



Deuterium-Enriched Water Increases the Cytotoxicity of Cyclooxygenase Inhibitors in Hep-G2 Hepatocellular Carcinoma Cells; Involvement of MAPK Pathway and Apoptosis

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Abstract

Background: One of the most prevalent forms of liver cancer globally is the hepatocellular carcinoma (HCC). Increasing evidence from both clinical settings and experimental studies indicates that cyclooxygenase (COX) enzymes could play a role in the formation of different types of cancer, including HCC. Deuterium-enriched water (DEW) and deuterium-depleted water (DDW) are involved in both the treatment and prevention of cancer. Employing a blend of COX inhibitors in conjunction with DDW/DEW might serve as a promising strategy to enhance the efficacy of both therapies in HCC.

Methods: The cytotoxicity of celecoxib and indomethacin, examined both individually and in combination with DDW and DEW, was assessed. To explore their antiproliferative effects, Hep G2 cells were treated with the drugs and various concentrations of deuterium in the forms of DDW or DEW, while cell viability was evaluated through the MTT assay. Furthermore, the levels of COX-2, MAPK pathway proteins, the anti-apoptotic protein Bcl2, the pro-apoptotic protein Bax, and the activity of caspase-3 were investigated using SDS-polyacrylamide gel (SDS-PAGE) and western blot techniques.

Results: The simultaneous administration of indomethacin or celecoxib alongside DEW significantly improved the cytotoxic effects and induced apoptosis in Hep G2 cells. The combination of DEWs with celecoxib or indomethacin enhanced the antiproliferative impact of the drug by about 20%–60% and 15%–45%, respectively. This improvement was linked to the activation of p38 and JNK MAPKs, along with a reduction in the pro-survival proteins Bcl-2, COX-2, and ERK1/2. Additionally, the simultaneous treatment led to the activation of caspase-3, an important mediator of apoptosis, and suppressed poly ADP-ribose polymerase (PARP).

Conclusion: The pairing of DEW with NSAIDs can influence the primary cellular signaling pathways, acting as a tactic in cancer chemotherapy.

Keywords: Hep G2 cell, DDW, DEW, Cyclooxygenase inhibitors, MAPK pathway, Apoptosis

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Introduction

One of the most prevalent forms of liver cancer is the hepatocellular carcinoma (HCC), representing 90% of all instances (1). Worldwide, HCC ranks as the sixth most prevalent cancer and is the third leading cause of cancer-related fatalities (2).

Using current HCC treatment chemotherapeutic agents like sorafenib, over an extended period, can lead to complications such as toxicity and/or ineffectiveness and could not significantly decrease the progression of this cancer (3). Thus, researchers have a growing focus on

developing low-toxic and potent anti-cancer drugs.

The stage of fibrogenesis predominantly occurs after the inflammation caused by liver disorders. Therefore, if the inflammation is inhibited, it can prevent the progress of this stage toward HCC (4). COX-2 is highly expressed in liver cancer, which contributes to tumor advancement and enhances the ability of cancer cells to cope with chemotherapy and radiotherapy (5). Various studies show that nonsteroidal anti-inflammatory drugs (NSAIDs) have both protective and therapeutic effects against multiple cancers, including those of the colon-rectum,



breast, pancreas, prostate, head and neck, lung, ovary, and liver (6).

NSAIDs promote apoptosis by triggering p38 and c-Jun N-terminal kinase (JNK), which are pro-apoptotic factors, while inhibiting the extracellular-signal-regulated kinase (ERK1/2), an anti-apoptotic factor, in the MAPK signaling pathway (7). Additionally, NSAIDs can help enhance the expression of tumor suppressor genes like Bax, which is associated with the B-cell lymphoma protein 2 (Bcl-2) family. Moreover, NSAIDs can decrease the levels of anti-apoptotic genes, such as Bcl-2. Proteins related to Bax are the primary factors that block the action of Bcl-2, leading to apoptosis through damage to the mitochondrial membrane. This damage allows for the release of other substances involved in apoptosis, such as cytochrome C, which initiates a series of caspase enzymes, ultimately resulting in cell death (8). Additionally, NSAIDs can cut and deactivate poly-ADP ribose polymerase (PARP), an essential enzyme involved in DNA repair processes (9).

While NSAIDs have demonstrated chemotherapeutic efficacy, their significant anti-cancer effects require high doses, which dampens enthusiasm for their application due to two primary concerns: drug resistance and undesired toxicities affecting the gastrointestinal, renal, liver, and cardiovascular systems (10).

To tackle this problem, one approach is to combine NSAIDs with another antineoplastic agent. In this context, combination therapy allows for the concurrent targeting of various molecular pathways essential for the survival of cancer cells and eradicates cellular mechanisms associated with adaptive resistance (11).

Deuterium-depleted water (DDW) has gained attention as a possible natural treatment for cancer (12). The stable isotopes of hydrogen, deuterium (D) and protium (H), not only vary in their physical characteristics but also in their biological and chemical behaviours. More evidence suggests that deuterium in aqueous solutions plays a crucial role in stimulating or inhibiting metabolic processes in living organisms (12). DDW contains a lower concentration of D compared to natural sea-level occurrences (lower than 150 ppm D) (13). It exhibits several surprising biological properties, including antidotal, antitumor, and metabolic effects (12,14). Indeed, tumor cells exhibit high sensitivity to DDW, resulting in tumor shrinkage and, in some instances, necrosis. Simultaneously, healthy cells can adapt to the reduced D content of water (15). The underlying mechanisms of this impact are attributed to DDW's structure, physicochemical properties, and alterations in ligand-receptor interactions within biological entities of varying hierarchical levels (14,16).

To our best knowledge, there has been no research conducted on the combined effects of NSAIDs and DDW in terms of their cellular pathways for the treatment of HCC. In addition, taking into account certain studies

that emphasize the anti-cancer properties of deuterium-enriched water (DEW) (12), we were interested in investigating whether higher D concentrations in water would amplify the anti-cancer effects of NSAIDs. Therefore, the objective of this study was to assess the cytotoxic effects of celecoxib and indomethacin, both separately and in combination with DDW or DEW, on HCC cells. Moreover, we examined changes in apoptosis and MAPK pathways to identify potential molecular pathways.

Methods

Materials and reagents

DDWs (31 and 127 ppm D) and DEWs (50000 and 300000 ppm D) were sourced from the Atomic Energy Organization of Iran. Dulbecco's modified Eagle medium (DMEM) (high glucose), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from PAA in Australia. Trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA) was supplied by Biosera in England. Antibodies for JNK, phospho-SAPK/JNK, anti-extracellular receptor kinase 1/2 (ERK1/2), phospho-ERK1/2, p38, phospho-p38, Bax, Bcl-2, Caspase-3, COX-2, and β -actin were provided by Cell Signaling Technology, based in the USA. PARP and secondary antibodies were acquired from Roche, Germany. Coomassie blue R-250, Coomassie blue G-250, Bromophenol blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and a caspase-3 colorimetric assay kit were made available by Sigma Chemical Company in the UK. Celecoxib (Cel) and indomethacin (Indo) were graciously obtained from a collaborative lab (with a purity of 98.8%) and dissolved in a small amount of dimethyl sulfoxide (DMSO), making sure the concentration of DMSO in the experiments stayed below 1%. The Western blot detection kit and polyvinylidene difluoride (PVDF) membrane were obtained from Roche Applied Science located in Germany. Dithiothreitol (DTT) and other reagents were procured from Merck.

Cell culture

The Hep G2 cells were sourced from the Iranian Biological Resource Centre (IBRC) located in Tehran, Iran, and were cultured in monolayer form using a complete medium consisting of 89% DMEM, 10% heat-inactivated fetal bovine serum, and 1% penicillin and streptomycin, maintained at 37 °C in a humidified atmosphere with 5% CO₂. The monolayer Hep G2 cells were removed from the flask surface utilizing a 2.5% trypsin-EDTA solution. All experiments were carried out on the cells during their logarithmic growth phase to reduce cellular reactions to stimulators and/or inhibitors.

Preparation of media containing different concentrations of deuterium

The components of the culture medium were dissolved

in DDWs (31 and 127 ppm D) and DEWs (50 000 and 300 000 ppm D) to make cell culture media containing different concentrations of D, and then, they were sterilized using 0.2 µm filters.

MTT assay for cellular proliferation

A suspension of 8×10^3 Hep G2 cells in 50 µL of culture medium was created, and then, they were distributed into each well of a flat-bottomed 96-well plate. The cells were allowed to incubate in a humid atmosphere containing 5% CO₂ for 24 hours to reach a confluence of 70%-90%. Subsequently, Cel (celecoxib, concentrations ranging from 2 to 400 µM), Indo (indomethacin, concentrations from 2 to 800 µM), and their combinations with DDW and DEW at the concentrations listed in Table 1 were added to each well. The control group consisted of cells treated with an equal volume of medium (DMEM). Additionally, wells containing DMEM without cells were designated as blanks. After 24, 48, and 72 hours of incubation, the medium was removed from every well, and after washing twice with PBS, a volume of 25 µL of MTT solution (5 mg/mL) was introduced into each well, after which the plates were incubated for 4 hours at 37 °C in an atmosphere containing 5% CO₂. The viable cells' mitochondrial activity is indicated by the conversion of the tetrazolium salt MTT to formazan crystals. The formazan crystals were solubilized by adding 100 µL of DMSO. The optical density of the ensuing solution was assessed at 570 nm with an ELISA plate reader. All experiments were conducted in triplicate (17).

Protein isolation and western blotting

Following a 48-hour treatment of Hep G2 cells with the specified conditions in Table 2, the cells were collected and rinsed with ice-cold PBS. Afterward, the cells were lysed with 100 µL of lysis buffer (50 mM HEPES (pH: 7.4), 5 mM CHAPS, 5 mM DTT) at 4 °C for 15 minutes. The cell extracts were then centrifuged at 14 000 g for 5 minutes, and the supernatants were placed into new tubes. The Bradford assay was used to determine the protein concentration of the supernatants. Next, 30 µg of each protein sample was mixed with an equal volume of 2X SDS-sample buffer and subjected to electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE). The proteins were subsequently transferred from the SDS-polyacrylamide

gel to PVDF membranes (Roche). Following these initial steps, the membranes were treated with 0.1% Ponceau S to confirm equal protein loading and were then blocked with a 0.5% blocking reagent in TBS (50 mM Tris, 150 mM NaCl) for one hour at room temperature. The membranes were then left to incubate overnight at 4 °C with various primary antibodies: polyclonal anti-COX-2 (1:1500), anti-Bax (1:2500), anti-Bcl-2 (1:1500), anti-PARP (1:2000), anti-caspase-3 (1:1500), anti-ERK1/2 (1:2500), anti-phospho-ERK 1/2 (1:2500), anti-SNAPK/JNK (1:1000), anti-phospho-SNAPK/JNK (1:1500), anti-P38 (1:1000), anti-phospho-p38 (1:1000), and anti-β-actin (1:1500). Afterward, the membranes underwent four washes with TBS-T (TBS containing 0.1% Tween-20), each lasting 15 minutes, followed by a TBS wash, after which the blots were allowed to incubate with a goat anti-mouse/rabbit-antibody-HRP conjugate (Roche) for 1 hour at room temperature. The blots were rinsed one last time, and the immunoreactive bands were detected by applying luminol substrate to the blots and exposing them to Fuji x-ray film (18).

Statistical analysis

Statistical analyses were conducted using SPSS software (version 11.0). The data were expressed as mean + SD. A one-way analysis of variance (ANOVA) was used to detect significant differences between the treatment groups. A significance level of $P < 0.05$ was set. The IC₅₀ was calculated using the Probit function in SPSS.

Results

Celecoxib and indomethacin reduced the proliferation of Hep G2 cells in a manner that depended on both dose and time

Cel and Indo dose-response curves for cell proliferation at 24, 48, and 72 hours are shown in Figure 1 (and Figure S1 in Supplementary file 1). Both medicines induced dose- and time-dependent cytotoxicity. The calculated IC₅₀s for Cel after 24, 48, and 72 h treatment were 38.71, 29.56, and 8.94 µM, respectively. These values for Indo were 233.17, 148.48, and 138.62 µM.

The combination of celecoxib and indomethacin with DEW but not DDW produces a more potent inhibitory effect on cell growth than either product alone

Since the IC₅₀ for Cel and Indo as single agents was almost

Table 1. The concentrations of celecoxib and indomethacin, both individually and combined with varying amounts of deuterium, utilized to treat Hep G2 cells for evaluating cytotoxic effects

Therapeutic agents		
Cel (µM)	Indo (µM)	Deuterium (ppm) (DDW/DEW)
10	50	31 (DDW)
20	100	127 (DDW)
50	175	50000 (DEW)
70	250	300000 (DEW)

Table 2. The concentrations of celecoxib, indomethacin, and deuterium used to treat the Hep G2 cells for western blot analysis.

Agent 1 (NSAIDs)	Agent 2 (Deuterium as DDW/DEW)
Cel (22 µM)	31 (ppm)
Indo (110 µM)	127 (ppm)
	50000 (ppm)
	300000 (ppm)

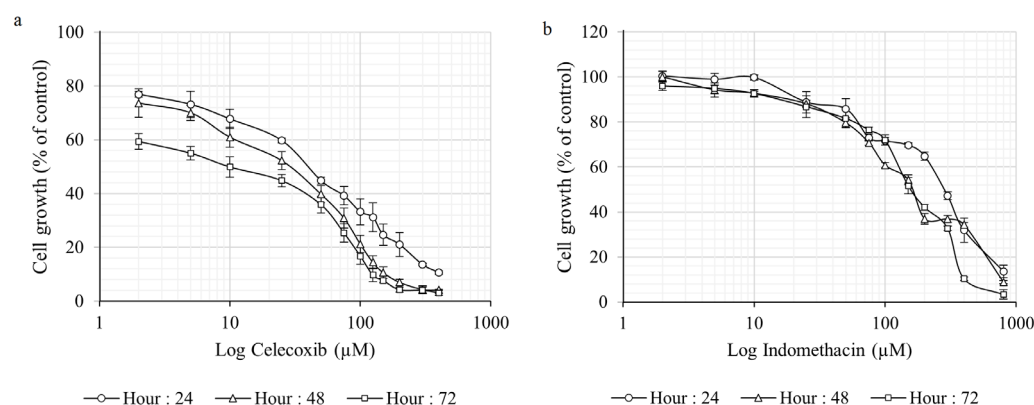


Figure 1. The inhibitory effect on growth, measured logarithmically, of celecoxib (a) and indomethacin (b) on the Hep G2 cell line. The viability of the cells subjected to treatment was assessed via mitochondrial activity using the MTT assay, with the results presented as a percentage of the control ($n=8$) + SD; SD refers to standard deviation

high, we decided to test combined treatment protocols, including simultaneous treatment with DDW or DEW for these two drugs. Thus, cells were treated with Cel (10, 20, 50, and 70 μM) and Indo (50, 100, 175, and 250 μM) combined with DDW, or DEW (Table 1) for 24, 48, and 72 h, and cytotoxicity was measured. Figure 2 (and Figure S2 in Supplementary file 1) and Figure 3 (and Figure S3 in Supplementary file 1) show that the cytotoxicity of Cel and Indo, in combination with DDWs (31 and 127 ppm D), had no significant increment. However, combinations of these two NSAIDs with DEWs (50 000 and 300 000 ppm D) remarkably reduced the survival of Hep G2 cells when compared to Cel and Indo administered separately, in both a dose- and time-dependent fashion.

When cells were treated with the combination of DEWs (50 000 and 300 000 ppm D) plus [Cel] $< \text{IC}_{50}$ (24 hours) (10, 20 μM), the viability was reduced by approximately 20%-40% and 35%-60% compared to Cel alone, respectively (Figure 2). The combination of the mentioned DEWs significantly augmented the antiproliferative effect of [Indo] $< \text{IC}_{50}$ (24 hours) (50, 100 μM) to almost 15%-30% and 30%-45%, respectively (Figure 3) ($***P < 0.001$, $**P < 0.01$, and $*P < 0.05$).

The findings demonstrated that the pairing of DEWs with all doses of Cel and Indo significantly reduces cell proliferation when compared to the use of NSAIDs alone ($***P < 0.001$, $**P < 0.01$, and $*P < 0.05$). Moreover, DEW (300 000 ppm D) exhibited the greatest synergistic toxic effect in a time- and dose-dependent fashion with Cel. Specifically, at 72 hours, the combination of DEW (300 000 ppm D) and Cel (70 μM) showed a cytotoxic effect that surpassed that of Cel (400 μM) (see Figure S2c in Supplementary file 1).

Western blotting analysis results

To explore the signaling pathways activated by our agents, we assessed the levels of proteins associated with apoptosis (Bax, phospho-p38, phospho-JNK/SAPK, and caspase-3) as well as those linked to survival (COX-2,

Bcl-2, phospho-ERK1/2, and PARP). In this analysis, we aimed to determine how varying deuterium levels in water influenced the effectiveness of indomethacin and celecoxib on protein expression; therefore, we used concentrations of indomethacin and celecoxib that were below their IC_{50} values to attribute the findings specifically to the modulation of deuterium in the culture water. To achieve this, Hep G2 cells were treated with celecoxib (22 μM), indomethacin (110 μM), and media with different deuterium concentrations (31, 127, 50 000, and 300 000 ppm D), either individually or in combination, for 48 hours, and the expression of the target proteins was analysed using the western blotting technique.

COX-2 protein expression in the cells treated with Cel, Indo, DDW, DEW, and their combinations

As mentioned previously, increased levels of COX-2 protein are present in HCC and play a role in helping cancer cells resist treatment (5). Since COX-2 inhibitors have demonstrated potential therapeutic effects in HCC (19), we began with preliminary studies to assess the influence of Cel and Indo, along with their combinations with DDWs and DEWs, on COX-2 expression. Both Cel and Indo were found to lower COX-2 protein levels compared to the control, with Cel exhibiting a stronger ability to inhibit COX-2 than Indo. However, DDWs (31 and 127 ppm D) and DEWs (50 000 and 300 000 ppm D) did not significantly alter COX-2 expression (Figure 4). The combination of (Cel + 31 ppm D) exhibited a notable inhibitory effect compared to the control but was not as effective as Cel used alone. On the other hand, DDW (127 ppm D) appeared to enhance the effectiveness of Cel further. The DEW (50 000 ppm D), when paired with Cel also increased the COX-2 inhibitory response, akin to the effect seen with DDW (127 ppm D). The combination therapy involving Hep G2 cells with Cel + 300 000 ppm D yielded intriguing results, as this particular combination demonstrated a strong synergistic effect through complete inhibition of COX-2 expression (Figure 4).

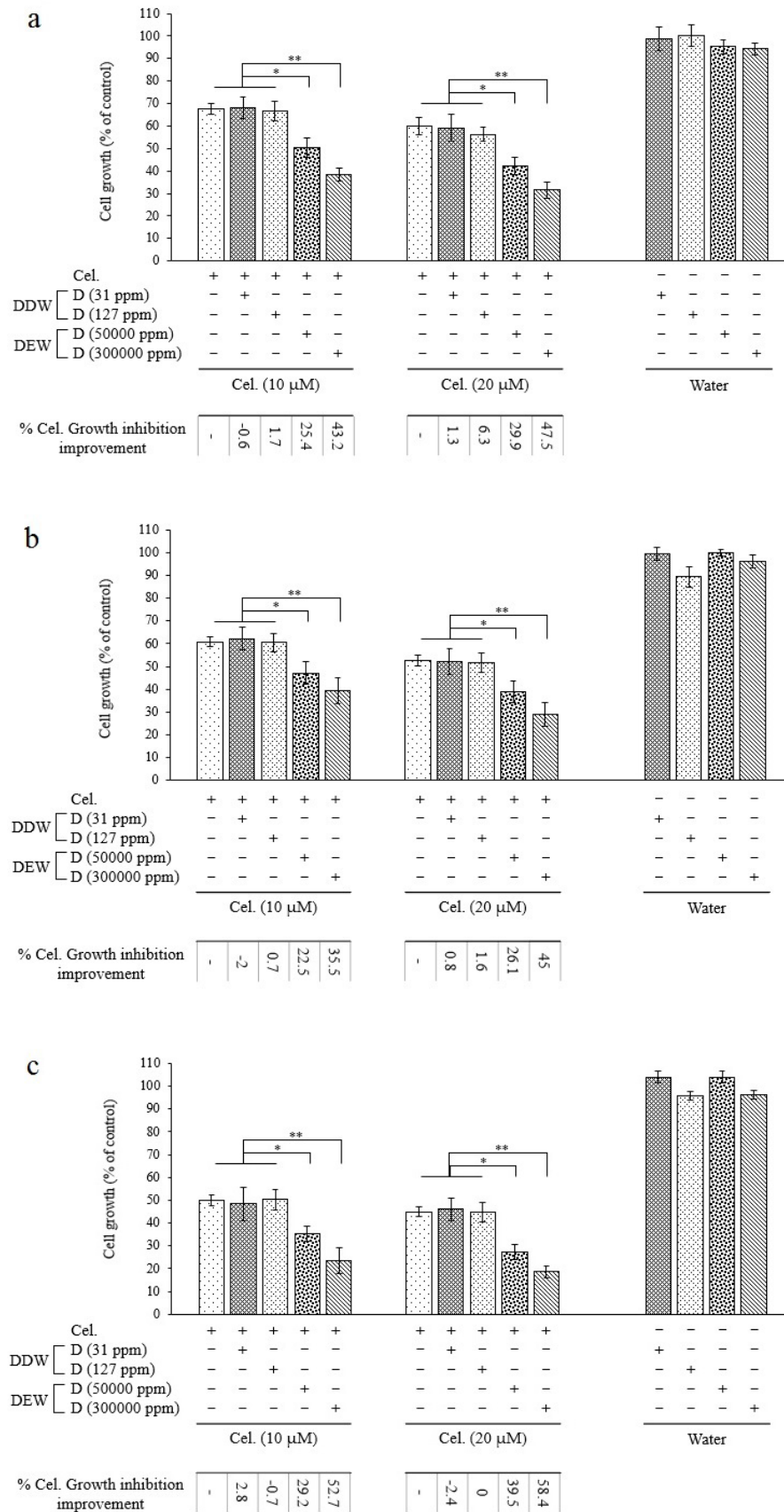


Figure 2. The impact of celecoxib on growth inhibition, when combined with DDWs and DEWs, was assessed over 24 (a), 48 (b), and 72 (c) hours on the Hep G2 cell line. The cell viability of the treated samples was determined using the MTT assay. Values are expressed as mean + SD obtained from triplicate wells in two independent experiments. The statistical significance of the differences was evaluated using one-way ANOVA. * $P < 0.05$, ** $P < 0.01$. Cells that received only celecoxib were considered the reference group

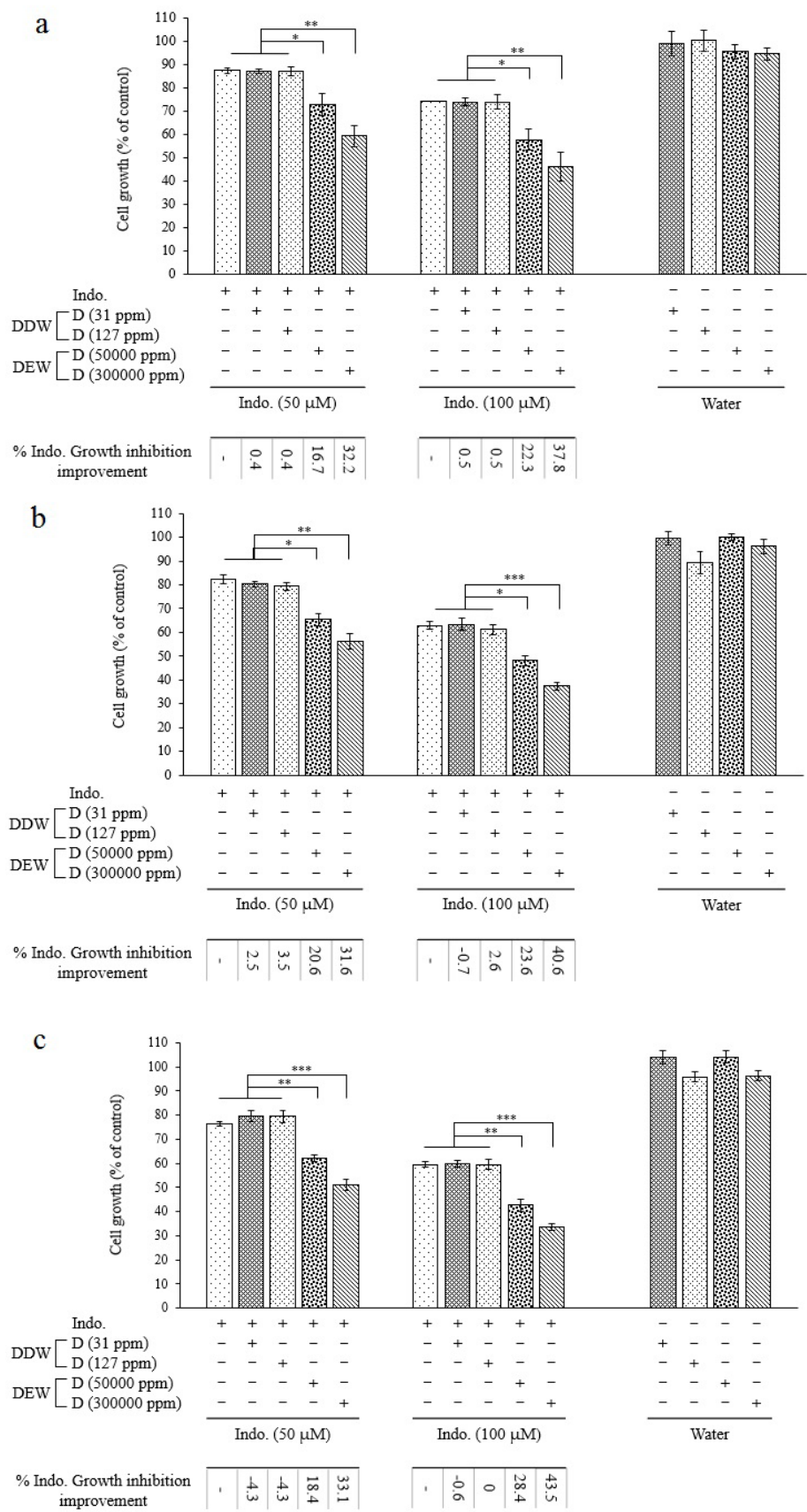


Figure 3. The impact of indomethacin on growth inhibition, when combined with DDWs and DEWs, was assessed over 24 (a), 48 (b), and 72 (c) hours on the Hep G2 cell line. The cell viability of the treated samples was determined using the MTT assay. Values are expressed as Mean+SD obtained from triplicate wells in two independent experiments. The statistical significance of the differences was evaluated using One-way ANOVA. * $P<0.05$, ** $P<0.01$. Cells that received only indomethacin were considered the reference group

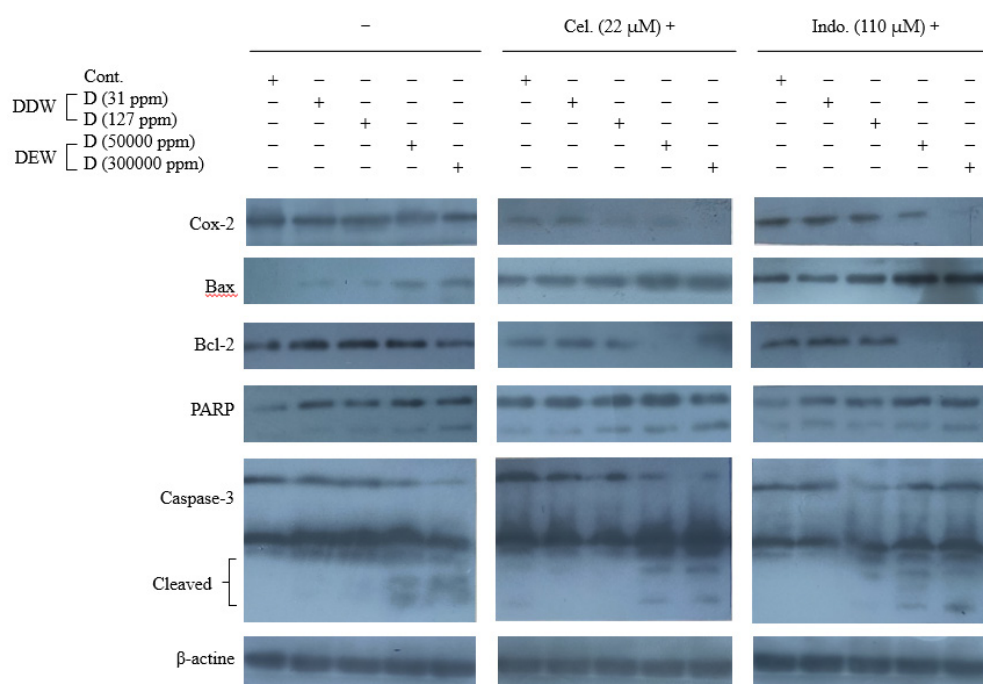


Figure 4. Western blot analysis was conducted to investigate the expression of COX-2, Bax, Bcl-2, poly ADP-ribose polymerase (PARP), and Caspase-3 proteins in Hep G2 cells treated with celecoxib and indomethacin, both individually and in combination, at different concentrations of deuterium

The outcomes of Indo when combined with DDW and DEW were comparable to those of Cel. It appears that as the concentration of D (within the water content of the culture medium) rises, the capacity to synergistically lower COX-2 expression intensifies.

Expression levels of Bax and Bcl-2 proteins in cells treated with Cel, Indo, DDWs, DEWs, and their combinations

Bcl-2 family proteins are essential in the intrinsic apoptotic pathway, and the ratio of Bax to Bcl-2 proteins determines a cell's fate—either survival or apoptosis (20). Consequently, we investigated whether our compounds could affect the expression levels of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) proteins.

As depicted in Figure 4, Cel and Indo could increase the level of Bax significantly. Moreover, DEWs but not DDWs enhanced the anti-apoptotic Bax expression.

In combined regimes, DDW (31 ppm D) could not augment any of Cel and Indo Bax protein expression. However, DDW (127 ppm D) positively influenced the Bax protein expression ability of Indo. The combination of DEWs with Cel and Indo exhibited a strong synergistic effect on Bax expression, and this enhancement was [D] dependent. Importantly, Cel alone and combined forms had higher Bax expression-stimulating effects than Indo formulations.

The level of Bcl-2, which acts as an anti-apoptotic protein, was reduced by Cel and Indo but DDWs and DEWs, except DEW (300000 ppm D), did not have a remarkable impact. Treatments consisting of Cel+DEWs

and or Indo + DEWs were able to inhibit Bcl-2 expression considerably. These results proved the enhancement of apoptotic ability by DEW, which was already illustrated by impacting Bax expression.

Assay of caspase-3 activation and PARP cleavage in cells exposed to Cel, Indo, DDWs, DEWs, and their combinations

The intrinsic or extrinsic signaling apoptotic pathways activate the ubiquitous caspase cascade. This cascade ends with caspase-3. Activation of caspase-3 cleaves and disables essential cellular proteins, such as the DNA repair enzyme (PARP) (21). As a result, we evaluated the role of caspases-3 and PARP in triggering apoptosis in Hep G2 cells that were treated with our agents.

As shown in Figure 4, in single NSAID therapy, Cel could activate caspase-3 slightly. Also, a weak PARP cleaving occurred by Cel and Indo. DDWs did not show apoptotic effects by caspase-3, but they could cleave PARP very slightly. In contrast, DEWs activated caspase-3. Furthermore, DEWs illustrated PARP cleavage ability [D]-dependently.

The combination therapy showed that Cel+DDWs regime did not have significant caspase-3 activity enhancement compared to Cel alone. In contrast, DEWs plus Cel resulted in elevated caspase-3 activity. As expected, the results of PARP cleavage confirmed the apoptotic activity improvement of Cel by DEWs previously shown by caspase activity. Furthermore, DDW (127 ppm D) could enhance the PARP cleavage activity by Cel.

There was no increase in caspase-3 activation in the Indo+DDWs treatment when compared to Indo on its own. Conversely, the combination of DEWs with Indo resulted in a significantly greater effect on caspase-3 activity. The PARP cleavage activity observed with the Indo combinations indicated that DEW (300 000 ppm D) could augment the apoptotic potential of Indo.

The expression levels of MAP Kinase proteins in the cells were assessed after treatment with Cel, Indo, DDWs, DEWs, and various combinations of these

The MAP Kinase pathway in mammalian cells encompasses p38, JNK, and ERK1/2 proteins (22). The ERK signaling pathway is predominantly activated in cancer, initiating cell cycle processes and fostering the proliferation of cancer cells, which aids in tumor development. In contrast, the functions of p38 and JNK are associated with the promotion of apoptosis (23). In this study, since the changes in the levels of total ERK, JNK, and p38 proteins were not clearly defined, we also investigated the phosphorylation status of these proteins. As depicted in Figure 5, Cel, along with a lesser extent of Indo and DDWs, primarily increased p-p38 levels. However, DEW resulted in an elevation of phosphorylated JNK and p38 (Figure 5).

DDW (31 ppm D) failed to enhance the apoptotic signal of Cel and Indo in MAP Kinase pathways. However, Cel+DDW 127 ppm D and Indo+DDW 127 ppm D could increase p-JNK compared to Cel and Indo alone. Both Cel+DEWs and Indo+DEWs combinations had more robust apoptotic activity than Cel and Indo alone by increasing p-JNK and p-p38 and decreasing p-ERK.

This issue demonstrates that [D] is critical in progressing apoptotic pathways (Figure 5).

Discussion

HCC is the most prevalent type of cancer and ranks among the top causes of mortality globally. The traditional approaches for treating liver cancer, including chemotherapy, surgical procedures, ablation, and radiation therapy, have limitations in their effectiveness to varying degrees (24). Therefore, a multifaceted approach is necessary for the effective treatment of HCC. A novel HCC treatment strategy involves searching for alternative chemotherapeutic agents with minimal adverse effects or incorporating natural substances as adjuncts. Chronic inflammation resulting from persistent damage is typically the cause of HCC. Therefore, one approach to address this cancer is by utilizing agents that have anti-inflammatory properties, like NSAIDs. Recent significant evidence from various clinical and experimental investigations has suggested that NSAIDs might reduce the risk of different cancer types, including HCC (5,6). This study investigated the cytotoxic effects of two NSAIDs: Cel, which primarily inhibits COX-2, and Indo, a non-selective COX-2 inhibitor, on the Hep G2 liver cancer cell line. Our results showed that both Cel and Indo decreased the viability of Hep G2 cells in a dose- and time-dependent manner (see Figure 1 and Figure S1 in Supplementary file 1). In light of these findings, Indo demonstrated weaker cytotoxicity (IC50 values at 24, 48, and 72 hours were 233.17, 148.48, and 138.62 μ M, respectively), as compared to Cel (with IC50 values at 24, 48, and 72 hours being 38.71, 29.56, and 8.94 μ M).

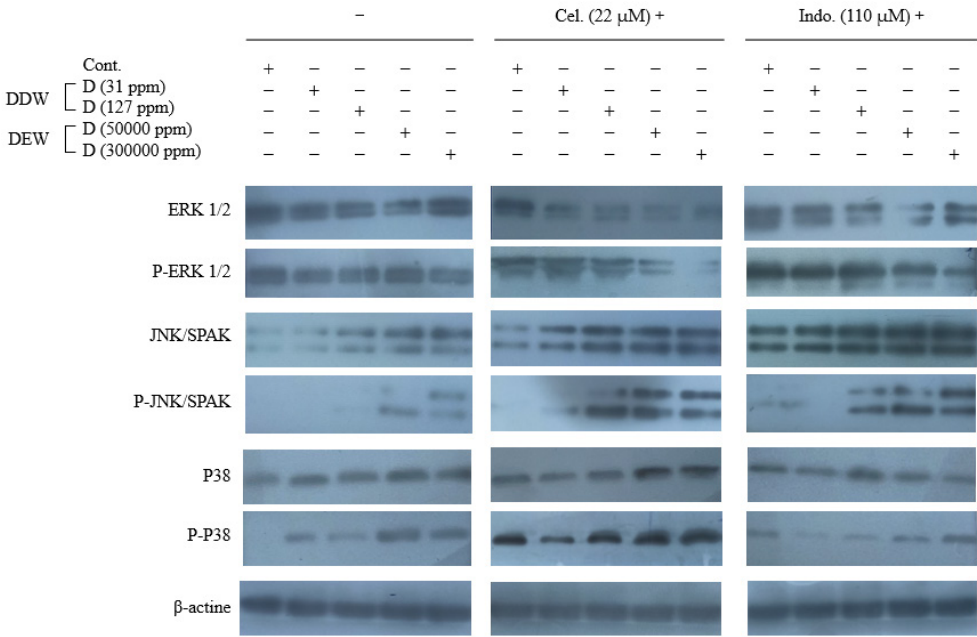


Figure 5. Western blot analysis was utilized to assess the expression levels of EK1/2 and the MAPK proteins, including p-ERK1/2, JNK, p-JNK, p38, and p-p38, in Hep G2 cells that underwent treatment with celecoxib and indomethacin, both individually and in combination with different concentrations of deuterium.

The primary mechanism through which NSAIDs function involves inhibiting the cyclooxygenase (COX) enzyme, which is crucial in tumor development. COX is responsible for producing PGE2, a substance that encourages angiogenesis, cellular invasion, metastasis, and cell survival (25). Moreover, PGE2 facilitates the prevention of apoptosis by boosting the production of anti-apoptotic proteins like Bcl-2, while simultaneously decreasing levels of pro-apoptotic proteins such as Bax (26). Numerous studies and a meta-analysis have indicated that elevated COX-2 levels correlate with worse outcomes for patients suffering from HCC (27,28). In this regard, Cel and Indo were found to lower COX-2 protein levels in Hep G2 cells when compared to the control group, with Cel exhibiting a stronger COX-2 inhibitory effect than Indo (Figure 4).

To better understand how Cel and Indo promote programmed cell death in the Hep G2 cell line, we examined the levels of Bax and Bcl-2, which are members of the Bcl-2 protein family. The intrinsic apoptosis pathway is considerably affected by mitochondria, which control and facilitate cell death through the actions of Bcl-2 family proteins. Bax is a protein that starts a series of events leading to cell death via the intrinsic apoptosis pathway. Once activated, Bax causes the release of cytochrome c from the mitochondria, which then activates caspases, including caspase-3. This activation results in the cleavage and inactivation of crucial proteins like PARP, which is vital for DNA repair, ultimately culminating in cell death (29). Our findings indicated that Bax expression in cells treated with Cel was greater than in those treated with Indo. As a result, there was a slight activation of caspase-3 and cleavage and deactivation of PARP observed with Cel treatment. Moreover, Cel demonstrated greater efficacy than Indo in reducing the levels of the anti-apoptotic protein Bcl-2 (Figure 4). Hossain et al presented findings that supported our conclusions, indicating that aspirin, which is another type of NSAID, promotes apoptosis in Hep G2 cells by enhancing the Bax/Bcl-2 ratio and activating the caspase pathway (9). In a similar vein, Yoshinaka et al found that celecoxib, a COX-2 inhibitor, significantly inhibits tumor growth and lung metastasis in a mouse model of mammary cancer by significantly increasing caspase-3 activity (30). Additionally, the heightened anticancer effects of both selective and non-selective COX-2 inhibitors in human liver cancer cells are linked to the activation of caspase-3, the concurrent cleavage of PARP, and a reduction in Bcl-2 protein levels (31). Furthermore, the COX-2 selective inhibitor meloxicam has been shown to trigger apoptosis in Hep G2 cells by raising the levels of pro-apoptotic proteins such as Bax (28).

Furthermore, numerous research studies have demonstrated that NSAIDs can suppress cell growth in various types of cancer by impacting the MAP kinase

pathways (7). MAPKs are a family of enzymes that attach phosphate groups to serine/threonine amino acids and relay signals from the cell membrane to the nucleus in response to diverse stimuli. This process alters gene transcription, resulting in physiological responses. When a cell encounters stress, the JNK and p38 pathways are typically activated, which results in apoptosis. Conversely, the ERK1/2 pathway is primarily stimulated by growth factors (22). Research shows that NSAIDs can influence the MAPK signaling pathway in various cancers, including gastric (7), liver, colorectal, and head and neck (6) cancers. For example, in experiments involving mice with liver cancer, celecoxib was found to inhibit ERK activity while promoting the activation of p38 and JNK signaling, which slowed down cancer progression and triggered apoptosis in cancer cells (32). Additionally, celecoxib was noted to enhance p38 signaling and reduce cellular proliferation in head and neck squamous cell carcinoma. When p38 signaling was blocked, the decrease in growth induced by celecoxib was considerably reversed (33). Moreover, Indomethacin obstructed the proliferation and division of cells and triggered programmed cell death (apoptosis) in MKN28 human gastric cancer cell lines by inhibiting the ERK2/MAPK signaling pathway (7). In our study, we assessed the changes in both total MAP Kinase protein expression and the activated (phosphorylated) versions of these proteins. Our findings revealed that celecoxib could induce apoptosis by activating p38 compared to the control. In contrast, Indomethacin activated p38 but also increased p-ERK1/2, acting as an antiapoptotic factor (Figure 5). Although ERK activation is typically associated with antiapoptotic roles, some studies have indicated that ERK activation can also be crucial for apoptosis induced by cytotoxic agents, depending on the specific cell type and treatment used.

As previously disclosed, the IC_{50} (24, 48, and 72 h) of Cel and Indo were 38.71, 29.56, and 8.94 μ M and 233.17, 148.48, and 138.62 μ M, respectively. It is essential to mention that this large quantity is applied directly to the cell culture medium. To achieve a lethal dose in Hep G2 cells for treating HCC, patients must consume much higher doses than the IC_{50} doses. Administering a high dosage of Cel and Indo may lead to drug resistance and exacerbate their adverse effects, as stated in previous studies (10).

A possible approach is to mix NSAIDs with a harmless natural anticancer substance to reduce the amount of NSAIDs required. DDW refers to a variety of water with a lower D/(D + H) ratio than typical water. Many laboratory and clinical investigations have shown DDW's ability to inhibit tumor growth. Studies have indicated that DDW greatly diminishes cell proliferation, their ability to form colonies, and their invasion potential. Furthermore, it influences cell division by lowering the number of cells in the S phase, increasing the percentage of cells in the G1 phase, and enhancing the generation of antioxidant

enzymes (34). Wang et al previously demonstrated these effects on nasopharyngeal cancer cells in a controlled laboratory environment using D levels between 50 and 100 ppm (35).

Considering the antineoplastic ability of DDW, we decided to use this agent as an NSAID adjuvant to HCC treatment. For this purpose, we treated Hep G2 cells with (Cel+DDW) or (Indo+DDW) combinations. Our MTT assay results revealed that DDWs (31 and 127 ppm D) were not able to enhance the cytotoxic effect of the mentioned NSAIDs in different [D] significantly, even after 72 hours. (Figure 2 and Figure S2 in Supplementary file 1, Figure 3 and Figure S3 in Supplementary file 1). Similarly, Soleyman-Jahi et al published a report demonstrating that exposing human cancer cell lines of the prostate, colon, breast, and stomach to varying concentrations of D in DDW did not result in any noteworthy limiting impacts. This was determined through cytotoxicity analysis based on MTT (34). Furthermore, Kleemann et al showed that exposing malignant melanoma cell lines A375, SK-Mel-28, and SK-Mel-30 to water with lower-than-normal D levels did not affect their growth (36). Moreover, these findings were consistent with the findings of our previous investigation, which revealed that DDW having 31 and 127 ppm D did not provide significant adjuvant effects to Cel and Indo in the fight against human lung cancer cells (A549) (37).

The western blotting analysis of COX-2, MAP Kinase pathway (p38, JNK, and ERK1/2), and intrinsic apoptosis pathways (Bax, Bcl-2, caspase -3, and PARP) proteins showed the expression of provided proteins in these pathways in Hep G2 treated with (Cel+DDW 31 ppm D) or (Indo+DDW 31 ppm D) for 48 hours has not changed efficiently, whereas DDW (127 ppm D) could change the ability of Cel and Indo to express p-JNK and Bcl-2 proteins slightly in agreement with cell death (Figure 4 and Figure 5). In contrast to our findings, Gyöngyi et al reported that DDW containing 25 ppm D could prevent the overexpression of the Bcl-2, Kras, and Myc genes induced by DMBA in the lungs of mice. As per their conclusion, this form of water could serve as a non-toxic dietary supplement with anticancer properties that may prolong the survival of individuals with lung cancer (38). Furthermore, Boros et al illustrated the supplementary inhibitory D-depletion effect when combined with cisplatin *in vitro* on MIA-PaCa-2 pancreatic cancer cells (12).

According to our results, the combination of Cel and Indo with DDW (31 and 127 ppm D) did not have a significant synergistic impact on inhibiting the growth of Hep G2 cells. Therefore, we deduced that the decreased level of D in water did not have a notable anticancer effect on this particular cell category, at least within 72 hours. Consequently, we opted to experiment with water varieties with a D concentration higher than the standard

level. We utilized DEW (50 000 and 300 000 ppm D) as a combined agent to achieve this objective.

Stress caused by deuterium disrupts the process of energy metabolism. Deuterium influences enzymes involved in energy metabolism, such as cytochrome c oxidase within the mitochondrial respiratory chain, and ATP synthase, which greatly diminishes the cellular ATP supply. This impact is particularly crucial for cancerous cells (39,40). Following this hypothesis, various studies have indicated the anticancer properties of DEW (37). Here, the addition of DEWs (50 000 and 300 000 ppm D) to [Cel] < IC₅₀ (24 hours) (10, 20 µM) during 24, 48, and 72 hours could improve the % Cel growth inhibition approximately 20%–40% and 35%–60%, respectively. Also, the DEWs (50 000 and 300 000 ppm D) in combination with [Indo] < IC₅₀ (24 h) (50, 100 µM) after 24, 48, and 72 hours could prevent the Hep G2 cell viability almost 15%–30% and 30%–45% compared with Indo alone. The synergistic growth inhibition of DEWs in combinations was time and dose-dependent, so the (Cel+300 000 ppm D) and (Indo+300 000 ppm D) induced the most growth prevention after 72 hours. Furthermore, the combination of (70 µM Cel+300 000 ppm D) showed cell growth inhibition even more than 400 µM Cel alone after 72 hours. In a previous study, we demonstrated that treating Hep G2 and A549 cells with DEWs (50 000, 100 000, 200 000, 300 000 ppm D) alone for an extended period (21 days) could inhibit cell growth in a [D]-dependent manner (41). Leonard and Mullins noted that PtK1 cells, when subjected to a culture medium containing up to 500 000 ppm D, were able to both begin and finish mitosis. However, the duration of the mitotic phase increased concerning the quantity of D₂O used (42). In addition, Bader et al demonstrated that when human pancreatic carcinoma cells (AsPC-1, BxPC-3, and PANC-1) were treated with both D₂O and gemcitabine at the same time, the IC₅₀ values for gemcitabine were decreased across all pancreatic cancer cell lines investigated, and synergistic effects were detected when D₂O was administered before gemcitabine (43).

To explore the cancer-fighting molecular mechanisms of DEWs (50 000, 300 000 ppm D) and their interaction with NSAIDs, we measured the expression levels of p38, JNK, and ERK1/2 (including their phosphorylated forms), along with the proteins Bax, Bcl-2, COX-2, and the activation of caspase-3, while inhibiting the PARP enzyme in Hep G2 cells. Notably, DEWs alone increased the expression of pro-apoptotic proteins such as Phospho-p38, Phospho-JNK, and Bax, while DEW (300 000 ppm D) significantly reduced the expression of the anti-apoptotic proteins COX-2 and Bcl-2. Likewise, DEW (300 000 ppm D) induced caspase-3 activation and PARP cleavage more effectively than DEW (50 000 ppm D). Kalkur et al demonstrated that DEWs (10 000–50 000 ppm D) exert significant cytotoxic effects on murine

astrocytoma cells stimulated by the Raus sarcoma virus. The mechanisms behind DEW-induced cytotoxicity were linked to the promotion of apoptosis and cell cycle arrest during the G2/M phase (44). Furthermore, it has been indicated that apoptosis in malignant astrocytoma cells triggered by DEWs occurs through the caspase activation pathway, with the rate of apoptosis positively correlating with DEW concentration (45). Furthermore, Bahk et al verified that DEW (at concentrations of 75% and 100%) exhibited anti-proliferative, anti-adhesive, and anti-invasive effects on bladder cancer cells (T-24) following an exposure period exceeding 2.5 hours. They suggested that the anti-proliferative action of DEW stemmed from the activation of the apoptotic pathway that was promoted by a decrease in Bcl-2 levels and an increase in Bax expression (46). In another investigation, it was found that increasing the D₂O concentration above normal levels in the culture medium of malignant melanoma cell lines, including A375, SK-Mel-28, and SK-Mel-30, resulted in a decrease in cell proliferation and hindered cell migration in a dose-dependent manner. Additionally, cell cycle analysis showed an increase in the number of cells in the sub-G1 phase. As highlighted in the study, markers for programmed cell death were activated, including DNA fragments associated with histones, the protein Bax, and PARP (36).

In the combination therapy, the outcomes from the western blot analysis were consistent with the MTT cytotoxicity results. Specifically, the combined use of either Cel or Indo with DEWs led to a marked dose-dependent activation of apoptosis pathways in Hep G2 cells, unlike their combination with DDWs. DEWs boosted the capacity of Cel and Indo to increase the levels of pro-apoptotic proteins (Bax, p-JNK, and p-p38) while reducing the levels of anti-apoptotic factors (COX-2, Bcl-2, and p-ERK1/2). Additionally, DEWs assisted Cel and Indo in activating Caspase-3 and inhibiting PARP. Additionally, the combined effect of DEWs was more pronounced with Cel compared to Indo in the majority of assessments of protein expression (see Figure 4 and Figure 5). Moreover, our earlier research demonstrated that pairing COX inhibitors (celecoxib and indomethacin) with DEW could increase cytotoxicity in the A549 lung cancer cell line. This enhancement was accomplished by activating the intrinsic apoptosis pathway, which includes p-JNK, p-P38, Bax, and caspase-3 (37).

The highest level of apoptosis activity was obtained using a combination of Cel and DEW at a concentration of 300 000 ppm D. Cel acts as a potent COX-2 selective inhibitor, offering greater suppression of this pro-survival enzyme compared to Indo, which subsequently deactivates anti-apoptotic pathways, such as Bcl-2, linked to COX-2 (47). Conversely, as previously demonstrated, the anti-cancer efficacy of DEW is dose-dependent on D, hence, the combination of Cel and DEW (300 000 ppm D)

produced the most synergistic effect against HCC.

Conclusion

In conclusion, this study shows that COX inhibitors such as celecoxib and indomethacin could have anti-HCC effects as a single treatment, but this ability occurs in high doses. To increase the antitumor effect of these compounds, DEW can be used in combination with them. The results of cytotoxicity and investigating apoptosis and MAP Kinase signaling pathways show that DEW can synergistically improve the anti-HCC effects of these two NSAID medicines [D]-dependently. The Cel + DEWs combination has stronger inhibitory effects than Indo + DEWs on Hep G2 cells, which brings hope as a new candidate for the HCC treatment.

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Competing Interests

The authors declare no conflict of interest, financial or otherwise.

Ethical Approval

This article was approved by the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC. 1391.383).

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Supplementary Files

Supplementary file 1 contains figure S1-S3.

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