Alfarama Journal of Basic & Applied Sciences

Faculty of Science Port Said University

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ISSN 2682-275X

January 2022 , Volume 3, Issue I DOI: 10.21608/AJBAS.2021.103988.1073

Submitted: 10-11-2021
Accepted: 30-11-2021 Pages: 35-44

# Effect of Doxorubicin and Cisplatin on Alpha-fetoprotein levels in Hepatocellular Carcinoma Cell lines

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## ABSTRACT

Hepatocellular carcinoma is a kind of cancer that affects the liver. HCC consider the main reason for the great ratio of cancer deaths worldwide. In Europe and the United States, its occurrence is quickly elevating, due to the present epidemic of hepatitis C virus and nonalcoholic fatty liver disease cases. Patients with HCC have a prognosis that is impacted by many factors as tumor stage, with therapeutic choices only obtainable for patients who have been diagnosed early. Currently, the main effective therapy for HCC is considered surgery, which includes transplantation resection. Moreover, rates of recurrence are increased and in the long run duration, the chance of surviving is slim. Traditional cytotoxic chemotherapy has not been proven to be effective in giving clinical advantages or lengthy patients' survival with developed HCC. Cisplatin is considered one of the main medications used in the conventional treatment of several cancers in different organs. Latterly, many hopeful results of utilizing have been demonstrated cisplatin in the therapy of patients with HCC has developed, in addition, Doxorubicin has a wide range of anti-tumor properties. The importance of early discovery through surveillance programs cannot be overstated. Therefore, the assessment of alpha-fetoprotein (AFP) and ultrasound for patients at risk of developing HCC are recommended in HCC guidelines all around the world. Therefore, the treatment with cisplatin and doxorubicin significantly reduced the elevation in the levels of AFP in HepG-2 cells. Moreover, The findings showed that doxorubicin and cisplatin can inhibit HCC cell proliferation by enhancing hepatocyte function.

**Key Words:**

Doxorubicin, Cisplatin, Hepatocellular carcinoma, Alpha-fetoprotein, MTT assay, Proliferation assay.

## 1. INTRODUCTION

Hepatocellular carcinoma is the most prevalent form of primary liver cancer[1]. Hepatocellular carcinoma is generally always found in people with chronic liver disorders, such as cirrhosis-related hepatitis B or C infection [2]. Patients with chronic liver illness are more likely to develop hepatocellular carcinoma, the most common kind of liver cancer. It's also increased if the liver has been damaged by hepatitis C or B infection [3]. Hepatocellular carcinoma is greatly prevalent in persons who are alcoholic and fatty liver patients [4]. There are many procedures and tests used to diagnose HCC like Imaging scans, blood tests to assess the optimal treatment for hepatocellular carcinoma will be determined by the size and location of the tumor, the state of the liver, and the patient's overall health. [5]. Surgery, liver transplant surgery, using heat or cold to kill cancer cells, and directly administering chemotherapy or

radiation to cancer cells are all treatments for hepatocellular carcinoma. Radiation therapy, targeted medication therapy, and immunotherapy are all options for treating cancer [6].

The best-studied serologic test, alpha-fetoprotein (AFP), is appealing for observation since it is relatively inexpensive and widely available [7]. However, the American Association for the Study of Liver Diseases (AASLD) recently recommended against utilizing AFP for early-stage HCC, citing its lack of specificity and sensitivity [8]. AFP has a specificity and sensitivity for HCC of about 80% and 60%, respectively, at a cut-off of 20ng/mL, the most commonly used cut-off in clinical practice [9].

Barnett Rosenberg, a bacteriologist In 1965 indicated that a platinum compound, extracted from platinum electrodes that were used in his experiments, had a suppression effect on the growth of Escherichia coli [10]. Following that, other platinum compounds were evaluated for anticancer action in the hopes of finding a medication that would slow the division of cancer cells, which are characterized by rapid multiplication [11]. Cisplatin was later discovered to be a powerful anti-cancer agent [12]. The US National Cancer Institute (NCI) began clinical research on cisplatin in 1972, and the efficacy of cisplatin as an anticancer drug was first demonstrated in the treatment of malignant tumors of the urinary system [13]. Cisplatin is currently the most commonly used medicine in routine cancer treatment regimens, including those of the genitourinary, respiratory, and digestive systems [14].

Alpha-fetoprotein (AFP) is considered a protein mainly created by the liver in (fetus) [15]. Normally the levels of AFP are increased when a baby is born and then decrease rapidly [16]. Other than birth and pregnancy, the high levels of AFP are due to certain cancers and liver damage [17]. The high levels of AFP are created by regenerating cancer cells and chronic liver diseases. certain tumors may produce high levels of AFP therefore, the AFP test is considered a tumor marker [18]. There are high amounts of AFP in most people with the popular type of liver cancer called HCC and hepatoblastoma that consider an uncommon type of liver cancer that happens in infants.[19]. In addition to there are found in patients with cancers of the ovaries or testicles [20]. This study aimed to an evaluation of alpha-fetoprotein levels as a tumor marker in HepG-2 cells after being treated with two of the most effective chemotherapy drugs (Cisplatin and Doxorubicin) and observed the effect of cisplatin and doxorubicin on the morphology of HepG-2 cells.

## 2. MATERIALS AND METHODS

### 2.1 Cell Lines and cell culture Samples collection and analysis:

HepG2 cells were obtained from Medical Research Institute (MRI), Smouha, and Alex, Egypt. Dulbecco's modified Eagle's medium (DMEM) with foetal bovine serum (FBS), penicillin, and streptomycin were used to keep HepG2 cell lines alive. At 37°C, all cells were cultured in 5% $CO_2$. The HepG2 cancer cells were removed after achieving confluence and planted into the same complete medium with 1x106 cells using 0.25 percent trypsin EDTA. Every three days, the DMEM medium was changed.

### 2.2 Reagents and drug treatments:

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were, purchased from GIBCO® (Invitrogen). Dimethylsulfoxide (DMSO), Standard anticancer drugs (Doxorubicin and Cisplatin) were purchased from Sigma-Aldrich.

### 2.3 Estimation of Alpha-fetoprotein (AFP) Concentration:

Alpha-fetoprotein was determined by Human, (ELISA) Immunoassay Kit, cloud clone corp. Company (USA). The manufacturer's instructions were followed for all ELISA techniques. The absorbance was measured using a Biotek plate reader (Gen 5th) at wavelengths ranging from 570 to 630 nm.

**2.4 Cell proliferation:**

Viable cell analysis (MTT Assay) was performed to examine the inhibitory actions of cisplatin and doxorubicin on HepG-2 cell growth [21]. Cells were diluted and counted to ensure that the population and density were appropriate. Then the cells were planted at a density of $2\times10^5$ cells/ml in 96-well plates (Flat Bottom) and allowed to adhere 24 h at 37 °C. Drug dilutions of (50, 25, 12.5, 6.25, 3.125, and 1.56 µg) were added to the cells in complete media. After three days of exposure to the tested drug, 10 µL of stock MTT solution (5 mg/mL) was applied to each well. After that, the cells were incubated at 37°C for another 4 hours. The absorbance was measured using a Biotek plate reader (Gen 5th) at wavelengths ranging from 570 to 630 nm.

**2.5 Statistical analysis:**

GraphPad Prism 8 was used to do the statistical analysis calculations (GraphPad, San Diego, CA, USA). The significance of the inhibition data was determined using a one-way analysis of variance (ANOVA). If the P-value was less than 0.05, the results were considered statistically significant. To represent all of the data, the mean and standard deviation are utilized (SD).

## 3. RESULTS

**3.1 Effect of treatments on (AFP) level:**

As shown in Table (1), the value of absorbance means was reduced significantly ($P<0.001$) after the treatment of hepG2 cells with Doxorubicin and Cisplatin as chemotherapy drugs in comparison to the control group (DMSO). A more potent reduction was noticed after treating with Cisplatin. Likely, the concentrations of AFP were reduced significantly ($P= 0.0018$) and ($P=0.0006$) after treatment with the previous chemotherapy treatments Doxorubicin and Cisplatin, respectively as shown in figure (1). The resulting absorbance and AFP levels were significantly decreased and this decrease was found to be dependent on the mechanism of action of each chemotherapy drug.

**Table (1):** AFP level after treatment of HepG-2 cells with Doxorubicin and Cisplatin.

| Drug | AFP (µg/ml) |
|---|---|
| Control (DMSO) | 6.0425±0.129 |
| Doxorubicin (DOXO) | 4.117**±0.239 |
| Cisplatin (CIS) | 3.065**±0.234 |

Data are expressed as means ± SD, Significant difference between the treatments is analyzed by one-way ANOVA test, then there's the t-test., where: ** significant at $P\leq 0.01$.

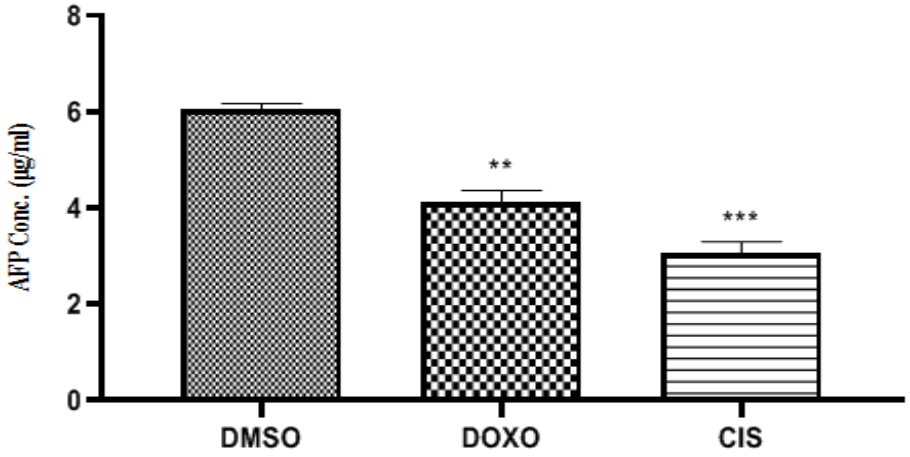


**Fig (1): AFP levels of treated hepG2 cells with a Doxorubicin and Cisplatin.** Data are expressed as means ± SD, ** significant at P≤ 0.01 and *** significant at P< 0.001.

**3.2 Morphological changes and cell proliferation inhibition of Hepg-2 cells by cisplatin and doxorubicin.**

As demonstrated in tables (2) and (3), cells treated with cisplatin and doxorubicin at various concentrations showed a significant reduction in proliferative activity of HepG-2 cells. An MTT assay was used to determine the viability or proliferation percent of HepG-2 cells treated with cisplatin and doxorubicin. As shown in table (4) and figure (4), cisplatin use at (4.843 µg) for an indicated time demonstrated a significant inhibition effect against HepG-2 cells when compared to doxorubicin and DMSO. As demonstrated in the table (4) and figure (4), the same findings were achieved in HepG-2 cells treated with doxorubicin at IC50 concentration (2.436 µg) when compared to DMSO. These findings showed that cisplatin has a considerable cytotoxic effect on the proliferation of HepG-2 cells. Furthermore, morphological variations were seen in HepG-2 cells treated with cisplatin, as shown in Figures (2) and (3), when compared to doxorubicin and DMSO, after treatment for 48 hours with 1.56 to 12.5 µg of doxorubicin did not cause notable changes in morphology or cell number. Furthermore, treatment with 25-50 µg of cisplatin resulted in significant morphological alterations and notably suppression. In comparison to negative control cells treated with DMSO, long treatments resulted in a decrease in cell count.

**Table 2.** Effect of the Cisplatin on cell proliferation percentage on HepG-2 cells.

| Cisplatin Conc. (µg) | Proliferation for HepG-2 (%) |
|---|---|
| 50 | 11±1 |
| 25 | 14±2 |
| 12.5 | 16±1 |
| 6.25 | 50±1 |
| 3.125 | 62±1 |
| 1.56 | 77±2 |

**Table 3.** Effect of the Doxorubicin on cell proliferation percentage on HepG-2 cells.

| Doxorubicin Conc. (µg) | Proliferation for HepG-2 (%) |
|---|---|
| 50 | 16±1 |
| 25 | 21±1 |
| 12.5 | 30±1 |
| 6.25 | 36±1 |
| 3.125 | 45±1 |
| 1.56 | 58±1 |

**Table 4.** IC50 values of HepG-2 after treatment with cisplatin and doxorubicin.

| Compound | Chemical name | IC50(µg) |
|---|---|---|
| CIS | Cisplatin | 4.843 |
| DOXO | Doxorubicin | 2.436 |

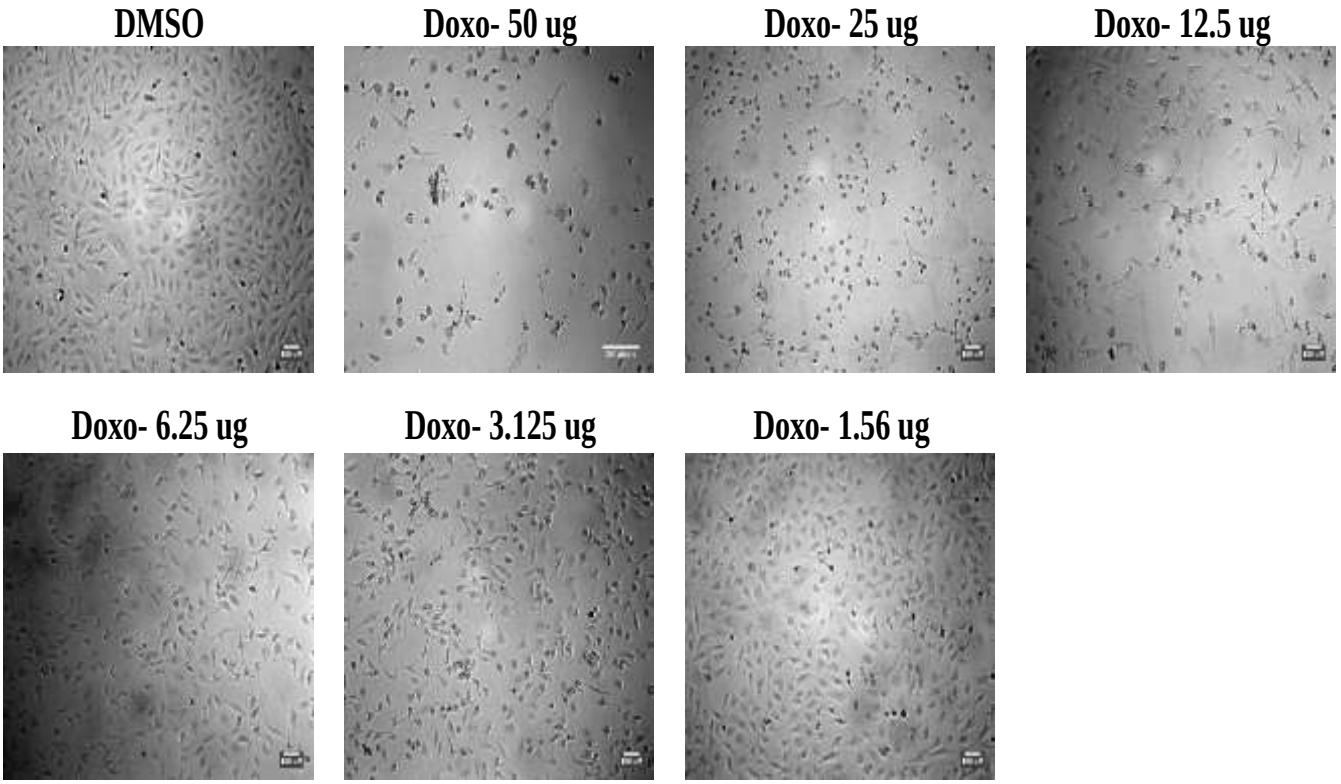


**Figure 2.** Micro photomicrograph showing the effect of treatment with doxorubicin on the morphology of HepG-2 cells (an inverted microscope).

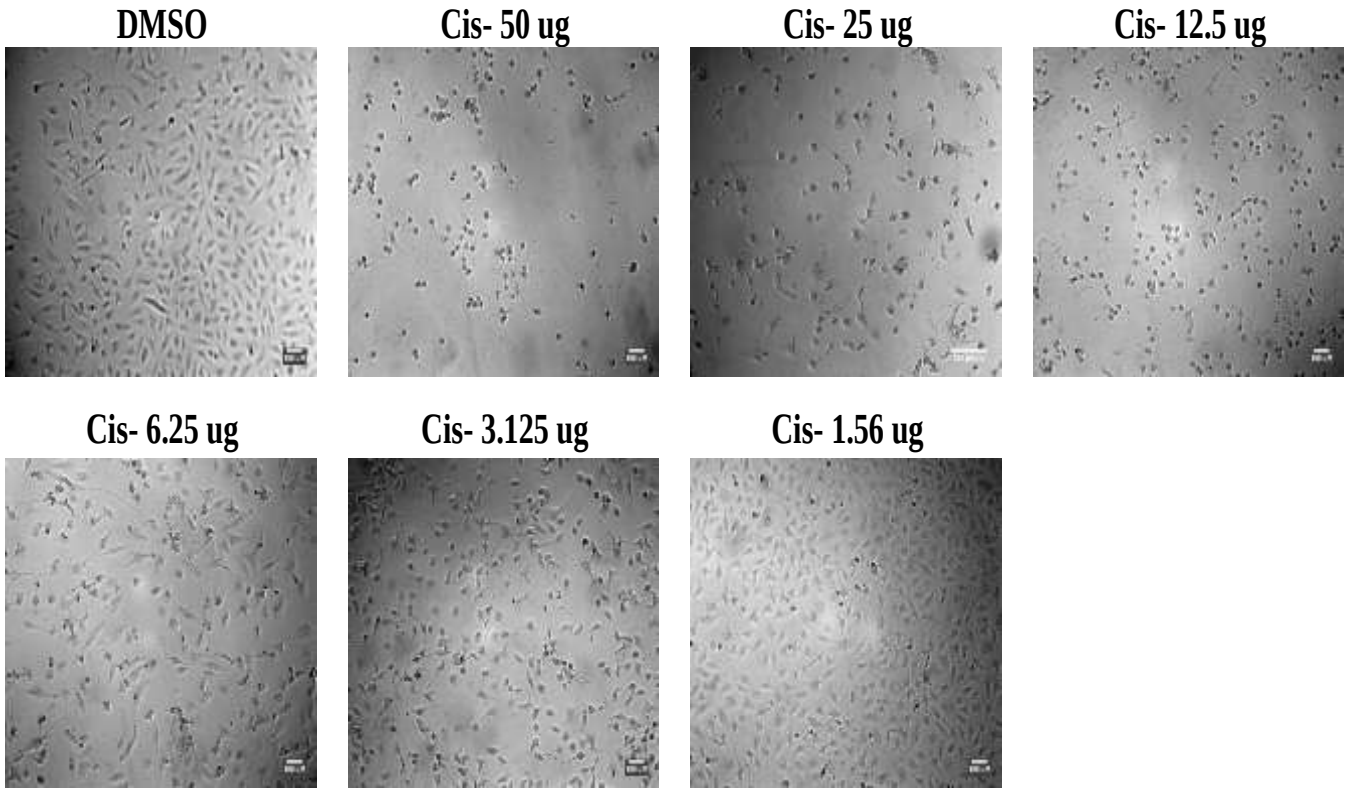


**Figure 3.** Micro photomicrograph showing the effect of treatment with cisplatin on the morphology of HepG-2 cells (an inverted microscope).

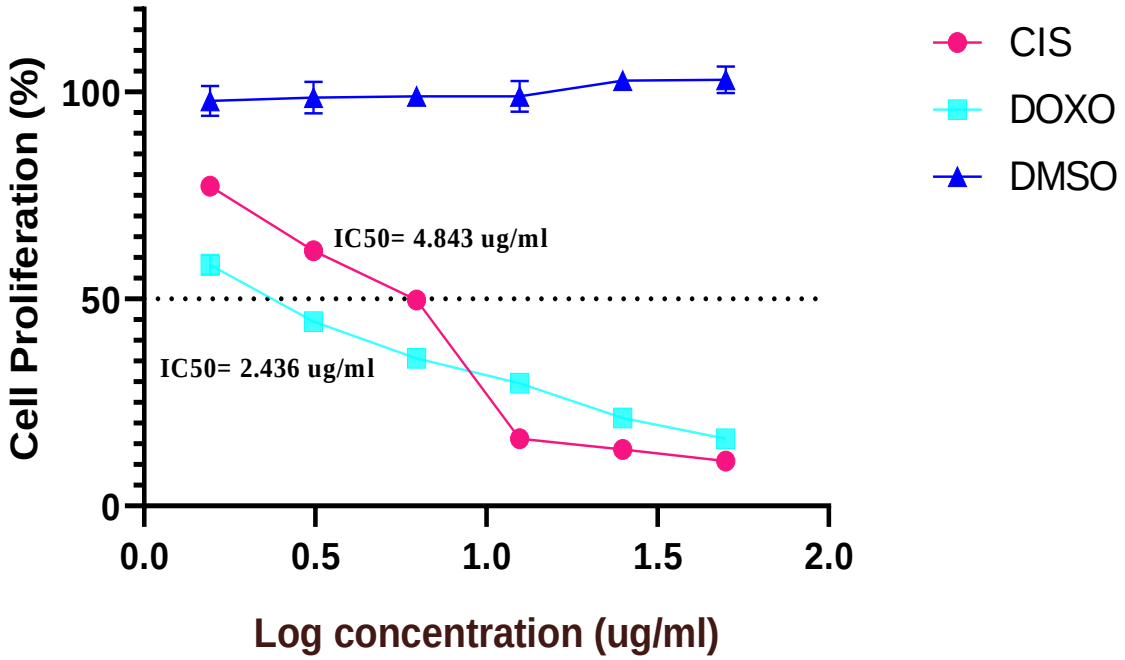


**Figure 4.** Effect of treatment with cisplatin and doxorubicin on the proliferation activity on HepG-2 cells.

## 4. DISCUSSION

The diagnosis of hepatocellular carcinoma constitutes several challenges that can vary between different centers and regions. Frequently every 6 months in HCC high-risk peoples, AFP and US imaging are utilized to keep an eye on them [22]. When there are high levels of AFP or suspiciousness of malignant tumor, the observation period must be limited and can be used multiphasic CT scan or MRI as more sensitive imaging techniques [23].

Many diagnostic techniques have been suggested, several of which are specific to both the region and the center [24]. The European Association for the Study of the Liver (EASL) has established standard diagnostic criteria for hepatocellular carcinoma that encompass both invasive and noninvasive tests [24]. Two strategies are used in the noninvasive criteria of imaging, each one of them indicating a focal lesion having a diameter of more than 2 cm and with characteristics of arterial hyper vascularization, as well as a single radiologic examination with these characteristics and a serum AFP level of more than 400 ng/ml [25]. The application of this and other criteria can be extremely beneficial, but the deficiency of proof-based research prevents their stringent usage in the examination of HCC. Moreover, the research projects carry on to enter of interest on growing methods to progress diagnostic methods and techniques aimed at detecting HCC in its early stages.

Due to the extensive success of cisplatin in the treatment of a variety of tumors, it has been identified as one of the most commonly utilized cytotoxic anticancer medications. For the treatment of solid tumors, cisplatin is administered intravenously as a short-term infusion with normal saline. Cancer is defined as aberrant cell division with the ability to infiltrate neighboring cells [26]. Carcinogenesis necessitates the genotypic expression of some key features in the cell. Cancer generally referred to a malignant tumor, that emerges when the normal control of cellular proliferation in body tissues is lost [27].

The mechanism by which cisplatin works is as follows. Cisplatin interacts with the cellular DNA to form a covalent compound after entering the target cells. The substance acts as a catalyst for reversible adenine and guanine alkylation and shapes intra- and interstrand cross-links in DNA, inhibiting DNA polymerase protraction (suppression of DNA transcription and replication). Additionally, the development of intrastrand cross-links affects cell formation. The anticancer impact of the medication is due to these alterations, which cause cancer cells to undergo apoptosis and necrosis. Cisplatin's anticancer effect is characterized by time and concentration-dependent characteristics [28].

Doxorubicin is a type of chemotherapy drug known as an anthracycline [29]. Doxorubicin works by inhibiting or stopping the proliferation of cancer cells by suppressing an enzyme called topoisomerase II [29]. Cancer cells require this enzyme to divide and multiply. Doxorubicin resistance is caused by a reduction in the drug's ability to gather in the nucleus, as well as a reduction in suppression of the subsequent processes that convert the DNA damage signal into apoptosis [30]. Doxorubicin is currently frequently used to treat a variety of cancers. Liposomal doxorubicin compositions (Doxil® or Lipodox®) have been developed to enhance the therapeutic index of doxorubicin to be used in traditional chemotherapeutic (systemic administration) while maintaining anti-tumor action [31]. The liposomal compositions have been used to treat metastatic breast/ovary malignancies as well as Kaposi's Sarcoma related to AIDS [31]. For the cure of hepatocellular carcinoma, doxorubicin in a heat-sensitive liposome composition has been presented an Orphan designation (EU/3/10/833) in Europe [32].

In both normal and malignant cells, doxorubicin may trigger a series of free radical reactions. The molecule interacts with the cell's electron transport chain, especially the cytochrome P450 reductase (P450R), in most cases [33]. The quinone moiety quickly absorbs a single electron move to generate the semiquinone free radical, which might be immediately lethal in hypoxic settings by covalent alteration of biological macromolecules, as seen in Fig. 5. The semiquinone radicals can transfer electrons to

molecular oxygen under aerobic circumstances, leading to an increase in superoxide anion radicals [34]. These poisonous intracellular radicals can be transformed into hydrogen peroxide and hydroxyl radicals, which can also harm DNA, RNA, lipids, and proteins directly [35]. The potential of doxorubicin to cause cell death, as well as its dose-limiting cardiotoxicity, appears to be influenced by this oxidative stress pathway. The doxorubicin ring structure is recovered to its former condition during electron shuttling, making it accessible for subsequent redox cycling processes.

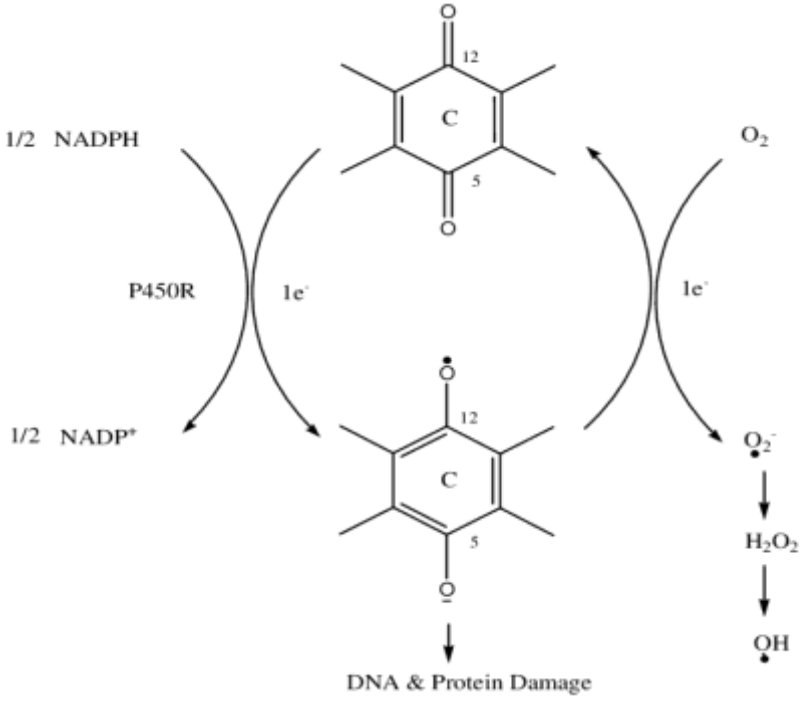


**Figure 5.** Simplified schematic of the redox cycling of doxorubicin [35].

The present study has shown that the control group's AFP level was substantially greater in HepG-2 cells before being subjected to any chemotherapy these results agreed with similar results that were obtained by many studies [36] and [37]. [38] who announced that AFP seemed in several parenchymatous liver diseases but the elevated levels were found greatest repeatedly in HCC more than chronic hepatitis and acute viral hepatitis. As well, in this study, we used cisplatin and doxorubicin as two of the most effective treatments in liver cancer. After noticing the effect of each drug on HepG-2 cell lines, we found highly decrease in the levels of AFP in two groups treated with cisplatin and doxorubicin. Nevertheless, there were different degrees in this inhibition as cisplatin has a high inhibition effect and is more effective than doxorubicin. In conclusion, in The present results of the diagnostic study, AFP was tested as a marker in the diagnosis and prognosis of HCC. As well as the AFP concentration was reduced significantly after treatment of HepG2 cells with both treatments indicating the ameliorating effect of both treatments on tumor cells. Searching for new chemo-sensitizers to combine with cisplatin and doxorubicin are recommended to decrease their toxicity and to increase their efficacy.

## 5. CONCLUSION

The present study might offer important evidence for the selection of therapeutic agent candidates for hepatocellular carcinoma management. According to our findings, this study demonstrated that Alpha-fetoprotein levels are significantly elevated in HCC and after treatment with chemotherapy drugs we found cisplatin drug more effective in the treatment of HCC than doxorubicin drug. Because of its great sensitivity and high specificity, AFP can still be employed in HCC surveillance.

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**List of abbreviations:**

Doxo: Doxorubicin
Cis: Cisplatin
HCC: Hepatocellular carcinoma
AFP: Alpha-fetoprotein
MTT: Methyl thiazole tetrazolium
DMSO: Dimethylsulfoxide