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<sup>[0]</sup> In vitro anticancer potential of Dill seed extract against human hepatocellular carcinoma (Huh-7) cells

#### Abstract

<sup>[0]</sup> Background: Hepatocellular carcinoma is the most prevalent type of primary liver cancer and remains the foremost cause of cancer-related deaths globally. Dill (*Anethum graveolens*) seeds, rich in phytoconstituents, is renowned for their pharmacological properties.

<sup>[0]</sup> Objectives: This study performed an in vitro evaluation to assess the cytotoxic effects of dill seed extract (DS-EE) on the Huh-7 hepatocellular carcinoma cell line. <sup>[0]</sup> Moreover, the study investigated its effects on cell viability, cellular morphology, oxidative damage, levels of intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and the expression of apoptosis-related genes in Huh-7 cells.

<sup>[2]</sup> Methods: Huh-7 cells were treated with DS-EE at concentrations ranging from 5 to 100 µg/mL for a duration of 24 hours.

<sup>[0]</sup> Results: The cytotoxicity findings showed that DS-EE decreased cell viability and suppressed the growth of Huh-7 cells in a dose-dependent way, with an IC<sub>50</sub> value of 60 µg/mL. <sup>[0]</sup> Exposure to DS-EE extract for 24 hours resulted in a significant elevation in lipid peroxidation (LPO) and a notable decrease in glutathione (GSH) content compared to the control. <sup>[0]</sup> Furthermore, DS-EE significantly increased ROS production while notably decreasing the MMP level in Huh-7 cells. <sup>[0]</sup> Moreover, DS-EE induces cell apoptosis by upregulating the expression of proapoptotic genes (p53, caspase-3, caspase-9, and Bax) and downregulating the expression of the antiapoptotic gene, Bcl-2.

<sup>[2]</sup> Conclusion: DS-EE exhibited a notable cytotoxic effect on Huh-7 cells by increasing oxidative damage and subsequently modulating the expression of apoptosis-related genes. <sup>[1]</sup> The results of this study highlight the anticancer effectiveness of DS-EE, indicating its potential as a promising agent for hepatocellular carcinoma management.

Keywords: Dill seeds, Huh-7 cells, cytotoxicity, oxidative damage, ROS generation, Gene expression

## 1. Introduction

Cancer is undeniably a significant health concern worldwide, and its prevalence varies across different regions and countries. <sup>[0]</sup> The global incidence of cancer escalated to almost 20 million new cases, accompanied by nearly 10 million fatalities (Al-Shamsi and Musallam, 2024). In developed countries, factors such as lifestyle, diets, environmental exposures, and access to healthcare may contribute to higher cancer rates (Minas et al., 2021). Liver cancer, specifically hepatocellular carcinoma, ranks among the six most commonly diagnosed and the third most common causes of cancer-related deaths globally (Rumgay et al., 2022). Early detection and intervention are crucial in improving outcomes for individuals with liver cancer (Guan et al., 2021). Additional data reveals that cancer remains a significant health challenge, with the burden continuing to rise annually (Ali et al., 2023). The field of cancer treatment has evolved into a dynamic area of research, encompassing both conventional and cutting-edge techniques. Various methods, including chemotherapy, radiation therapy, and surgery, are employed in combating cancer (Debela et al., 2021). Nonetheless, each of these approaches comes with its own set of drawbacks and limitations. Chemotherapy, though widely employed in cancer treatment, often induces severe side effects due to its non-selective action (Nurgali et al., 2018). Intrinsically, there is an urgent call to investigate alternative approaches for managing and preventing hepatocellular carcinoma. Natural products, with their diverse array of compounds and biological activities, present a compelling avenue for anticancer drug discovery (Chunarkar-Patil et al., 2024). Their potential to provide more targeted and tolerable therapies offers hope for improved outcomes in cancer treatment (Desai et al., 2008). Herbal medicine has emerged as a safe, non-toxic, and readily accessible reservoir of compounds with potential for treating cancer (Oyenihini et al., 2021). Herbs are thought to counteract the effects of diseases within the body due to their diverse array of characteristics (Isbill et al., 2020; Charneca et al., 2023). Research conducted thus far has assessed the anticancer potential of numerous plants and plant-derived compounds (Chandra et al., 2023). Several of these botanicals and their constituents demonstrate notable efficacy against one or multiple types of cancer (Khan et al., 2019). Dill, more commonly referred to as *Anethum graveolens*, is an aromatic herb that belongs to the Apiaceae (Umbelliferae) family. Originating from the Mediterranean and Southwest Asia, this plant is known for its distinct fragrance (Singh et al., 2005). Dill seeds have been renowned for its medicinal and therapeutic uses in traditional medicine for an extended period. *A. graveolens* has been recognized for its biological and

pharmacological properties, including antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and anticancer effects (Afshari et al., 2019; Haidari et al., 2020;<sup>[0]</sup> Al-Oqail et al., 2021). Conventionally, in Saudi Arabia, native people utilize the Dill seeds as appetizers, carminatives, antispasmodics, and aphrodisiacs (Youssef, 2013). Phytochemical studies conducted on the Dill seeds have identified a diverse array of phytoconstituents, including tannins, flavonoids, terpenoids, glycosides, phenolic acids, as well as fatty acids such as capric, palmitic, stearic, and oleic acids (Jana and Shekhawat, 2010; Dahiya and Purkayastha, 2012).<sup>[7]</sup> Hence, based on previous research on medicinal importance of dill seed, this study was aimed to assess the anticancer potential of dill seed extract against human hepatocellular carcinoma cells (Huh-7) as well as to elucidate the mechanism(s) involved in cell death.

## 2. Materials and methods

### 2.1. Reagents

All specified chemicals, reagents, and diagnostic kits were obtained from Sigma Chemical Company Pvt. Ltd., St. Louis, MO, USA, unless noted otherwise. The DMEM, antibiotic/antimycotic solution, and fetal bovine serum were sourced from Sigma company, USA. Culture vessels and other plastic consumables utilized in the study were acquired from Nunc, Denmark.

### 2.2. Plant material and extraction

Dill (*Anethum graveolens*) seeds were bought from a local market in Riyadh, Saudi Arabia. The dill seeds underwent manual screening before being ground into a coarse powder. Approximately 500 g of the grounded seeds were immersed in 1.5 liters of ethanol for a duration of 3 days, with periodic agitation.<sup>[15]</sup> Afterward, the extract underwent filtration using Whatman #1 filter paper. Then, the resulting filtrate was concentrated to dryness using a rotary evaporator operating at 40 °C under reduced pressure. The resulting ethanolic extract, designated as DS-EE, was then preserved at 4 °C for subsequent utilization.

### 2.3. MTT assay<sup>[7]</sup>

Cell viability was assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, following the protocol outlined by Siddiqui et al. (2008). To start, cells ( $1 \times 10^4$ ) were permitted to adhere within 96-well culture plates for 24 hours in a CO<sub>2</sub> incubator at 37°C. After exposure, MTT solution (5 mg/ml in PBS) was added (10 µl per well in 100 µl of cell suspension), and the plates were then incubated for 4 hours. Next, the supernatant was aspirated,

and 200  $\mu$ l of DMSO was carefully added to each well, followed by gentle mixing. The resulting color was measured at 550 nm. Control sets without treatment were run under identical conditions for comparison.

#### 2.4. NRU assay

The neutral red uptake (NRU) assay was performed according to the methodology described by Siddiqui et al. (2008). After exposure, the cell culture medium was aspirated, and the cells were rinsed twice with PBS. Then, the cells were cultured for 3 hours in medium containing neutral red (50  $\mu$ g/ml). Following the incubation period, the medium was promptly aspirated, and the cells were rinsed with a solution containing 0.5% formaldehyde and 1% calcium chloride. Subsequently, the cells were further incubated for 20 minutes at 37°C in a blend of acetic acid (1%) and ethanol (50%) to extract the dye. The absorbance of the extracted dye was recorded at a wavelength of 550 nm using a microplate reader. The acquired values were compared with those of the control group.

#### 2.5. Morphological assay

Morphological changes induced by DS-EE in Huh-7 cells were assessed following exposure to increasing concentrations (5-100  $\mu$ g/ml) of DS-EE for 24 hours. Cell morphology was examined using an inverted phase contrast microscope (Olympus, CKX41, Japan) at 20  $\times$  magnification to visualize alterations caused by the DS-EE treatment.

#### 2.6. Intracellular glutathione (GSH) content

The GSH content was quantified following the method described by Chandra et al. (2002). Following exposure, cells were harvested, and cellular proteins were precipitated by treating 1 ml of sonicated cell suspension with 1 ml of 10% trichloroacetic acid (TCA) on ice for 1 hour, then centrifuged at 3000 rpm for 10 minutes. The supernatant was then mixed with 2 ml of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA, and 0.01 M 5,5'-dithionitrobenzoic acid (DTNB) was added to reach a final volume of 3 ml. The tubes were placed in a water bath with shaking at 37 °C and incubated for 10 minutes, after which the absorbance of the resulting yellow color was measured at 412 nm.

#### 2.7. Lipid peroxidation (LPO)

LPO was assessed using the thiobarbituric acid-reactive substances (TBARS) protocol described by Buege and Aust (1978). Following exposure, Huh-7 cells were collected by centrifugation, sonicated in ice-cold potassium chloride (1.15%), and centrifuged at 3000 rpm for 10 minutes. The

resulting supernatant (1 ml) was mixed with thiobarbituric acid (TBA) reagent (comprising 15% trichloroacetic acid (TCA), 0.7% TBA, and 0.25N HCl) and heated at 100 °C for 15 minutes in a boiling bath.<sup>[19]</sup> Subsequently, the samples were cooled, centrifuged at 1000 × rpm for 10 minutes, and the absorbance of the supernatant was measured at 550 nm.

## 2.8. ROS generation<sup>[10]</sup>

For assessing reactive oxygen species (ROS) generation, we employed the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma Aldrich, USA) according to a previously outlined protocol (Farshori et al., 2022).<sup>[14]</sup> Following a 24-hour exposure to DS-EE, cells were rinsed with PBS and then incubated at 37°C for 60 minutes with 20 µM of DCFH-DA dye in incomplete culture medium. Following this, intracellular fluorescence was examined using a fluorescence microscope.

## 2.9. Mitochondrial membrane potential (MMP)

MMP level was assessed following the protocol outlined by Zhao et al. (2022). In brief, both control and treated cells were washed with PBS. Following that, cells were subjected to a 1-hour incubation at 37 °C in darkness with 10 µg/mL of Rhodamine-123 fluorescent dye. After incubation, cells were again washed twice with PBS, and the fluorescence intensity of Rhodamine-123 was assessed using a fluorescence microscope (CKX41; Olympus, Japan), capturing images at a magnification of 20×.

## 2.10. RT-qPCR analysis

Huh-7 cells were exposed to 60 µg/ml of DS-EE for 24 hours before RNA extraction. Total RNA was isolated from harvested cells using the RNeasy mini kit (Qiagen) in accordance with the guidelines provided by the manufacturer. The concentration of RNA was determined using the NanoDrop spectrophotometer (Thermo Scientific), and RNA purity was assessed by the OD260/OD280 absorbance ratio. Subsequently, 2 µg of total RNA was utilized for cDNA synthesis, performed using MLV reverse transcriptase kit (GE healthcare, UK) as per the method provided with kit. Amplification was conducted with the SYBR Green I (Roche) and the specific primers for p53, caspase-3, caspase-9, Bax, and Bcl-2 are reported in our previously study (Al-Oqail et al., 2017). The relative mRNA expression level for each gene, presented as fold change compared to GAPDH, serving as the housekeeping gene.

## 2.11. Statistical analysis<sup>[7]</sup>

The results were expressed as the mean  $\pm$  standard deviation derived from three separate experiments.<sup>[7]</sup> Statistical evaluation involved employing one-way analysis of variance (ANOVA), followed by post-hoc Dunnett's test to compare values between control and treated groups. A significance threshold of  $p < 0.05$ <sup>[16]</sup> was considered as statistically significant.

### 3. Results

#### 3.1. MTT assay

The cytotoxic effect of DS-EE in Huh-7 cells using the MTT assay is depicted in Fig. 1. A concentration-dependent decrease in percentage cell viability was observed following 24 hours of DS-EE exposure, which was statistically significant ( $p < 0.001$ ). Specifically, cell viability of 85%, 71%, 50%, 36%, and 23% was recorded at DS-EE concentrations of 20  $\mu\text{g/ml}$ , 40  $\mu\text{g/ml}$ , 60  $\mu\text{g/ml}$ , 80  $\mu\text{g/ml}$ , and 100  $\mu\text{g/ml}$ , respectively, after 24 hours of exposure in Huh-7 cells.

#### 3.2. NRU assay

Fig. 2 depicts the cytotoxic impact of DS-EE on Huh-7 cells as determined by the NRU assay. A notable reduction in cell viability, correlated with increasing concentrations of DS-EE, was observed after 24 hours of exposure, demonstrating a concentration-dependent effect ( $p < 0.001$ ). Precisely, the cell viability of Huh-7 cells by NRU assay was recorded as 87%, 73%, 53%, 39%, and 29% at 20  $\mu\text{g/ml}$ , 40  $\mu\text{g/ml}$ , 60  $\mu\text{g/ml}$ , 80  $\mu\text{g/ml}$ , and 100  $\mu\text{g/ml}$  of DS-EE, respectively, following the 24-hour exposure period.

#### 3.3. Morphological assay

Fig. 3 displays the morphological changes observed in Huh-7 cells following 24-hour exposure to DS-EE. Using a phase-contrast inverted microscope, alterations in the morphology of Huh-7 cells were visualized. The most pronounced effects were observed after exposure to DS-EE, with changes in cell morphology occurring in a concentration dependent manner.<sup>[7]</sup> Huh-7 cells exposed to DS-EE concentrations of 40  $\mu\text{g/ml}$ , 60  $\mu\text{g/ml}$ , and 80  $\mu\text{g/ml}$  reduced cell adhesion capacity, cells become rounded, and inhibited the growth of the cells compared to the control.

#### 3.4. Intracellular glutathione (GSH) content<sup>[3]</sup>

Fig. 4A summarizes the depletion of glutathione (GSH) levels in cultured Huh-7 cells exposed to concentrations of 40  $\mu\text{g/ml}$ , 60  $\mu\text{g/ml}$ , and 80  $\mu\text{g/ml}$  of DS-EE for 24 hours. The results indicate that DS-EE decreased GSH level in a concentration-dependent manner.<sup>[3]</sup> A significant decrease in GSH level was observed, with reductions of 12%, 34%, and 43% at 40  $\mu\text{g/ml}$ , 60  $\mu\text{g/ml}$ , and 80  $\mu\text{g/ml}$  of DS-EE exposure for 24 hours, respectively, compared to the control.



### 3.5. Lipid peroxidation (LPO)

The lipid peroxidation level induced by DS-EE in Huh-7 cells following a 24-hour exposure are presented in Fig. 4B. <sup>[18]</sup> A concentration-dependent, statistically significant increase in lipid peroxidation was observed. Specifically, there was an increase of 110%, 141%, and 177% at DS-EE concentrations of 40 µg/ml, 60 µg/ml, and 80 µg/ml, respectively, in Huh-7 cells exposed for 24 hours.

### 3.6. ROS generation

Significant ROS generation (p 0.01) was observed in Huh-7 cells exposed to DS-EE at concentrations of 40 µg/ml, 60 µg/ml, and 80 µg/ml for 24 hours (Figs. 5A and 5B). The increase in ROS generation exhibited a concentration-dependent pattern, with levels reaching 128%, 146%, and 204% compared to the untreated control following exposure to 40 µg/ml, 60 µg/ml, and 80 µg/ml of DS-EE, respectively.

### 3.7. Mitochondrial membrane potential (MMP)

The effect of DS-EE exposure on mitochondrial membrane potential (MMP) was assessed in Huh-7 cells. A concentration-dependent, statistically significant (p 0.01) decrease in MMP level was observed after 24 hours of DS-EE exposure. The decrease in MMP level was measured as 13%, 34%, and 45% at concentrations of 40 µg/ml, 60 µg/ml, and 80 µg/ml of DS-EE, respectively, compared to the untreated control (Figs. 6A and 6B).

### 3.8. RT-qPCR analysis

The investigation focused on the impact of DS-EE on apoptosis-related genes in Huh-7 cells. Using real-time PCR, the mRNA levels of p53, caspase-3, caspase-9, Bax, and Bcl-2 were evaluated. <sup>[11]</sup> As depicted in Fig. 7, the mRNA expression of p53, caspase-3, caspase-9, and Bax genes were increased by 1.6-, 2.0-, 2.1-, and 1.7-fold following 24 hours of incubation with DS-EE, respectively. Conversely, Fig. 7 revealed a decrease in Bcl-2 gene expression by 0.46-fold after 24 hours of incubation with DS-EE. <sup>[20]</sup> These findings suggest that the DS-EE extract induced apoptosis in Huh-7 cells primarily through the modulation of apoptotic related genes.

## 4. Discussion

For centuries, medicinal plants have been integral to folk medicine, offering natural healing remedies with well-documented therapeutic benefits (Karunamoorthi et al., 2013). These encompass a wide range of areas, from cardiovascular disease prevention to anti-inflammatory, antimicrobial, antiaging, and anticancer activity (Anand et al., 2019). The crude extract and a vast

array of natural compounds, such as tannins, flavonoids, terpenoids, glycosides, phenolic acids, from dill seeds, have been discovered to possess anticancer properties (Jana and Shekhawat, 2010; Dahiya and Purkayastha, 2012). These researches highlight the significance of traditional dill seeds as invaluable reservoirs for the discovery of anticancer potential.<sup>[0]</sup> Based on this premise, we initiated this study to primarily assess the cytotoxic activities of dill seed extract (DS-EE), and the mechanism(s) involved in cell death of Huh-7 cells.<sup>[0]</sup> In this study, as indicated by MTT and NRU assays, DS-EE showed a potent cytotoxic activity with IC<sub>50</sub> value of ~60 µg/ml against human hepatocellular carcinoma cell line (Huh-7).<sup>[0]</sup> Previous reports corroborated by our study, which highlighted that the essential oil extracted from dill seeds exhibits a dose-dependent anticancer and antiproliferative effect against human hepatocellular carcinoma cells (HepG2), with an IC<sub>50</sub> value of 59.6 µg/ml (Al-Sheddi et al., 2019). These findings also align with other reports indicating that the *A. graveolens* demonstrated cytotoxic and inhibitory effects on other human cancer cell lines such as human breast cancer, human cervical cancer, and human colon cancer (Sharopov et al., 2013). The findings were further corroborated through morphological examination of Huh-7 cells treated with DS-EE. The light microscopy images depicted a loss of normal growth, cellular rounding, and inhibition of cell proliferation in Huh-7 cells exposed to DS-EE, indicative of apoptosis (Brady, 2004). Likewise, Al-Oqail and Farshori (2021) demonstrated that dill seed extract induces apoptosis in MCF-7, A-459, and HeLa cells, as evidenced by morphological alterations.

<sup>[0]</sup> Further we explored the underlying mechanism of DS-EE-induced Huh-7 cell death.<sup>[0]</sup> For this, Huh-7 cells were subjected to concentrations ranging from 40 to 80 µg/mL of DS-EE for 24 hours, and analyses were conducted on oxidative stress markers (GSH and LPO), ROS generation, MMP, and expression of apoptotic marker genes.<sup>[9]</sup> The increase in ROS leads to elevated LPO levels, which are known to reduce endogenous antioxidants like glutathione (GSH) (Gao et al., 2024).<sup>[0]</sup> The results of the present investigation suggest that the administration of DS-EE at concentrations of 40, 60, and 80 µg/mL substantially increased lipid peroxidation (LPO) levels while simultaneously decreased glutathione (GSH) content in a dose dependent manner.<sup>[0]</sup> Earlier research has documented that the exposure to plant extracts leads to heightened lipid peroxidation (LPO) and diminished levels of glutathione (GSH) in cancer cells (Al-Oqail 2021).<sup>[0]</sup> Additionally, it has been documented that the stimulation of plant extracts can trigger excessive generation of reactive oxygen species (ROS), resulting in oxidative stress and apoptotic cell death in cancer cells (Rostamabadi et al.,

2023).<sup>[8]▶</sup> As evidenced in this study, DS-EE treatment led to a dose-dependent increase in ROS production.<sup>[8]▶</sup> These findings highlight the involvement of oxidative stress and ROS generation in DS-EE-induced cell death in Huh-7 cells.<sup>[5]▶</sup> The decreased mitochondrial membrane potential (MMP) has been linked to the initiation of apoptosis (Ly et al., 2003). As depicted in Fig. 6,<sup>[0]▶</sup> there was a dose-dependent decline in mitochondrial membrane potential (MMP) subsequent to DS-EE treatment. This suggests that DS-EE induces mitochondrial dysfunction, consequently triggering the apoptotic pathway.<sup>[0]▶</sup> Given that the apoptosis pathway is the most commonly induced type of cell death by plant extracts (Chaudhry et al., 2022),<sup>[4]▶</sup> the study examined the ability of DS-EE to induce apoptosis in order to elucidate its potential mechanism of action.<sup>[4]▶</sup> Apoptosis, a genetically regulated process, plays a crucial role in selectively eliminating cells, essential for cell turnover and normal development.<sup>[11]▶</sup> It is tightly controlled through the expression of various genes, with the tumor suppressor gene p53 being paramount as the guardian of the genome. Triggered by either intrinsic or extrinsic pathways, apoptosis ultimately activates caspases, the enzyme responsible for executing key apoptotic features.<sup>[0]▶</sup> This study utilized RT-qPCR gene expression profiling to investigate the cytotoxic impact of DS-EE on Huh-7 cells.<sup>[0]▶</sup> The results indicate that DS-EE induces apoptosis, as evidenced by the notable increase in expression levels of proapoptotic marker genes, including p53, Caspase-3, Caspase-9, and Bax and a decrease in expression level of antiapoptotic gene Bcl-2.<sup>[11]▶</sup> Consistent with our findings, prior investigations have also demonstrated that plant extracts induce apoptosis, exhibiting alterations in the apoptosis related genes in human hepatocellular carcinoma cell line (Pouraminaei et al., 2020).

## 5. Conclusion

In conclusion, this study demonstrates the concentration-dependent cytotoxic effects of dill seed extract (DS-EE) on human hepatocellular carcinoma (Huh-7) cells through oxidative stress.<sup>[0]▶</sup> The research indicates a notable rise in lipid peroxidation (LPO) level and a decline in intracellular glutathione content (GSH), suggesting the involvement of DS-EE in triggering oxidative stress.<sup>[0]▶</sup> The notable rise in intracellular ROS production and decline in mitochondrial membrane potential (MMP) suggest the contribution of DS-EE in cellular death mechanisms.<sup>[0]▶</sup> Furthermore, present findings indicate that DS-EE extract induces apoptosis in Huh-7 cells, accompanied by alterations in the expression of pro-apoptotic and anti-apoptotic genes.<sup>[0]▶</sup> Additionally, this study has also contributed to a deeper understanding of how DS-EE induces apoptosis in Huh-7 cells, indicating its potential as a novel therapeutic option for hepatocellular carcinoma management.<sup>[1]▶</sup>

## Acknowledgment

The authors extend their appreciation to the Deanship of Scientific Research, King Saud University for funding through Vice Deanship of Scientific Research Chairs;<sup>[1]</sup> Chair for DNA Research.

## <sup>[3]</sup> Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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