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Investigation of the Effects of Metformin and Ifosfamide Applications on Cell Proliferation and Apoptosis in Hepatocellular Carcinoma Cells

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Abstract

Cancer is a concern, for health ranking as the second leading cause of death after cardiovascular diseases. Among types of cancers hepatocellular carcinoma (HCC) is particularly noteworthy being the most common type following lung and stomach cancers. Various treatment approaches are used for managing HCC like chemotherapy. In this study the HepG2 cancer cell line was used as a model to investigate the effects of ifosfamide and metformin on cancer cells. Cell viability tests and RT PCR analysis targeting genes associated with the PI3K/AKT/mTOR pathway were conducted to evaluate these effects. These results offer valuable insights for advancing further research for their potential application in the treatment not only of HCC but also of other cancer types.

Keywords: Hepatocellular Cancer, Metformin, Ifosfamide, Cell Proliferation, PI3K/AKT/mTOR pathway.

Introduction

Hepatocellular carcinoma (HCC) ranks third in terms of lethality among all known cancer types worldwide (1). HCC accounts for 80% of primary liver cancer cases (2). Since it develops and progresses rapidly, early diagnosis is crucial for effective treatment(1). Therefore various surgical treatment methods are being developed by experts such as oncologists and radiologists (3). Among the primary causes are Hepatitis B and hepatitis C viruses, especially prevalent in developing countries, while excessive alcohol consumption is more common in developed countries. Additionally, aflatoxin contamination found in food crops in some parts of the world contributes to the development of liver cancer (4). Various methods, including surgery, immunotherapy, hormone therapy,

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radiotherapy, chemotherapy, and injection of various RNA molecules, are employed in treating cancer diseases. Chemotherapy is considered the most effective method, playing a significant role in treatment (5). In this study, ifosfamide and metformin were administered both separately and in combination to target HCC. Ifosfamide (3-(2-Chloroethyl)-2-[(2-chloroethyl) amino] tetrahydro-2H-1,3,2-oxaza phosphorine-2-oxide) is an alkylating, cytostatic, and antineoplastic drug (6). It has been utilized in combination with other chemotherapy agents for treating types such as testicular cancer and soft tissue cancer. Moreover, research has been conducted on its efficacy in treating cancers such as osteosarcoma, small cell lung cancer, and cervical cancer (6).

Metformin is responsible for decreasing blood glucose levels by reducing hepatic glucogenesis, thereby decreasing glucose output from the liver (7). It has been used for many years in the treatment of diabetes, either as monotherapy or in combination with other drugs (8). Its therapeutic properties for various types of cancer include colorectal cancer, liver cancer, and breast cancer, attributed to its ability to regulate glucose levels, have been demonstrated (9).

In this study, research was conducted using HEPG2, a liver cancer cell line, with literature information on ifosfamide and metformin as a reference. The study commenced by examining the effects of these substances on the viability of HepG2 cells, followed by an investigation of their impact on genes involved in processes such as cell viability and apoptosis through RT-PCR tests.

Materials and Methods

Cell culture applications: The Hepatocellular carcinoma cell line, HepG2, is stored in a -196 °C liquid nitrogen tank at Erzurum Technical University, High Technology Research Center, Molecular Cancer Biology Laboratory. The DMEM (Dulbecco's Modified Eagle Medium) medium was prepared with 10% FBS (Fetal Bovine Serum), 2% L-Glutamine (Gibco, USA), and 1% Penicillin-Streptomycin. The parental HepG2 cell line was cultured in 25cm² flasks (Esco Co, Korea) within an incubator set at 37 °C, with 95% humidity and 5% CO2.

Drug treatment: Ifosfamide and metformin were utilized to assess their cytotoxic and proliferative effects on the HepG2 cell line. These effects were evaluated both separately and in combination groups. To compare with the control group, to which no substance was applied, individual groups were treated with Ifosfamide at doses of 3.5 µg (Ifosfamide 1) and 7 µg (Ifosfamide 2), as well as with Metformin at doses of 8 µg (Metformin 1) and 40 µg (Metformin 2). Additionally, combined groups were treated with Ifosfamide and Metformin at the following doses: ifos1+met1, ifos1+met2, ifos2+met1, ifos2+met2.

Cell cytotoxicity assay: MTT analysis is a method used to quantify cell proliferation by measuring enzyme activity based on MTT reduction. This method allows for the determination of cytotoxic or proliferative effects of therapeutic agents on cells. In this study, Ifosfamide and metformin were tested at predetermined concentrations using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to investigate their cytotoxic effects on the HepG2. Initially, 1,500 cells were seeded into 96-well plates, and after 24 hours, the drugs were applied at the predetermined concentrations. Following an additional 24-hour incubation period, the MTT kit (Sigma Co., USA) was added to assess viability. After 3 hours of further incubation with MTT, the absorbance of the cells was measured at 570 nm using a microplate reader spectrophotometer (µQuant, BioTek Instruments, Winooski, Vermont, USA) in duplicate measurements (26).

Quantitive Real Time PCR (q-RT-PCR) Analysis

RNA isolation: Total RNA isolation from treated-HepG2 was conducted using a commercial kit (Ambion Purelink RNA mini kit, catalog numbers 12183018A and 12183025). The procedure involved steps such as cell lysis, homogenization, and purification following the protocol provided with the kit. Subsequently, the concentration of purified RNAs was measured spectrophotometrically using a nanodrop device (EPOCH Take3 Plate, Biotek). The isolated RNAs were then stored at -20°C for subsequent cDNA synthesis (27).

cDNA synthesis: The RNA samples stored at -20 °C were reverse transcribed into cDNA using a commercial kit (Maxime RT Premix kit). In each PCR tube, 5 µL of RNA and 15 µL of RNase-free water were combined and then placed into the Veriti 96 Well Thermal Cycler (Applied Biosystems). The PCR was conducted on the device at 45°C for 60 minutes followed by a denaturation step at 95°C for 5 minutes. Subsequently, the concentrations of the synthesized cDNAs were determined spectrophotometrically, and the cDNA samples were stored at -20°C for future use.

qRT-PCR analysis: mRNA expressions of Akt3, Bcl-2, PIK3CA, mTOR, BAX, and GAPDH genes were assessed using quantitative real-time PCR (qRT-PCR) with specific primers as detailed in Table 1. The amplification, detection, and data analysis were conducted utilizing the Qiagen Rotor-Gene Real-Time PCR System (Rotor-Gene Q 5plex HRM System). The amplification conditions were set as follows: initial enzyme activation at 95°C for 3 minutes, followed by denaturation at 95°C for 5 seconds (for 40 cycles), and amplification at 60°C for 10 seconds (for 40 cycles). Relative gene expression levels were calculated using the ΔΔCT method, allowing for insights into the molecular mechanisms underlying cellular responses to drug treatments.

Table 1: Sequences of primers used for qRT-PCR

Primers		5´-3´ sequences of primers
AKT3	Forward	GGAAGAATGGACAGAAGCTATTCCA
	Reverse	TCCACTTGCCTTTCTCTGAAC
BCL2	Forward	CGCATCCTGAGGCACCG
	Reverse	TTTCATCCAGGATCGAGCAGGG
PIK3CA	Forward	TGGATGCTTCACAGGGCTTTCT
	Reverse	TATCTTGCCGTAAATCATCCCCCA
mTOR	Forward	GTCAGTGGGACAGCATGGAAG
	Reverse	CCCATATGCCCGACTGTAACTC
BAX	Forward	CGCATCCTGAGGCACCGG
	Reverse	TTTCATCCAGGATCGAGCAGGG
GAPDH	Forward	GGTCAATGAAGGGGTCATTGATGG
	Reverse	ATTTGGTCGTATTGGCGCCT

Statistical analysis: In the study, the MTT test was conducted four times, and the resulting data were averaged and visualized using the Microsoft Office Excel program. For RT-PCR analyses, averages were computed using the Microsoft Office Excel program, and the data were then graphed using the GraphPad program.

Results

Antiproliferative effects of ifosfamide and metformin on HepG2 cells: The antiproliferative effects of ifosfamide and metformin on HepG2 cells were evaluated through separate and combined applications of ifosfamide (3.5 µg and 7 µg) and metformin (8 µg and 40 µg). Following a 24-hour incubation period, the viability of HepG2 hepatocellular carcinoma cells was compared between the drug-treated groups and the control group. The results depicted in Figure 1 illustrate the dose-dependent effect of ifosfamide and metformin on cancer cell viability. Upon examination of Figure 1, it is evident that both ifosfamide and metformin exhibit a reduction in cell viability with increasing concentrations. The significant decrease in absorbance which indicate the cell viability observed in the ifos2+met1 combination group compared to the other treatment groups is particularly striking, but overall, no statistically significant differences were observed between the groups.

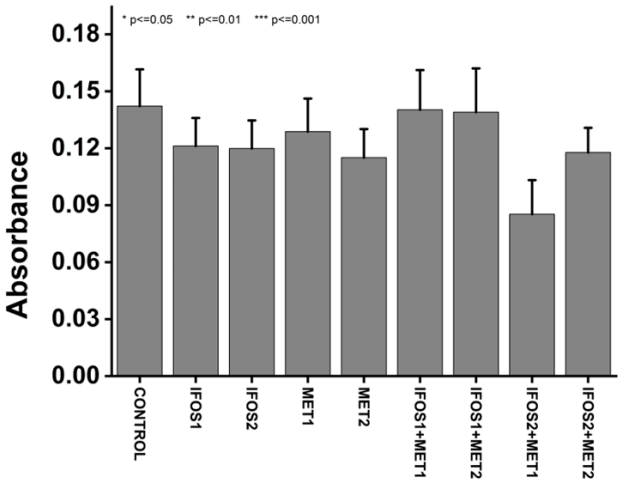

Figure 1: Cytotoxic effect of ifosfamide and metformin on HepG2 cells over 24 hours.

This combination therapy may have a synergistic effect, meaning that both drugs used together may have produced a stronger antiproliferative effect. Furthermore, this combination therapy may prevent the development of resistance or break existing resistance. The combination of the two drugs was effective at lower doses than when each drug was used alone. This can help reduce side effects and improve treatment tolerance. However, the different effects observed in combinations of different concentrations may be related to the way drugs interfere with cellular mechanisms. The fact that ifosfamide and metformin affect different cellular signaling pathways may have more effectively inhibited the ability of cancer cells to survive and proliferate when used together. For these reasons, the combined use of ifosfamide and metformin may be an important strategy in cancer treatment.

qRT-PCR Analysis Results: After conducting MTT analysis, the impact of ifosfamide and metformin on HepG2 cancer cells, as well as their effects on AKT3, BCL-2, PIK3CA, mTOR, BAX, and GAPDH genes, was investigated using RT-PCR with a 24-hour incubation period. GAPDH gene served as a control throughout the analysis. In the AKT3 (Protein Kinase B, PKB) gene expression analysis illustrated in Figure 2, a notable increase was observed in the ifos1 group compared to the control group, indicating a significant alteration. However, no substantial changes were detected in the other experimental groups. The AKT3 gene is involved in the overgrowth and survival of cancer cells. Therefore, over-activation of AKT3 promotes cancer development, while decreased expression of this gene prevents cancer development.

Regarding the Bcl-2 (B-cell Lenfoma-2) gene expression analysis depicted in Figure 2, expression levels across the applied groups appeared similar to the control group, except for the ifos1+met2 group, where a notable increase in Bcl-2 gene expression was observed. The Bcl-2 gene is responsible for the production of a protein that regulates apoptosis in cells. Increased expression of Bcl-2 in cancer cells leads to inhibition of normal cell death processes and excessive survival of cancer cells, while its low expression leads to apoptotic death.

In the PIK3CA (p110α subunit of phosphatidylinositol 3-kinase) gene expression analysis, a significant increase in expression level was noted in the ifos1+met2 group compared to the control group without drug application. Conversely, expression levels decreased in the ifos1, ifos2, met1, met2, ifos1+met1, ifos2+met1, and ifos2+met2 groups compared to the control group. Such alterations underscore the significance of PIK3CA mutations in cancer pathogenesis, with reduced expression potentially exerting a preventive effect against cancer progression. Mutations in PIK3CA can lead to constitutive activation of the PI3K/AKT/mTOR signaling pathway, which is central to cancer development and progression. This aberrant activation promotes cell survival, proliferation, angiogenesis, and metastasis, while also conferring resistance to apoptosis and chemotherapy.

Examining the mTOR (Mammalian Target of Rapamycin) gene expression analysis, a significant increase was observed in the ifos2+met1 group compared to the control group, while expression levels

in other groups closely resembled those of the control group (Figure 2). Overactivation of the mTOR gene promotes the overgrowth and survival of cancer cells, but a decrease in mTOR expression slows the proliferation of cancer.

Finally, in the BAX gene expression analysis, all experimental groups exhibited a significant decrease compared to the control group (Figure 2), with the most significant reduction observed in the ifos2 group. Activation of the BAX protein initiates cell death, which may prevent the uncontrolled growth of cancer cells. However, in some cancers, alterations in the BAX gene can lead to inhibition of apoptosis and survival of cancer cells.

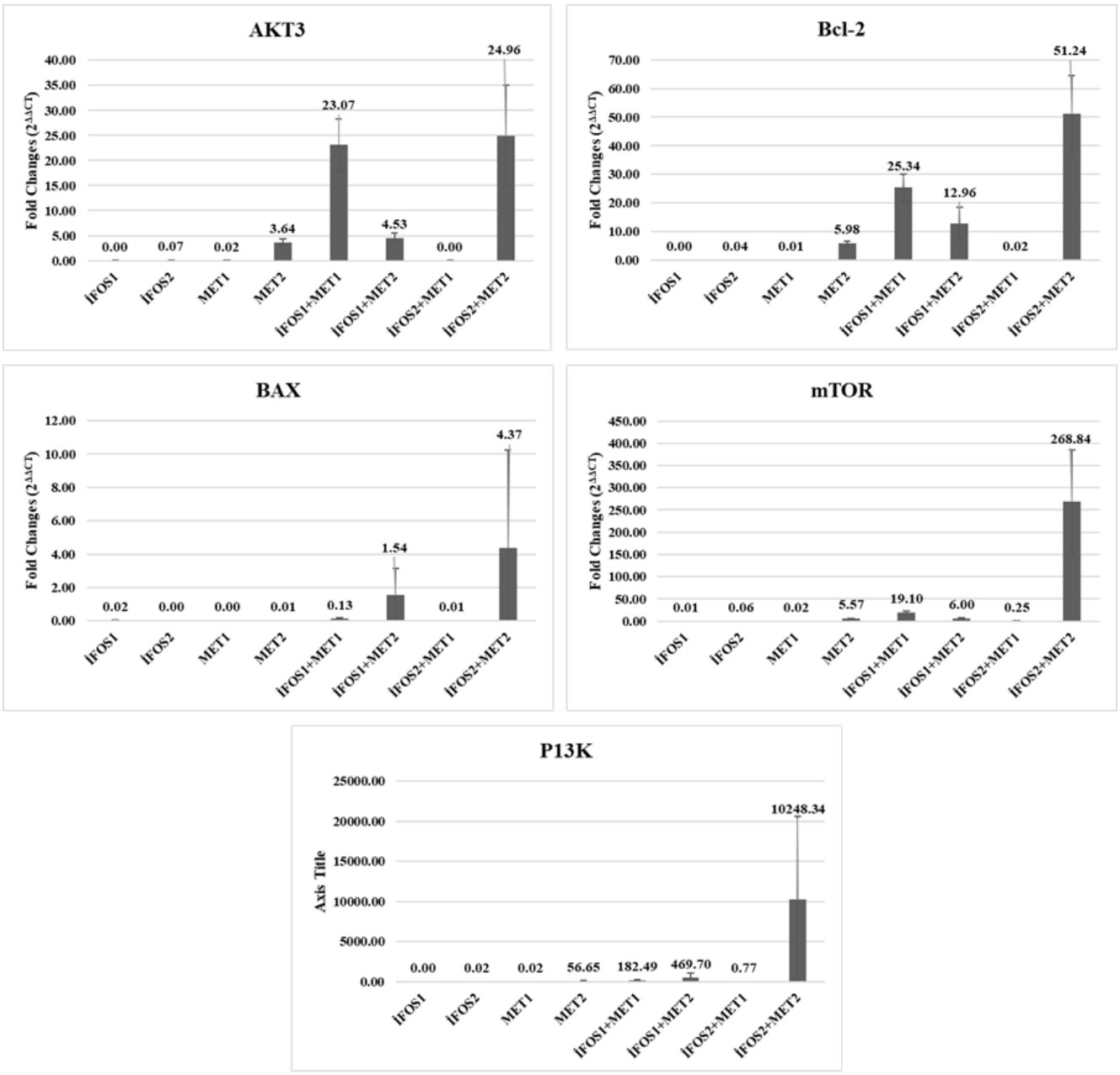

Figure 2: Comparison of AKT3, BCL-2, PIK3CA, mTOR, and BAX gene expression levels in HepG2 cells treated with ifosfamide and metformin.

These findings suggest that ifosfamide and metformin treatments may differentially modulate the expression of AKT3, BCL-2, PIK3CA, mTOR, and BAX genes in HepG2 cells. The observed alterations in gene

expression levels could influence key cellular processes involved in cancer development and progression, such as cell growth, survival, apoptosis, and proliferation. This suggests that combination therapy targets different signaling pathways to influence the ability of cancer cells to grow and survive. Further research is warranted to elucidate the precise molecular mechanisms underlying these effects and explore the therapeutic implications for hepatocellular carcinoma and other cancers.

Discussion

Hepatocellular carcinoma (HCC) stands as the predominant form of liver cancer, originating from hepatocytes and accounting for 85-90% of liver cancer cases. Following lung and stomach cancers, it ranks third in lethality (10). Major contributors to its development include viral hepatitis, excessive alcohol consumption, and cirrhosis (11). While numerous treatment modalities exist for HCC, chemotherapy emerges as a leading approach (11,12). In this study, we explore the therapeutic potential of metformin and ifosfamide for HCC treatment, focusing on their efficacy against HepG2 cells, a commonly used model for HCC research.

Metformin, primarily utilized in treating Type-2 diabetes by regulating hepatic glucose release, has garnered attention in cancer research due to its demonstrated efficacy across various cancer types. A 2020 study on colorectal cancer revealed metformin's anti-proliferative and anticarcinogenic effects (13). Similarly, investigations into lung cancer showcased its clinical benefits, particularly in enhancing survival rates (14). In breast cancer research, metformin exhibited inhibitory effects on cancer cell proliferation (15), highlighting its potential therapeutic role beyond diabetes management. Furthermore, metformin is often combined with other agents for enhanced treatment outcomes. For instance, studies exploring the synergy between metformin and statins in colon cancer treatment reported a significant reduction in mortality rates (16). In 2023, research into ovarian cancer demonstrated the effectiveness of metformin and statins in treatment (17), further underlining their therapeutic potential in combating cancer. Our study aligns with existing literature findings. MTT results indicate a decline in cell viability with increasing metformin concentrations (Figure 1). Particularly, HepG2 cell viability decreased notably with 8 µg of metformin compared to the control group, with further reductions observed at 40 µg. These findings verify the anti-cancer properties of metformin, as reported in the literature, suggesting its potential in impeding cancer cell viability and growth.

Ifosfamide, an alkylating agent commonly employed in cancer chemotherapy, shares structural similarities with cyclophosphamide and is frequently used in various cancer types (18). Its efficacy has been extensively explored across different malignancies. Studies on cervical cancer have demonstrated its therapeutic effectiveness either as a standalone or combined treatment (20). Research conducted in 2021 investigated the cytotoxic effects of ifosfamide on breast cancer (MCF-7) and cervical cancer (HeLa) cell lines, revealing reduced cell viability and an inclination towards apoptosis (21). Similarly, its cytotoxic activity against breast cancer (MDA-MB-231) cells has been documented (22). Investigations in 2020 evaluated the combined use of ifosfamide and irinotecan in lung cancer, showcasing their cytotoxic effectiveness against cancer cell lines (23). However, literature regarding ifosfamide’s efficacy in hepatocellular carcinoma is scarce. In our study, ifosfamide was administered at concentrations of 3.5 µg and 7 µg to investigate its impact on cell cytotoxicity (Figure 1). Our findings demonstrate a concentration-dependent reduction in cell viability of HepG2 hepatocellular carcinoma cells following 24-hour incubation with ifosfamide. Furthermore, in the MTT assay, the combined application of ifosfamide and metformin was

examined. Remarkably, the group exhibiting the most substantial decrease in cell viability was ifos1 + met2 (ifosfamide 3.5 µg + metformin 40 µg).

Following the MTT assay, we conducted RT-PCR tests using the same groups and HepG2 cells. The PI3K/Akt3/mTOR pathway plays a pivotal role in essential cellular functions such as viability, proliferation, survival, migration, and apoptosis (24). Apoptosis, a regulated form of cell death, is orchestrated through two mechanisms: the intrinsic (mitochondrial pathway) and extrinsic (death receptor pathway) pathways. The intrinsic pathway is modulated by the Bcl-2 family of proteins, which can be categorized into anti-apoptotic (e.g., Bcl-2, Bcl-x, Bcl-w) and pro-apoptotic (e.g., Bax, Bak, Mtd) genes (25). In our study, we examined the effects of a 24-hour incubation period on Akt3, Bcl-2, PIK3CA, mTOR, and BAX genes using RT-PCR (Figure 2). We utilized the GAPDH gene as a control gene, and analyses were conducted accordingly to assess the expression levels of these genes in response to the experimental conditions. The results were revealed distinguished changes in gene expression levels within the HepG2 cell line following the application of ifosfamide and metformin. Specifically, an increase in Akt3 gene expression was observed in the ifos1 and ifos2+met2 groups post-treatment. Regarding the Bcl-2 gene, the ifos1+met2 group exhibited elevated expression levels compared to the control, while other groups showed levels similar to the control. Higher expression levels of the PIK3CA gene were noted in the ifos1 + met2 group, whereas other groups exhibited significantly reduced levels compared to the control. Analysis of mTOR gene expression revealed a significant increase in the ifos2 + met1 group. Conversely, the expression levels of the BAX gene decreased significantly with drug applications, with the most significant decrease observed in the ifos2 group. These findings highlight the challenges in effectively managing cancer while highlighting the critical importance of treatment for HCC. The compounds utilized in this study not only reduced the viability of hepatocellular carcinoma cells but also prompted apoptotic pathways. Moreover, varying the concentration of these compounds opens avenues for both in vivo and in vitro investigations, suggesting promising directions for further research and potential therapeutic strategies.

Conclusion

This study focused on evaluating the effects of metformin, a longstanding antidiabetic agent, and ifosfamide, a chemotherapy drug used in diverse cancer treatments, on HepG2 liver cancer cells. Our findings demonstrate that both ifosfamide and metformin decrease HepG2 cell viability in a dose-dependent manner. Moreover, their combined application exhibits enhanced efficacy in reducing cell vitality. Through RT-PCR analysis, we observed significant alterations in the expression levels of key genes involved in cell proliferation and apoptosis, including PIK3CA, Akt3, mTOR, BAX, and Bcl-2. While the results are encouraging, further investigations are warranted to deepen our understanding. Future studies could explore the effects of these compounds on gene expressions in both in vivo and in vitro settings, allowing for a more comprehensive evaluation. Additionally, examining their impact on various signaling pathways could unveil additional mechanisms underlying their anticancer effects. Moreover, exploring different concentrations and combinations of these drugs may offer valuable insights into optimizing cancer treatment strategies.

Author Contributions:

BNG, HTK, KS, DU, AK contributed to project conception, laboratory works and study design. Writing the article (BNG, HTK, KS, DU, AK). All authors read and approved the manuscript.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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