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Assessment the effect of Cisplatin and Doxorubicin on Nitric Oxide levels on HepG2 cell line

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ABSTRACT

Background: Mortality due to HCC is the fourth most common cause of mortality among Americans. Furthermore, HCC is most common in Asia and Africa, where hepatitis C and B are prevalent. Hepatocellular cancer therapy relies heavily on chemotherapy, from between the chemotherapy medicines cisplatin and doxorubicin, two of the most widely prescribed for the treatment of liver cancer. **Aim of the study:** To determine the cytotoxicity of cisplatin and doxorubicin in HepG2 cell line by the methyl thiazole tetrazolium (MTT) assay and assess nitric oxide (NO) levels and their significance as oxidative status markers in the progression of HCC. **Material and Methods:** HepG2 cells were incubated with different drugs concentrations. The half-maximal inhibitory concentration (IC50) values were determined the methyl thiazole tetrazolium (MTT) for cisplatin and doxorubicin. **Results:** IC50 values for HepG2 were found for doxorubicin and cisplatin (1.679 µg and 4.323 µg), respectively. The NO levels in the HepG2 cell line treated with Cisplatin and Doxorubicin were measured. We found limited elevated NO levels after treating HepG 2 cell lines by doxorubicin. Moreover, there was significant inhibition in NO levels after treating with cisplatin. **Conclusion:** The findings suggest that nitric oxide levels can be utilized as a new diagnostic marker with predictive value for HCC.

Key Words:

Doxorubicin, Cisplatin, Hepatocellular carcinoma, Nitric oxide, Cytotoxicity, MTT assay.

1. INTRODUCTION

Each year, nearly 500,000 people are diagnosed with hepatocellular carcinoma (HCC) [1]. It is Egypt's fourth most prevalent type of cancer [2]. Numerous hospital-based investigations have revealed an increase in the incidence of HCC [3]. Improved screening and diagnostic technology, improved survival rates for cirrhotic patients, and an increase in the prevalence and implications of hepatitis C virus (HCV), Egypt's most significant risk factor for developing liver cancer, are all likely explanations for the increase in incidence [4]. HCC is the fourth leading cause of cancer-related death worldwide [5]. In 2012, it was responsible for more than 10% of all deaths (746,000 deaths). It is the leading cause of cancer-related death and morbidity in Egypt [6].

Although there are numerous therapeutic options for HCC, chemotherapy is a critical component of treatment for patients with advanced illnesses. It is used to treat people who cannot have surgery, local

ablative therapy, or transarterial chemoembolization (TACE), like those with extrahepatic metastases, vascular invasion, or resistant TACE [7]. Additionally, it is used to treat patients with extrahepatic metastases who are not considered unfit for TACE. In comparison, treatment efficacy remains unsatisfactory, and the prognosis for individuals with HCC remains poor in the majority of instances. HCC is treated in two ways: systemic chemotherapy and hepatic arterial infusion chemotherapy (HAI) (HAIC) [8].

Barnett Rosenberg, a bacteriologist, discovered in 1965 that a platinum compound eluted from platinum electrodes used in his research inhibited the growth of Escherichia coli [9]. Later, several platinum compounds were tested for anticancer action with the hope of discovering a medication that would limit the division of cancer cells, which proliferate at a high rate [10]. Cisplatin (cis-diamminedichloroplatinum; CDDP) was later discovered potent anticancer action [11]. Clinical research on CDDP began in 1972 at the National Cancer Institute (NCI) of the United States [12]. Its anticancer activity was first demonstrated in treating malignant tumors of the urinary system. CDDP is currently a critical component of standard regimens used to treat a variety of malignancies, encompassing respiratory, digestive, and genitourinary systems [13].

One of the first anthracyclines, doxorubicin, was found in Streptomyces peucetius over 40 years ago [14]. It has been acclaimed as one of the most effective cancer chemotherapy drugs [15]. Clinical usage of this medicine is complicated by cumulative cardiotoxicity, with the risk increasing with cumulative dosages of 550 mg/m^2 and above [16]. Clinical trials of doxorubicin in treating hepatocellular carcinoma (HCC) found that it had only a minimal effect on clinical outcomes. As a result, 30 to 70% of patients observed tumor reduction and partial responses when the medication was administered via the hepatic artery route [17]. Leukemia, lymphoma, bronchogenic, and traditional cell bladder carcinoma, Wilms' tumors, neuroblastoma, sarcoma, and carcinoma of the breast, ovary, and stomach, are among the most prevalent malignancies treated with doxorubicin [18]. It has been found that the therapeutic indices of doxorubicin can be improved by using liposomal doxorubicin formulations (Doxil® or Lipodox®) while maintaining its anti-tumor action [19]. They have been employed to treat metastatic breast/ovary malignancies and AIDS-related Kaposi's Sarcoma [20]. An Orphan designation (EU/3/10/833) for treating hepatocellular cancer has been granted in Europe for the heat-sensitive liposomal formulation of doxorubicin [21].

The two essential characteristics of HCV infection's natural history are viral persistence and liver destruction [22]. Nitric oxide (NO) is a highly versatile mediator in managing viral infections, as it is the first antiviral response of the host [23]. NO acts as both an inducer and inhibitor of Apoptosis in specific cell types, including hepatocytes [24]. Additionally, it was discovered that patients with HCV have increased inducible nitric oxide synthase (iNOS) expression, signaling excessive NO production that correlates favorably with viral load and hepatic inflammation [25]. The most conspicuous characteristic of hepatitis C is its strong proclivity for chronicity. NO may decrease antiviral response by reducing the activity of type 1 helper T cells [26].

Additionally, NO promotes viral escape mutations, allowing for viral persistence. NO promotes viral persistence by inhibiting Apoptosis in hepatocytes, whereas HCV promotes liver cell survival by inhibiting Apoptosis via activation of the NF-kB signaling pathway [27]. In chronic HCV infection, the upregulation of the INOS gene results in oxidative stress, and reactive NO species (RNOS) such as peroxynitrite and nitrogen oxide cause cytotoxicity and DNA damage [28]. Preliminary studies indicate that the nonstructural HCV protein NS5A and the core protein can promote INOS gene expression. HCV and NO interact synergistically to give a robust oncogenic signal to infected hepatocytes [29].

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. Penicillin, streptomycin, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM).

They were used to maintain HepG2 cell lines. At 37°C, all cells were cultivated in 5% CO_2. HepG2 cells were detached using 0.25 percent trypsin EDTA after attaining confluence, and $1x10^6$ cells were planted into the same complete media. Every three days, the DMEM medium was replenished.

2.2 Reagents and drug treatments:

Standard anticancer drugs (Doxorubicin and Cisplatin), Dimethylsulfoxide (DMSO) were, purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GIBCO® (Invitrogen). All other compounds were bought from Sigma Chemical CO. (St. Louis, MO, USA) with analytical grade.

2.3 Cytotoxicity assay:

The methyl thiazole tetrazolium (MTT) assay was used to determine the cytotoxicity of cisplatin and doxorubicin. After allowing the cells to attach for twenty-four hours, they were treated with the specified cisplatin and doxorubicin concentrations and durations. Before treatment, the drugs' stock solution (10 mM) was diluted to the required concentrations. For the prescribed time to add treatment, the cells were given varying cisplatin concentrations and doxorubicin as a positive control or DMSO alone as a negative control. After 48 hours of treatment, MTT (5 mg/mL PBS) was added to the cells. Then, 100 µL of acidified sodium dodecyl sulfate solution was added to solubilize formazan crystals. The absorbance of the 96-well plate was measured using a Biotek plate reader (Gen5TM) at wavelength 570-630 nm after another 4 hours of incubation at 37° C and 5% CO_2. The data is shown as the mean ± SD of the percentage cell viability of treated cells versus untreated controls. The IC50 (half-maximum inhibitory concentration) was calculated [30].

2.4 Measurement of Nitric oxide (NO):

At a density of 3×10^5 cells per well, cells were plated in a growth medium until they achieved 80% confluence in 6-well plates and then subjected to the dosage of chemotherapy drugs for 24 h. We immediately add 100ul Griess reagent per 100ul sample volume after treatment. The sample and reagent were combined directly in the wells of a 96-well plate. Then, we add 100ul deionized water + 100ul Griess reagent to serve as a blank in another well. For 30 minutes, we left the sample reagent mixture and blanked it to develop in the dark. Using a Biochrom EZ Read 800 Microplate Reader, we measured the absorbance of the sample mixture and blank solutions at 548nm. The absorbance of the blank and sample may be monitored concurrently using the plate reader, and nitrite was calculated using a standard curve produced from $NaNO_2$ (0–100 M). Each experiment was carried out three times [31].

2.5 Statistical analysis:

The statistical analysis computations were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). One-way analysis of variance was used to determine the significance of the inhibition results (ANOVA). The findings were considered statistically significant if the P-value was less than 0.05. The mean and standard deviation represent all of the data (SD).

3. RESULTS

3.1 Effect of treatments on (NO) level:

As shown in Table (1), the value of absorbance means was reduced significantly after treating HepG2 cells with Doxorubicin and Cisplatin as chemotherapeutic drugs compared to a control group (DMSO). A more potent reduction was noticed after treatment with cisplatin. Likely, the concentrations of NO were reduced significantly (P= 0.0117) and (P= 0.0004) after treatment with the previous chemotherapy treatments Doxorubicin and Cisplatin, respectively, as shown in Figure (1). The resulting absorbance and NO levels were significantly decreased, and this decrease depended on each chemotherapy drug's mechanism of action.

Table (1): NO level after treatment of HepG-2 cells with Doxorubicin and Cisplatin.

Drug	NO (µg/ml)
Control (DMSO)	9.135±0.103
Doxorubicin (DOXO)	6.820*±0.096
Cisplatin (CIS)	5.841***±0.112

Data are expressed as means ± SD; the significant difference between the treatments is analyzed by one-way ANOVA test, then, there is the t-test, where: *P≤0.05 and ***P≤0.001.

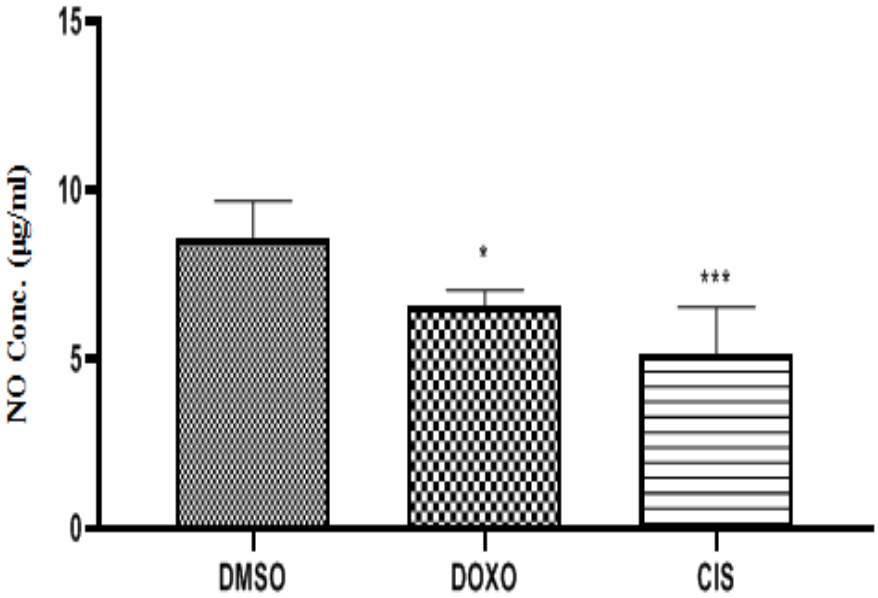

Fig (1): NO levels of treated hepG2 cells with a Cisplatin and Doxorubicin. Data are expressed as means ± SD, * significant at *P≤0.05 and *** significant at P< 0.00

3.2 Cytotoxicity effect of cisplatin and doxorubicin on HepG-2 cells:

The MTT assay was performed to evaluate cisplatin cytotoxicity at various concentrations ranging from 50 to 1.56 µg. The concentration that induced 50% inhibition in cell growth (IC50) was found to be 4.323 µg using a semi-logarithmic plot of cell viability vs. concentrations as shown in (Figure 2, Table 2, and Table 4). Cisplatin inhibited the development of HepG-2 cells in a dose-dependent manner. Furthermore, the cytotoxicity of doxorubicin at various concentrations ranging from 50 to 1.56 µg anticancer activity of doxorubicin was investigated using the MTT test on the HepG-2 cell line, and the concentration that induced 50% inhibition in cell growth (IC50) was determined to be 1.679 µg as shown in (Figure 2, Table 3 and Table 4).

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

Cisplatin Conc. (µg)	Viability for HepG-2 (%)
50	8±1
25	10±2
12.5	12±1
6.25	48±1
3.125	62±2
1.56	72±1

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

Doxorubicin Conc. (µg)	Viability for HepG-2 (%)
50	14±1
25	19±1
12.5	28±1
6.25	33±1
3.125	45±3
1.56	49±2

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

Compound	Chemical name	IC50(µg)
CIS	Cisplatin	4.323
DOXO	Doxorubicin	1.679

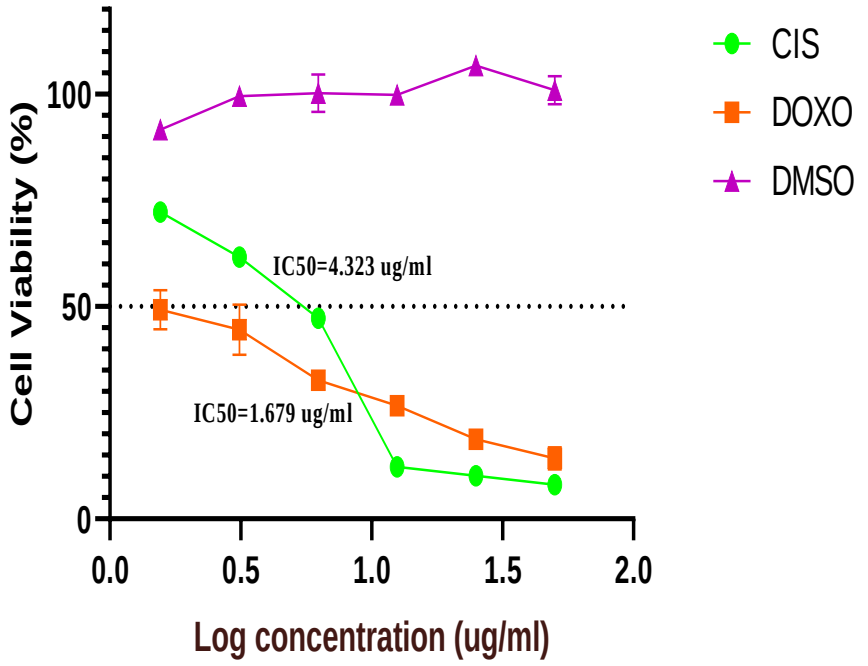

Figure 2. The effect of treatment with cisplatin and doxorubicin on the viability of HepG-2 cells by the MTT assay.

4. DISCUSSION

Cirrhosis, dysplastic nodules, and early cancer growth can all be reliably cured if detected before blood vessel invasion [32]. HCC usually develops in an ordered pattern. Early identification of HCC provides the most significant opportunity for curative treatment in those patients [33]. Despite advances in medical technology and research, there has been a significant increase in the number of patients eligible for potentially curative therapy such as surgical resection, transplantation, or percutaneous ablation in the 30–40 percent range [34]. Thus, most patients with advanced HCC will be given systemic chemotherapy treatments. Even though numerous single agent or combination therapies have been attempted, the role of systemic chemotherapy in advanced HCC is recognized to be quite restricted [35].

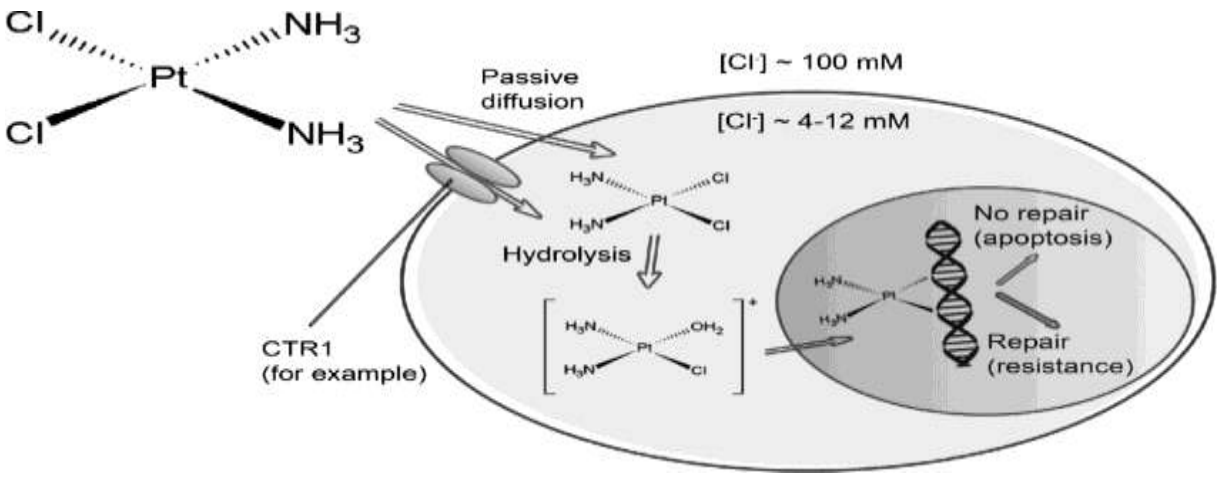

Figure 3: Cisplatin structure and mechanism of action.

The chemotherapeutic agent cisplatin (cis-Diamminedichloroplatinum II) is commonly used to treat many forms of solid cancer, including breast, prostate, and lung cancer [36]. Cisplatin's cytotoxic effect involves nuclear and cytoplasmic components, but its biochemical and molecular action methods remain

a mystery [37]. Figure 3 depicts cisplatin's structure and method of action. The most common route of cytotoxicity is the formation of platinum-DNA adducts on purine bases, which results in substantial DNA damage and triggers death in the cell. The toxicity of cisplatin is directly related to its accumulation in the cell. The more cisplatin DNA adducts there are the more cytotoxic effects in the cell [38].

The copper membrane transporter CTR1 is the primary route through which cisplatin enters the cell via passive diffusion and active absorption [39]. The high concentration of chloride ions (∼100 mM) in the blood keeps cisplatin reasonably stable and neutral. A water molecule replaces a chlorine ion after entering the cell due to the low chloride ion concentration (∼4−12 mM). The term "equation" refers to this. As illustrated in Figure 1, the aqua-cisplatin complexes are difficult to disperse from the cell, and the monochloride form is a powerful electrophile that reacts quickly with nucleophiles like DNA [39]. As a result, DNA loses a water molecule due to this binding to nitrogen in the N7 position on purine bases. The cisplatin can then be cross-linked to another purine using the remaining chloride, resulting in an equivalence. Cisplatin's cytotoxicity relies on cross-linking between neighboring guanine residues [40].

Adjuncts disrupt DNA replication and transcription, resulting in cell cycle arrest and perhaps activation of pro-apoptotic signaling. DNA repair processes, such as nucleotide excision repair, are activated when the cell cycle is halted (NER) [41]. By excising the damaged area, the NER complex can repair DNA adducts of cisplatin and allow for cell survival. On the other hand, Apoptosis is the most likely conclusion if the DNA damage is too substantial to repair. Cisplatin's apoptosis-inducing properties are not limited to DNA damage [41]. It has been found that cisplatin's interactions and reactions with other proteins might cause cell harm. Particularly damaging effects include mitochondrial damage and malfunction, glutathione depletion and depletion, lipid peroxidation, and activation of the apoptotic pathway that are brought on by cisplatin treatment, inducing oxidative stress [42]. This potent combination of apoptotic effects is achieved in treating aggressive solid tumors.

Human anticancer therapy makes extensive use of the anthracycline antibiotic doxorubicin [43]. As far as we know, there is currently no clear understanding of how the drug doxorubicin works. Doxorubicin inhibits macromolecular production by intercalating with DNA. The enzyme topoisomerase II slows down, and the DNA's supercoils relax, allowing transcription. After the topoisomerase II complex has broken the DNA chain for replication, doxorubicin stabilizes it, preventing the DNA double helix from resealing and terminating the replication process [Figure 4] [44]. Doxorubicin HCl's propensity to create free radicals damages DNA and the cell membrane, which is still another mechanism at work.

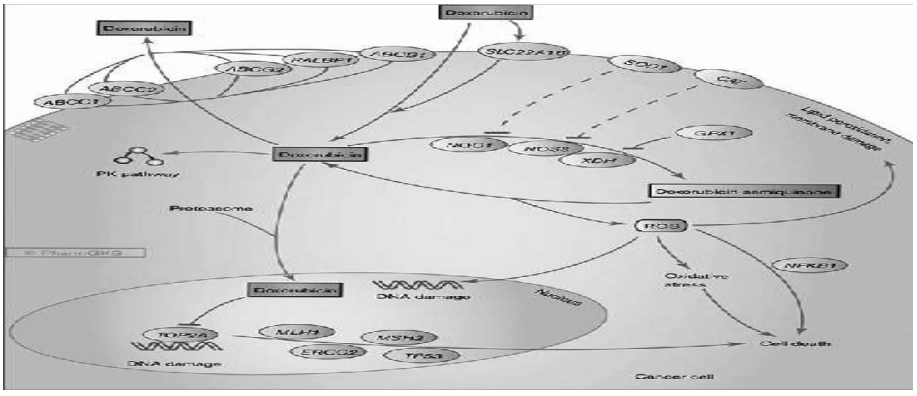

Figure 4: Mechanism of action of doxorubicin.

Nitric oxide (NO) is a lipophilic, tiny free radical gas having various biological effects on cell damage and carcinogenesis [45]. Nitric oxide synthase (NOS) produces NO through a sequence of redox processes involving L-arginine [46]. The half-life of endogenous NO is roughly one second after NOS synthesizes it. As a result, the activity of NOS plays a significant role in regulating the endogenous generation of NO. Neuronal (nNOS or NOS-1) and inducible (iNOS or NOS-2) genes, as well as

endothelial (eNOS or NOS-3) genes, encode the NOS isoenzyme in mammals. nNOS and eNOS are present in peripheral nerves and vascular endothelial cells, respectively [47]. However, iNOS is induced by mesenchymal and parenchymal cells by stimulating endotoxins and cytokines, primarily in pathological situations. NOS expression has been discovered in various malignancies, including the breast, bladder, stomach, prostate, lung, colon, pancreas, and kidney. NOS expression is unclear in liver cancer. One study compared iNOS expression in cancer tissue to non-tumor liver tissue and discovered that HCC had reduced iNOS expression [48]. However, because most HCC cases originated in the context of cirrhosis, the study mentioned above did not compare iNOS expression in HCC to that in cirrhosis patients without HCC. Additionally, eNOS and nNOS, required for NO production, were not evaluated parallel. As a result, NOS expression and serum NO levels in patients with HCC should be further explored.

The present study showed that 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 a significant decrease in the levels of NO 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. Similar to our findings, Zhang et al. [49] showed that NO levels increase in people with HCC due to increased eNOS and iNOS expression in tumor tissues.

Additionally, Moussa et al. [50] showed that CHC patients with HCC had increased plasma nitrites/nitrates levels. However, Zhou et al. [48] measured NO levels in tumor tissues and noncancerous liver tissue from people with HCC. They found that NO levels were considerably more significant in noncancerous tissue than malignant tissue. This discrepancy is attributed to the sampling differences between serum and malignant tissue and the exceptionally short half-life of endogenous NO.

5. CONCLUSION

The present study might offer substantial evidence for selecting therapeutic agent candidates for hepatocellular carcinoma management. According to our findings, this study revealed that nitric oxide levels are relatively high in HCC. Moreover, after adding chemotherapy drugs, we found cisplatin drug more effective in treating HCC than doxorubicin drug.

List of abbreviations:

Doxo: Doxorubicin
Cis: Cisplatin
HCC: Hepatocellular carcinoma
NO: Nitric oxide
MTT: Methyl thiazole tetrazolium
DMSO: Dimethylsulfoxide

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