-purification tests indicate that the yeast sequence YET2, which is homologous to the human BAP31 gene and has an unknown function that may interact with ribosomes, aligns with the human sequence BCAP31. YET2 also has a paralog, YET1, which originated via whole genome duplication (Figure 9).

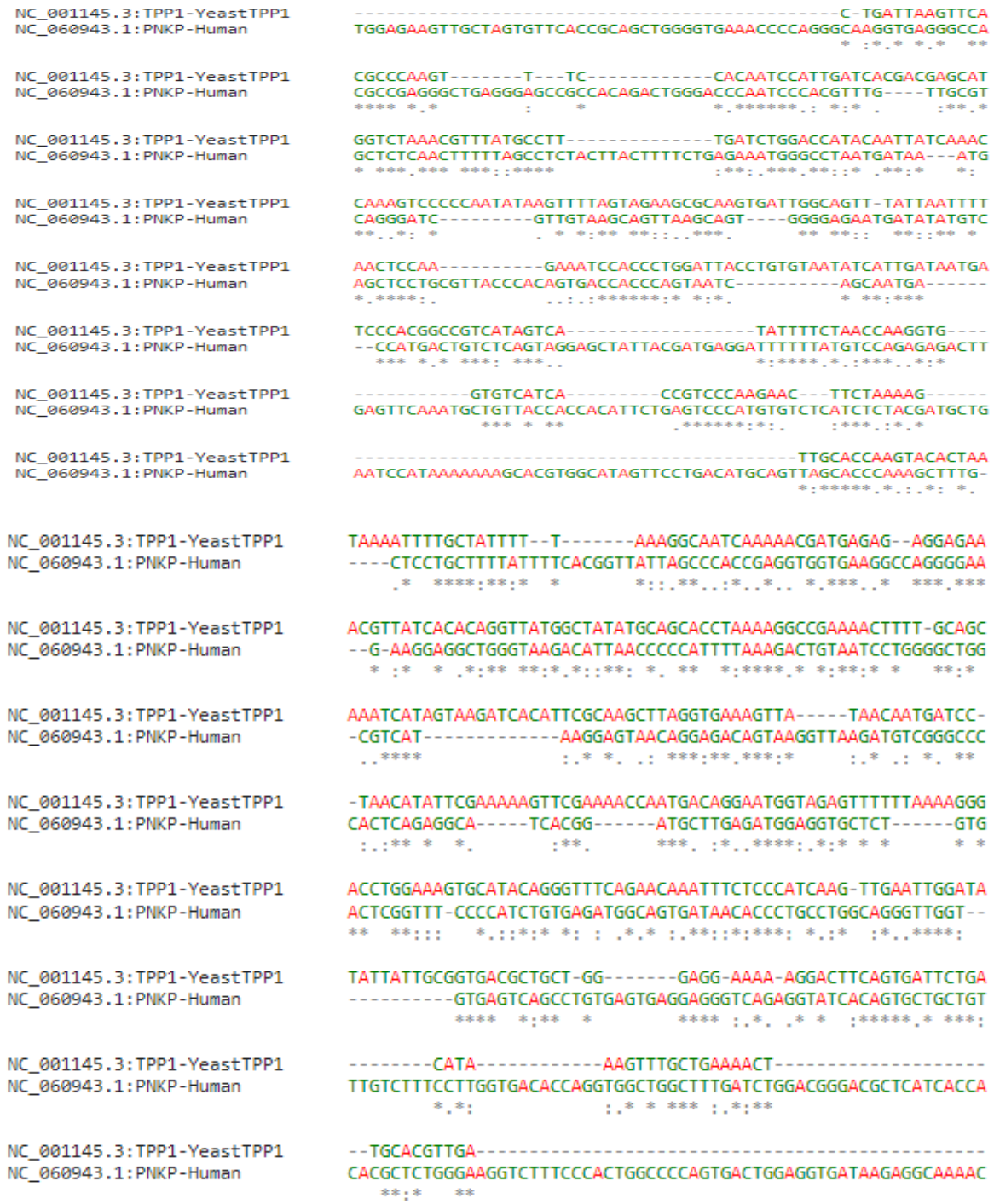

Figure (7): Gene alignment between human gene *PNKP* and yeast *TPP1* gene in the Clustal Omega web site ('*' indicates identical between two aligned, '-' indicates gaps missing of one) and ('.' indicates low similarity, ':' indicates more similarity used to denote the level of similarity that are not identical) at position.

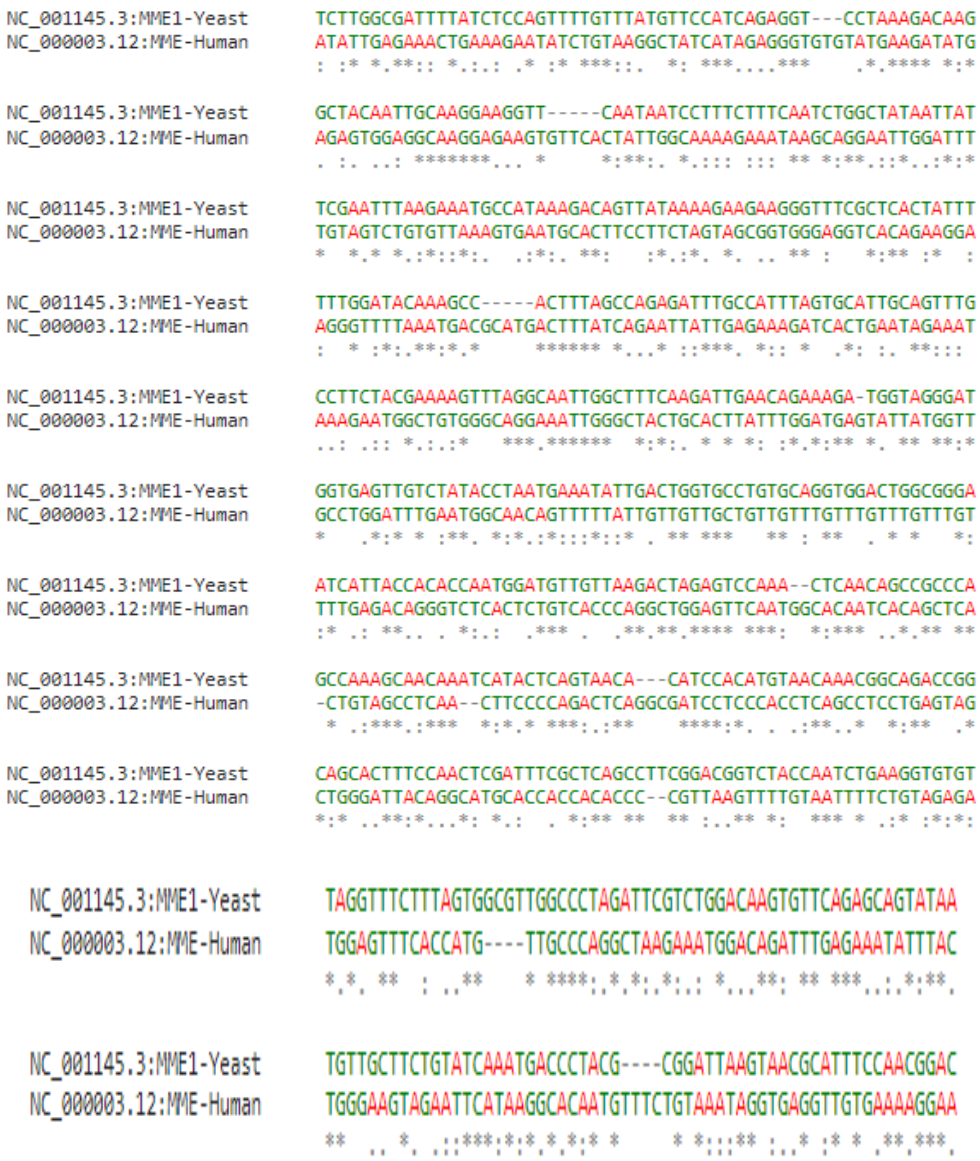

Figure (8): Gene alignment in the Clustal Omega web site between human gene *MME* and yeast gene *MME1* ('*' indicates identical between two aligned, '-' indicates gaps missing of one) and ('.' indicates low similar, ':' indicates more similar used to denote the level of similarity that are not identical) at position.

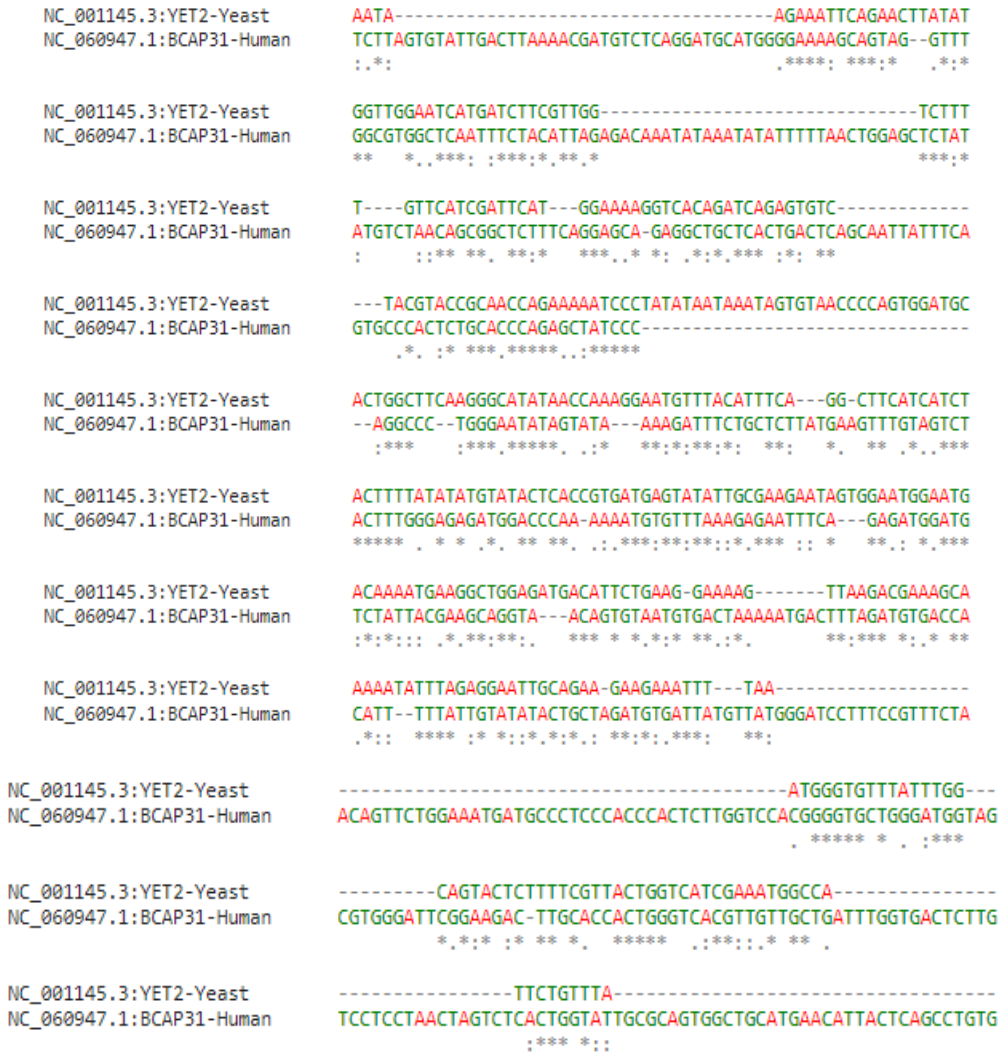

Figure (9): Gene alignment in the Clustal Omega web site between human gene *BCAP31* and yeast gene *YET2* ('*' indicates identical between two aligned, '-' indicates gaps missing of one) and ('.' indicates low similar, ':' indicates more similar used to denote the level of similarity that are not identical) at position.

7. Yeast protein-protein interaction prediction (Networking).

The degree of similarity between two species' cancer-related genes might be determined by predicting protein-protein interactions in yeast and humans (Figure 10). Every data set chosen for the investigation is shown in Gene MANIA prediction server together with its prognostic value. Currently separate sequencing executes prediction approaches on yeast and humans are supported in addition to or above two organisms (*Homo sapiens* and *Saccharomyces cerevisiae*), associate in Nursing Intuitive Computer Programmers, and depth of knowledge [17].

Gene MANIA displays four yeast queries (Figure 10). Four distinct relevant yeast genes that are connected by a pathway to the query list and many totally different interactions result in fully distinct networks. Other degrees of question customization (0.59%) include co-expression (6.89%), co-localization (2.10%), other (1.02%), genetic interaction (36.83%), related protein domains (0.34%), route (0.22%), and common macromolecule domains are among the factors that contribute to physical connection (48.13%). The influence of knowledge set selections on topology by Gene MANIA presents outcomes of gene queries. Mistreatment of the yeast default question's default settings, a yeast default question is the misuse of the default network weight technique. We selected YKOs deficient in genes related to human cancer genes. Forecasting human and yeast interactions between proteins may enable researchers to evaluate the degree to which some cancer-related genes are deliberately similar in the two species. This result confirms that of [32] who found that the Gene MANIA approach on saccharomyces and mouse benchmarks works as good as, if not better than, other function of genes prediction techniques. GeneMANIA is a helpful tool for any biologist because of its enormous database, user-friendly interface, and high precision for prediction.

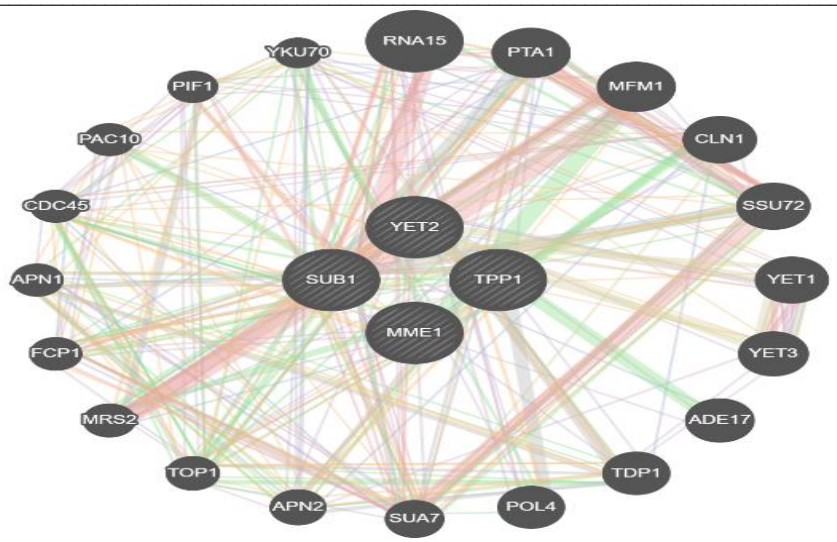

Figure (10): shows the default settings for the yeast cell-cycle inquiry and the default settings for the yeast cell-cycle query.

The yeast cell-cycle standard query is used by the system's default scaling strategy. The outcomes of four human investigations are shown by Gene MANIA (Figure 11). Four distinct relevant human genes that are connected by a pathway to the query list are spread throughout four completely different networks that are completely different from one another. Other degrees of question customisation (0.59%) include co-expression (6.89%), co-localization (2.10%), other (1.02%), genetic interaction (36.83%), shared protein domains (0.34%), route (0.22%), and common macromolecule domains are among the factors that contribute to physical connection (48.13%). Genes Inquiries Outcomes are displayed in Impact of Knowing Set Selection on Topology. by GeneMANIA. All default parameters in the human default question were mistreated, a human default question, mistreatment default network weight approach. We selected YKOs deficient in genes related to human cancer genes. Researchers may be able to assess the degree to which some cancer-associated genes are purposefully identical between the yeast and humans by forecasting protein-protein interactions among both organisms.

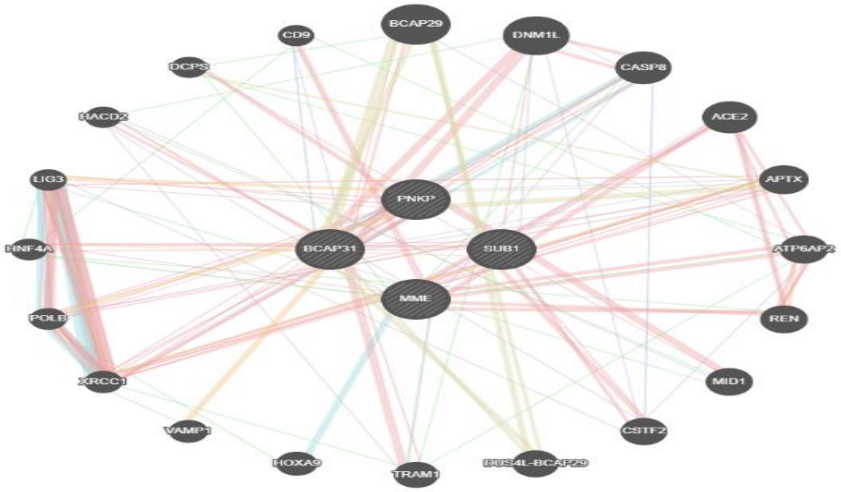

Figure (11): Utilizing all default parameters, the human default query. utilising the default network weighting method and the human default query.

4. Conclusions

The medication doxorubicin can prevent cell division in human cell lines and also clarified the dose-dependent cellular cytotoxicity effects of doxorubicin exposure. The results identified doxorubicin's apoptotic and toxic effects on human cell lines. In the current work, the flow cytometric analysis average DNA content % of PC3 cell line was affected by doxorubicin at dosage *50* µg /ml. The G0/G1 phase showed a decline in DNA content % from 51.03% (control) to 46.23% (treatment). The real time-PCR was used to measure doxorubicin-induced cytotoxicity on PC3 prostate cancer cell line cell lines affected apoptosis was investigated, Real-time PCR (qPCR) was used to determine Apoptosis-related genes including Bcl-2, p53, and casp3 are expressed at different levels. in PC3 cell line. In comparison to the control, *Casp3* increased by 1.50597074, and *p53* by 1.21935421. The results of *Bcl-2* dropped by 0.39046099 The results indicate that those receiving treatment had higher levels of casp3 and p53 genes expressed than the untreated

group. These results showed that doxorubicin killed PC3 cell line primarily through upregulating the genes *casp3* and *p53* during apoptosis, while downregulating *Bcl-2*. According to the alien object assay, doxorubicin has various degrees of yeast-specific genotoxic effects on YKO. The genotoxic impact of doxorubicin was demonstrated at doses of 25, 50, and 75 µg /ml. genotoxicity was high for the *SUB1, YET2, TPP1, MME1,* and *MME1* genes, but low for the *MMT1* gene sequence, displays the breakdown of the share of identified comets for doxorubicin. These findings imply that doxorubicin is genetically toxic to yeast genes and that reactive oxygen species generation may be the main mechanism by which DNA damage is caused. As a result, the use of doxorubicin may pose biohazards to human health. The threshold values that should be allowed for medications containing doxorubicin should be strictly controlled in light of the potential biosafety risks to people.

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6. References

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