Alfarama Journal of Basic & Applied Sciences
https://ajbas.journals.ekb.eg
ajbas@sci.psu.edu.eg
Faculty of Science Port Said University
http://sci.psu.edu.eg/en/
ISSN 2682-275X
July 2022, Volume 3 Issue II
DOI:10.21608/AJBAS.2022.113807.1082
Submitted: 10-01-2022
Accepted: 13-03-2022
Pages: 210- 219

Assessment of Alpha-fetoprotein levels and the effect of 5- Fluorouracil on Cytotoxicity of HepG2 cell line

Nabil M. Abdel-Hamid[1], Mohamed S. El- Gharieb [2], Fardous F. El- Senduny[3], Nadia A. Alnakib[2,*]

[1]Chemistry Department, Faculty of Pharmacy, Kafr-Elsheikh University

[2]Chemistry Department, Faculty of Sciences, Port-Said University

[3]Chemistry and Molecular Biology, Faculty of Sciences, Mansoura University

*** Corresponding author:** nadiaelnakib@yahoo.com

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide nowadays. In Egypt, the cancer is frequently discovered at an advanced stage, at which no therapy, even surgery, is successful. Early detection of the disease is critical because it enables the patients to be treated prior to enlarging or metastasizing to distant organs. A tumor marker is a serological agent whose blood level may indicate the existence of the tumor in the early stages. The gold standard and more reliable biomarkers for HCC is alpha-fetoprotein (AFP). **Aim of the study:** To determine the cytotoxicity and Selectivity index of 5-Fluorouracil in HepG2 and WI-38 cell lines by the methyl thiazole tetrazolium (MTT) assay and assess alpha-fetoprotein (AFP) levels as a tumor marker in the progression of HCC. **Material and Methods:** HepG2 and WI-38 cells were incubated with different concentrations of 5-Fluorouracil as a standard chemotherapy drug. The half-maximal inhibitory concentration (IC50) values were determined the methyl thiazole tetrazolium (MTT) for 5-Fluorouracil. **Results:** IC50 values for HepG2 and WI-38 were found for 5-Fluorouracil (32.533±0.777µM and 63.400±0.624µM), respectively. Moreover, the selective index value of 5-Fluorouracil was (1.949±0.027µM). The AFP levels in the HepG2 cell line treated with 5-Fluorouracil were measured. We found significant inhibition in AFP levels after treating HepG 2 cell lines by 5-Fluorouracil. **Conclusion:** The change in AFP levels following chemotherapy with 5-fluorouracil is beneficial for predicting treatment response in the HepG-2 cell line.

Key Words:
Hepatocellular carcinoma (HCC), 5-Fluorouracil (5-FU), Alpha-fetoprotein (AFP), Cytotoxicity, MTT assay.

1. INTRODUCTION

Chronic hepatitis B/C virus carriers and individuals with liver cirrhosis are the two most significant causes of cancer-related death globally [1]. The annual incidence of HCC, which has tripled in the past three decades, is most prevalent in Africa and Asia [2]. As a result, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis are becoming increasingly common causes of HCC in Western and more industrialized countries [3]. Because of this, HCC has become a severe and dangerous disease that requires rigorous treatment worldwide [4]. Moreover, HCC survival rates are low in the advanced stages, and patients tend to wait until they are in that stage before seeking therapy [5]. For that, early diagnosis of HCC with a surveillance program has been deemed an unquestionable technique for preventing the patient from progressing to an advanced stage of the illness [6].

Alpha-fetoprotein is abbreviated AFP. It is a protein produced in a developing infant's liver. When a newborn is born, AFP levels are typically elevated but fall too low by the time the child reaches the age of one [7]. AFP levels in healthy persons should be extremely low. Increased AFP levels do not always indicate cancer, while normal AFP levels do not permanently exclude cancer [8]. As a result, an AFP tumor marker test is rarely used alone to screen for or diagnose cancer [9]. However, when combined with another testing, it can diagnose cancer. Additionally, the test may be used to evaluate the success of cancer treatment and to determine whether cancer has returned after treatment is completed [10].

According to various international guidelines, using AFP biomarkers with abdominal ultrasonography is a practical approach to surveillance [11]. In most hospitals worldwide, high-risk patients for hepatocellular carcinoma (HCC)are routinely screened for the disease every six months [12]. In order to monitor HCC, the liver is examined using USG, and AFP is measured in blood. In clinical practice, alpha-fetoprotein (AFP) is the most often utilized serum marker for screening and initial diagnosis of HCC [13]. However, AFP has a sensitivity of around 60% at a 20 ng/mL cutoff value and low specificity [14]. Furthermore, AFP levels remain normal in 15%–30% of patients with advanced-stage disease and increase in some patients with chronic hepatitis, liver cirrhosis, and other liver disorders, resulting in a high risk of false-negative and false-positive results [15]. As a result, novel indicators that supplement the limitations of AFP are required for more reliable screening and diagnosis of HCC [16].

One of the most commonly used anticancer drugs is 5-fluorouracil (5-FU) [17]. 5-FU's mechanism of action has been better understood over the past 20 years, which has led to the development of new ways to enhance its anti-cancer properties. Despite these advancements, resistance to 5-FU continues to be a substantial impediment to its therapeutic usage [18]. Recent advances in DNA microarray profiling offer the potential to uncover new genes responsible for 5-FU resistance [19]. Utilizing these genes as chemotherapy targets or indicators in response to chemotherapy regimens that depend on 5-FU could be therapeutically beneficial [18]. Antimetabolite medications work by suppressing critical biosynthetic processes or absorbed into macromolecules, such as DNA and RNA, and preventing their normal function [20]. A fluoropyrimidine 5-fluorouracil (5-FU) can accomplish both of these things [21]. FLUOROPYRIMIDINES were created in the 1950s because hepatomas in rats, utilize uracil as one of the four bases of RNA significantly more rapidly than normal tissues, implying that uracil metabolism may be targeted for antimetabolite chemotherapy[22]. Misincorporation of 5-FU fluoronucleotide RNA and DNA and inhibition of thymidylate synthase have been implicated in 5-FU's ability to cause cell death in vitro and animal studies (TS) [23].

In treating a variety of malignancies, including colorectal, liver, breast tumors, and those of the respiratory system, 5-FU is commonly employed [24]. When used with other chemotherapeutic medicines, 5-FU can enhance breast, liver, head, and neck cancer response and survival rates [25]. To forecast or overcome tumor resistance to 5-FU, one must first understand the mechanisms by which the drug kills cells and how cancers develop resistance to it [26]. The DNA MICROARRAY technology offers the potential to find new genes that are crucial in modifying 5-FU-based chemotherapy resistance [18]. 5-FU chemosensitivity could be predicted using these genes as biomarkers or new biological targets for combating drug resistance [18].

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, Alex, Egypt. The HepG2 cell lines were maintained using penicillin, streptomycin, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM). All cells were cultured in 5% CO_2 at 37°C. After achieving confluence, 0.25 percent trypsin EDTA was used to detach the HepG2 cells, and $1x10^6$ cells were seeded into the same complete media. The DMEM media was replenished every three days.

2.2 Reagents and drug treatments:

Dimethylsulfoxide (DMSO) and 5-fluorouracil were bought from Sigma-Aldrich. Fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco's modified Eagle's medium (DMEM)were procured from GIBCO® (Invitrogen). From Sigma Chemical Co. (St. Louis, MO, USA), all additional compounds used in this study were of analytical quality were purchased.

2.3 Estimation of Alpha-fetoprotein (AFP) Concentration:

Used the Human (ELISA) Immunoassay Kit, cloud clone corp, to measure alpha-fetoprotein.Company (USA). The manufacturer's instructions were carried out in all ELISA procedures. Biotek plate reader (Gen 5th) measured the absorbance at wavelengths ranging from 570 to 630 nm.

2.4 Cytotoxicity assay:

The cytotoxicity of 5-fluorouracil was determined using the methyl thiazole tetrazolium (MTT) test. Cells were allowed to adhere for 24 hours before being treated with the specified 5-fluorouracil 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 5-fluorouracil 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% CO2. 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 [27].

2.5 Selectivity index (SI):

It is the ratio that measures the cytotoxicity between normal and cancer cells [28]. The higher the selectivity index ratio, the more effective and safer the drug [29]. Each SI value was determined using the following formula: $\mathbf{SI} = (\mathbf{IC50})\mathbf{normal}/(\mathbf{IC50})\mathbf{cancer}$

2.6 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 results were deemed 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 5- Fluorouracil on AFP (α-Fetoprotein) Tumor Marker:

Demonstrated a statistically significant reduction ($P \leq 0.01$) in the concentrations of (AFP) when Hepg-2 cell lines were treated with (5- FU) compared to the control group (DMSO) depending on the chemotherapy drug's mechanism of action, as illustrated in Table (1) and Figure (1).

Table (1): AFP level after treating HepG2 cells with 5-Fluorouracil.

Parameter / Drug	AFP (µg/ml)
Control (DMSO)	6.0425±0.129
5- Fluorouracil (5- FU)	2.458****±0.321

Data are expressed as means ± SD; the significant difference between the treatments is analyzed by t-tests, then there is the t-test., where: **** significant at P≤ 0.0001.

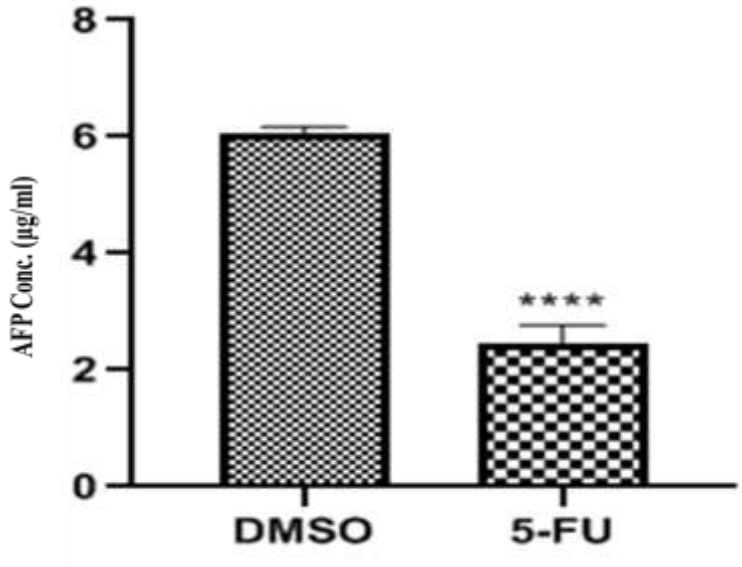

Fig (1): AFP levels of treated hepG2 cells with a 5-Fluorouracil. Data are expressed as means ± SD, **** significant at P≤ 0.0001.

3.2 Cytotoxicity effect of 5-Fluorouracil on HepG-2 cells:

The MTT assay was performed to evaluate 5-Fluorouracil cytotoxicity at various concentrations ranging from 50 to 1.56 µg as shown in Table (2). The concentration that induced 50% inhibition in cell growth (IC50) was found to be (7.433 µg/ml) using a semi-logarithmic plot of cell viability vs. concentrations as shown in Figure (2).

Table 2. Effect of the 5-Fluorouracil on cell viability percentage on HepG-2 cells.

5-Fluorouracil Conc. (µg)	Viability for HepG-2 (%)
50	32±2
25	36±2
12.5	48±3
6.25	53±1
3.125	63±1
1.56	56±2

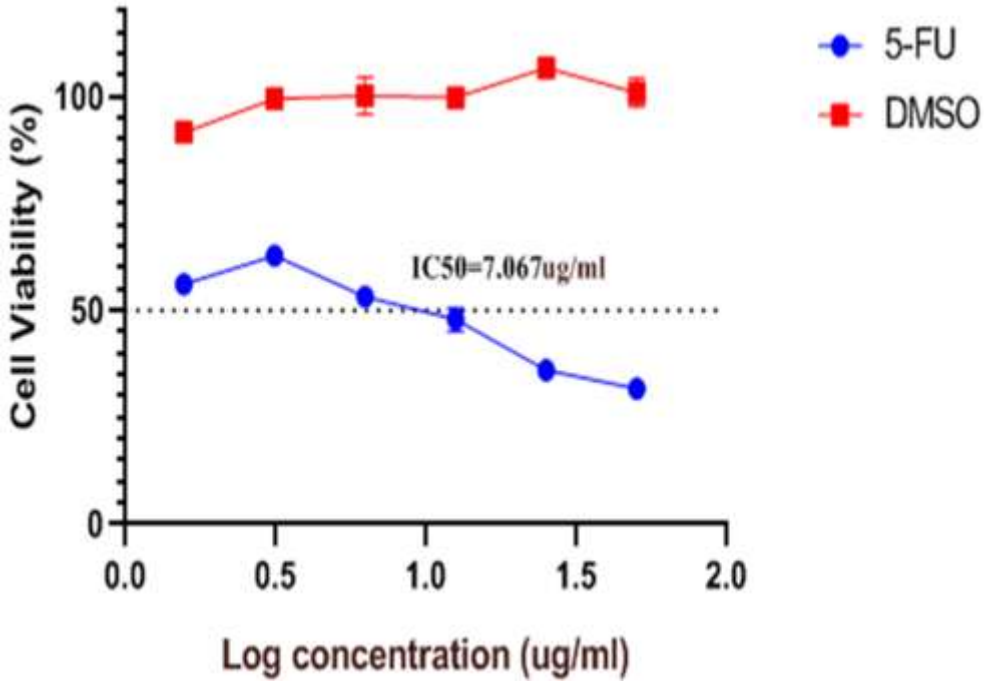

Figure 2. The effect of treatment with 5-Fluorouracil on the viability of HepG-2 cells by the MTT assay.

3.3 Selectivity index of 5-Fluorouracil:

Selectivity index was evaluated through testing the cytotoxic effect on normal cell line WI-38 and determination of their IC50. After 48 hours of incubation with normal lung fibroblast cells WI-38, the viability was measured by MTT assay, and the selectivity index was calculated according to (**SI** = (**IC50**)**normal**/(**IC50**)**cancer**) Equation. The result showed that the IC50 of the compound 5-Fluorouracil against HepG-2 cancer cell line was (32.533±0.777 µM/ml) and the normal lung fibroblast cells WI-38 was (63.400±0.624 µM/ml). The selectivity index of 5- Fluorouracil was (1.949±0.027 µM/ml).

4. DISCUSSION

Hepatocellular carcinoma (HCC) was the fifth most frequent type of cancer worldwide, accounting for 55% of occurrences [30]. HCC metastasized easily and was difficult to diagnose in its early stages [31]. Less than 20% of people with liver cancer can be treated surgically [32]. As a result, systemic chemotherapy became the primary method of treating liver cancer [33]. HCC is a well-known malignant tumor with low chemosensitivity to anticancer treatments. Generally, cancerous tumors are defined by uncontrolled cell division, absent in normal tissue [34]. When "normal" cells come into contact with similar cells, they cease to divide, a process known as contact inhibition [35].

Cancerous cells are unable to retain this ability. Cancer cells lack the typical checks and balances that regulate cell division [36]. The cell cycle is the mechanism through which cells divide, whether normal or malignant. The cell cycle progresses from rest to active growth and mitosis (division) [37]. Chemotherapy's ability to kill cancer cells dependent on its capacity to halt cell division. A common mechanism for these drugs is to damage the RNA or DNA that instructs the cell to replicate itself during cell division [38]. The cells die if they are unable to divide. The more rapidly the cells divide, the more likely chemotherapy kills them and shrinks the tumor.

Additionally, they induce cell apoptosis (self-death or apoptosis) [39]. Chemotherapy treatments that affect only dividing cells are referred to as cell-cycle specific. Chemotherapy treatments that affect cells at rest are cell-cycle non-specific treatments [40]. Chemotherapy is scheduled according to the type of cells, the rate at which they divide, and the period during which a particular medicine is likely to be effective. For this reason, chemotherapy is usually administered in cycles [41]. Chemotherapy is especially effective against quickly dividing cells. Unfortunately, chemotherapy is unable to distinguish between malignant and normal cells. While the "normal" cells will regenerate and become healthy, side

effects will develop in the meantime [42]. Chemotherapy most frequently affects "normal" cells in the blood, mouth, stomach, colon, and hair follicles, resulting in decreased blood counts, mouth sores, nausea, diarrhea, and hair loss. Varied medications may have different effects on different body areas [43]. To date, fluoropyrimidine fluorouracil, such as 5-FU, remains the first-line therapy for HCC. However, its application is limited because of the rapid development of acquired resistance.

5-Fluorouracil (5-FU) was discovered as antimetabolite chemotherapy in 1957 and was licensed by the FDA in 1962 to treat colorectal cancer (CRC) based on the fact that tumoral tissues metabolized uracil more rapidly than normal tissues [44]. Since its initial licensure, 5-FU has been widely used alone or in combination with other medications to treat a variety of solid malignancies of the digestive tract (colorectal, anal, pancreatic, oesophageal, gastric, and ampullary tumors), as well as cancers of other organs (i.e., breast, cervix, and head and neck cancers) [45]. Today, 5-FU is used to treat several of the most lethal forms of cancer, including pancreatic ductal adenocarcinoma(PDAC), as well as colorectal cancer (CRC), which are both types of cancer in the digestive system [45]. While treatment regimens (dosage, timing, and delivery) vary according to the tumor's genesis, 5-FU remains a critical medicine for cancer management [46].

Fluorine is substituted for hydrogen in the C-5 position of 5-FU, making it an analog of uracil (Fig. 3) [47]. Using the exact accelerated transport mechanism as uracil quickly penetrates the cell. Intracellularly, 5-FU is converted to a wide variety of active metabolites, including fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate (Fig. 3) [48]. The synthesis of RNA and the activity of TS are both impaired by these active metabolites, which is why TS is not effective against 5-FU-resistant cells [49]. Dihydropyrimidine dehydrogenase (DPD), where it converts 5-FU to dihydrofluorouracil and is the rate-limiting enzyme in the catabolism of 5-FU (DHFU) [50]. Usually, more than 80% of given 5-FU is catabolized in the liver, where DPD is abundantly expressed [51].

According to Zheng et al., 5-FU is currently the top choice for hepatocellular carcinoma treatment, because of its significant killing effects on cancer cells. 5-FU has the ability to harm proliferative cells, shrink tumor masses, and halt tumor growth [52]. The effects of 5-FU are unsatisfactory because 5-fluorouracil has a lower concentration in tumor tissue and substantially larger quantities in blood following intravenous injection. Furthermore, the drug's side effects are severe, and many patients cannot endure them [52]. In the present study after noticing the effect of the 5-Fluorouracil drug on HepG-2 cell lines, we found a significant decrease in AFP levels. Similar to our work, Chan et al. [53] discovered that serial AFP measurements effectively predict and monitor treatment response in patients with HCC receiving doxorubicin-based chemotherapy. They indicated that including AFP response in treatment outcome criteria should be handled in clinical practice and clinical trials of new medicines to treat HCC. Additionally, Our findings are consistent with Chou et al. [54], who showed that changes from baseline AFP levels have a prognostic effect in HCC patients with extra-hepatic spreading and oxaliplatin-based chemotherapy.

In contrast to retrospective research published in 2012 by Zhang et al. [55], roughly 30%-40% of HCC patients in China were AFP negative, making this markerless effective in these settings. Additionally, the scientists discovered that the Barcelona Clinic Liver Cancer stage had the most significant potential for predicting prognosis in patients with normal baseline AFP levels. A normal AFP level may signal earlier-stage tumors. Zhang et al. [55] included patients with all stages of HCC, making it difficult to demonstrate the actual predictive value of AFP in a mixed sample.

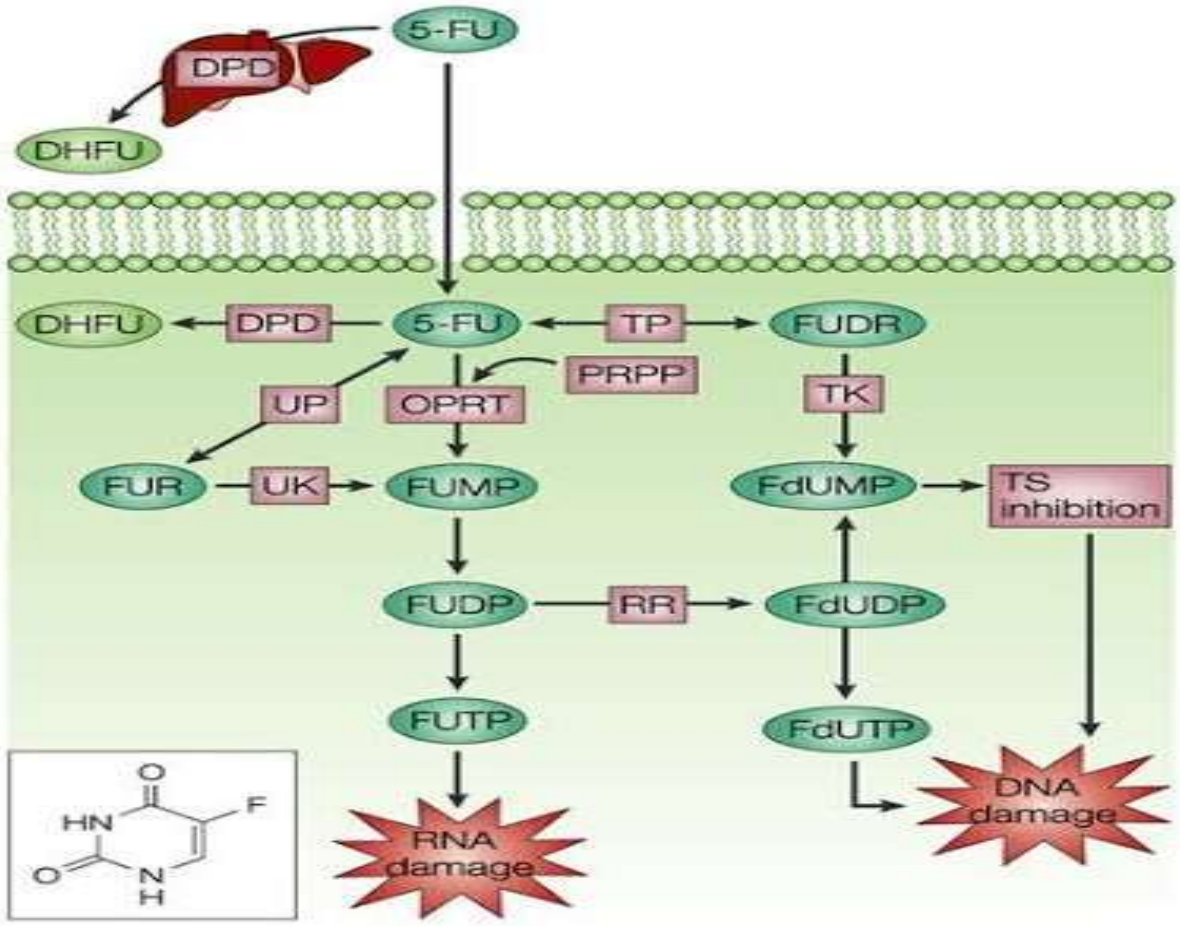

Figure 3. There are three primary active metabolites of 5-Fluorouracil (5-FU; see structure): fluorodeoxyuridine monophosphate, fluorouridine triphosphate (FUTP), and Fluorodeoxyuridine triphosphate (FdUTP). The primary mechanism of 5-FU activation is the transformation of fluorouridine (FUR) into fluorouridine monophosphate (FUMP), which can be accomplished either indirectly through the sequential activity of uridine phosphorylase (UP) and uridine kinase, or directly by orotate phosphoribosyltransferase (OPRT) using phosphoribosyl pyrophosphate (PRPP) as a cofactor. FUMP is then phosphorylated to generate fluorouridine diphosphate (FUDP), which can then be further phosphorylated to form fluorouridine triphosphate (FUTP) or converted into fluorodeoxyuridine diphosphate (FdUDP) by the enzyme ribonucleotide reductase (RR). FdUDP, in turn, can be phosphorylated or dephosphorylated to create the active metabolites FdUTP and FdUMP, which are both seen in high concentrations.Thymidine phosphorylase catalyzes the conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated to become FdUMP by the enzyme thymidine kinase (TK). The transformation of 5-FU into dihydrofluorouracil (DHFU) via dihydropyrimidine dehydrogenase is the rate-limiting step in 5-FU catabolism in both normal and malignant cells (DPD). DPD degrades approximately 80% of the 5-fluorouracil given in the liver [56].

At the moment, the alpha-fetoprotein (AFP) level in serum is a diagnostic sign for HCC detection. In individuals with chronic liver disease, it has been demonstrated that a sustained rise in AFP serum levels is one of the risk factors for HCC and has been utilized to identify a high-risk subgroup of chronic liver disease [57]. Fluctuations in AFP levels in patients with liver cirrhosis disease may indicate the development of HCC, sudden onset of viral hepatitis, or a progression of the potential liver disease. It is proven that various factors can influence the AFP level, complicating determining the threshold. A low cutoff value of 20 ng/ml of AFP resulted in strong sensitivity but poor specificity.

In comparison, a high cutoff value of 200 ng/ml resulted in high specificity but a marked reduction in sensitivity [58]. AFP 400 ng/mL was utilized as the diagnostic threshold in China's 2001 and 2017 diagnostic staging standards for HCC [59]. However, Saad et al. [60] indicate that the diagnostic efficacy of AFP 200 ng/mL may be higher, in part because some early HCC cases may be missed in populations with low AFP concentrations (20 to 200 ng/mL) if 400 ng/mL is still utilized as the HCC screening cutoff. As a result, the appropriate AFP cutoff for diagnosing HCC remains debatable until today [61].

5. CONCLUSION

In conclusion, The findings of this study may provide vital information for the selection of therapeutic agent candidates for the treatment of hepatocellular carcinoma. According to our findings, we demonstrated the cytotoxic activity of 5- fluorouracil on the HepG-2 cell line, and Selectivity was evaluated by measuring the cytotoxicity against a normal cell line. Moreover, we found Alpha-fetoprotein levels are relatively high in HCC, consistent with previous research. For that, after including5-fluorouracil as a chemotherapeutic medication, we discovered that the 5-fluorouracil had a strong inhibitory effect on HepG-2 cell lines. However, because of the quick development of acquired resistance, its use is restricted. Therefore we continued our research and our results are still under publishing for showing the effect of a combination of 5- fluorouracil with natural drugs which consider antioxidants with hepatoprotective effect to reduce the side effects of chemotherapy.

List of abbreviations:

HCC: Hepatocellular carcinoma
AFP: Alpha-fetoprotein
MTT: Methyl thiazole tetrazolium
DMSO: Dimethylsulfoxide
5- FU: 5-Fluorouracil

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