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Oleuropein changes the biological characteristics of the HepG2 cells by regulating the IL-6 / STAT3 pathway

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ABSTRACT

Aim: Oleuropein (Ole) possesses a wide array of pharmacological benefits. Despite Ole's well-established anti-inflammatory and anti-tumor effects, there remains a gap in the research concerning its specific mechanism of action.

We aimed to evaluate the effects of Ole treatment on various characteristics, such as the viability of the cells, wound healing, colony formation, and the protein levels of the IL-6 / STAT3 pathway were evaluated in vitro in HepG2 HCC cells.

Methods: Cell viability, wound healing, and colony formation assays were performed. IL-6 levels measurement by ELISA, STAT3, and pSTAT3 protein expression measurement by Western blotting were performed in Ole-treated HepG2 cells.

Results: Following the treatment of cells with Ole, there was a dose-dependent reduction in the viability of the cells and wound healing parameters. Colony formation was completely inhibited compared to control cells. Although IL-6 and STAT3 levels were strongly suppressed in Ole-treated cells compared to control cells, this difference did not achieve a statistical significance. Only pSTAT3 expression in Ole100-treated cells was significantly downregulated compared to control cells.

Conclusion: Based on our study, Ole treatment seems to have an impact on the characteristics of HCC cells through its effect on increased cell death, reduced cell migration, and the downregulation of the IL-6 / STAT3 pathway.

Keywords: Hepatocellular carcinoma, oleuropein, IL-6, STAT3.

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1. Introduction

Liver cancer is a significant global health burden, ranking as the sixth most widespread malignancy (4.6% of new annual cases) and the third primary driver of cancer mortality, responsible for 7.8% of global cancer fatalities in 2022. The majority of these cases (75-85%) are hepatocellular carcinoma (HCC). Main risk factors for HCC development encompass chronic Hepatitis B and C infections, aflatoxin exposure, alcohol consumption, obesity, and type 2 diabetes [1]. The lack of early symptoms in most HCC patients, combined with its high invasiveness and metastatic potential, limits the effectiveness of the current treatment strategies [2].

Despite advancements in the treatment of Hepatitis B and C infections, chronic liver inflammation is a continuing major problem. In

patients with liver inflammation, there is a typical progression from inflammation to cirrhosis, then dysplasia, and finally HCC [3]. Inflammation within the tumor microenvironment drives tumor cell growth, spread, and the development of resistance to chemotherapy [4]. Primarily synthesized by macrophages and lymphocytes, Interleukin-6 (IL-6) contributes to chronic inflammation, which in turn facilitates the establishment of a pro-tumorigenic environment. Induction of the signal transducer and activator of transcription 3 (STAT3) by IL-6 leads to the activation of inflammatory cascades, oncogenic pathways, and the promotion of malignant cell growth [5]. Upon binding its tyrosine binding site, STAT3 undergoes phosphorylation at its C-terminal Tyr705 residues. This phosphorylation process leads to STAT3 activation and accumulation in the cytoplasm and finally the formation of homodimers. Subsequently, STAT3 dimers bind to the promoter regions of target genes, which are involved in apoptosis, proliferation, angiogenesis, and immune response, and regulate their transcription [6]. IL-6-induced STAT3 activation reactivates the nuclear factor-κB (NF-κB), creating a positive feedback loop that amplifies and sustains IL-6 / STAT3 signaling axis [7].

Olea europaea (olive, Fam. Oleaceae) yields various preparations recognized for their general health benefits and these have been the subject of extensive recent research [8]. The pharmacological properties of the different parts of the olive tree, including roots, stem barks, leaves, and fruits, have been investigated due to their rich and varied chemical composition. Notably, Oleuropein (Ole) is found in high concentrations in olive leaves and is also present in unripe olive fruits [9]. Oleuropein is the main secoiridoid in olive leaves and demonstrates anticancer, cardioprotective, neuroprotective, hepatoprotective, and antiobesity properties. The observed properties are attributed to its putative antioxidant and anti-inflammatory effects [10].

Although the anticancer properties of Ole are partly attributed to its anti-inflammatory effects, its specific mechanism of action, particularly its influence on the IL-6 / STAT3 inflammatory pathway, remains unclear. Thus, this study was designed to clarify the impact of Ole treatment on the biological properties of HepG2 cells by evaluating proliferative and migratory characteristics, as well as protein expression of the IL-6 / STAT3 pathway.

2. Materials and methods

2.1. Cell culture: HepG2 cell (ATCC, HB-8065) was cultured in Minimum Essential Medium Eagle (MEM) (M5650, Sigma) with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin-Neomycin and incubated at 37 °C with 5% CO_2.

2.2. Cell cytotoxicity assay: For the determination of cytotoxicity of Oleuropein (Ole), HepG2 cells were resuspended in MEM and were seeded in a 96-well cell culture plate at a density of 10000 cells per well, incubated overnight, and treated with Ole (Cayman Chemical) (3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µM) in MEM for 24 h. 10 µL of MTT solution at a concentration of 5 mg/mL in phosphate-buffered saline (PBS) was added to well. Following 4 h incubation, the well content was aspirated and 100 µL of DMSO was added to dissolve the formed formazan crystal on the bottom of the well. Absorbance was measured at 570 nm using a microplate reader (Biotek, Synergy H1m) [11].

2.3. Determination of cell viability: HepG2 cells were seeded in a T25 flask and cultured

overnight. Cells were treated with 500 or 1000 µM Ole for 24 h, and the viability of the cells was determined using Trypan Blue. Cell suspension and trypan blue solution mix were loaded onto a cell-counting slide and counted on the Luna cell counter (Logos) [12].

2.4. Wound healing assay: HepG2 cells were seeded in a petri dish and cultured overnight. A scratch was created on a confluent cell monolayer with a pipette tip and to remove dislodged cells dish was washed with PBS. Cells were treated with 500 or 1000 µM Ole for 24 h, and an image of the scratch was recorded at regular time intervals. Gap fill and migration rate data were calculated by the integrated software of the imager (Leica, Paula) [13].

2.5. Colony formation assay: A thousand cells per well were seeded to 6-well plates, incubated overnight, and treated with 500 or 1000 µM Ole for 24 h. The medium was aspirated; wells were washed with PBS, replaced with MEM, and changed every 3 days. At the end of the incubation period, fixation of the cells was performed using methanol:acetic acid. Following staining with 0.5% crystal violet and washing with tap water, colonies were counted microscopically (Leica, DMi8) [14].

2.6. Measurement of IL-6 level: The cells were treated with 500 or 1000 µM Ole for 24 h, the IL-6 level was measured using a Human IL-6 ELISA Kit (BT Lab, E0090Hu) according to the kit protocol. 40 µL of the sample, 10 µL of biotinylated primary antibody, and 50 µL of streptavidin-horseradish peroxidase solution were added to the well. Following incubation, the content of the wells was aspirated and washed. 50 µL of Substrate A and B was pipetted into the well and incubated in the dark. The reaction was stopped by the addition of 50 µL of stop solution and absorbance was measured at 450 nm.

2.7. Western blotting: After treating cells with 500 or 1000 µM Ole for 24 h, cells were washed with PBS and lysed in ice-cold radioimmunoprecipitation assay buffer containing 1% protease and phosphatase inhibitors. The cells were scraped, collected, centrifuged, and the supernatant was collected. BCA Protein Assay Kit (Thermo Fisher, 23225) was used to determine the amount of protein. Equal volumes of sample and Laemmli sample loading buffer (Serva, 42526) were mixed and heated at 95 °C for 5 min. Samples were loaded onto a 4–20% Precast Gel and transferred onto polyvinylidene fluoride (PVDF) membrane by using PVDF Transfer Kit (Bio-Rad, 1704272). The membranes were blocked with EveryBlot and probed with primary antibodies, STAT3 (Cell Signaling, 8768), pSTAT3 (Cell Signaling, 9145), and β-Actin (Cell Signaling, 4970). Then, incubated with HRP-linked Anti-rabbit IgG (Cell Signaling, 7404). A Western ECL Substrate (Bio-Rad, 1705062), ChemiDoc imaging system, and Image Lab software were used for the visualization and quantification of the bands.

2.8. Statistical analysis: The Kolmogorov-Smirnov test was used for the test of normality. The continuous variables did not show a normal distribution. Therefore, results were expressed as median ± interquartile range. Protein expressions were represented as fold change. Continuous variables were compared using a pairwise Kruskal-Wallis test. A *p*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software version 27 (IBM).

3. Results

3.1. The Determination of Ole Cytotoxicity in HepG2 Cell Line: To determine the toxicity of Ole on HepG2 cells, an MTT assay was

performed. For Ole, A concentration-dependent toxicity was not observed. Cell viability for 500 µM Ole (Ole500) was 73.68% and 1000 µM Ole (Ole1000) was 44.73%, respectively (Figure 1). To see the effect of two different doses, Ole500 and Ole1000 were selected as application doses for the following experiments.

3.2. The viability of HepG2 cells treated with Ole: At the end of the 24-hour treatment period with Ole500 or Ole 1000, the viability of the HepG2 cells was determined through the Trypan blue exclusion test. The number of live cells was $1.58x10^6$, $7.76x10^5$, and $4.58x10^5$ for control, Ole500, and Ole1000, respectively. Viable and dead cells were photographed, as shown in Figure 2.

3.3. Wound healing assay of HepG2 cells treated with Ole: The results of the migratory characteristics of HepG2 cells treated with Ole500 or Ole1000 for 24h were summarized in Figure 3, and images of the scratch region at the

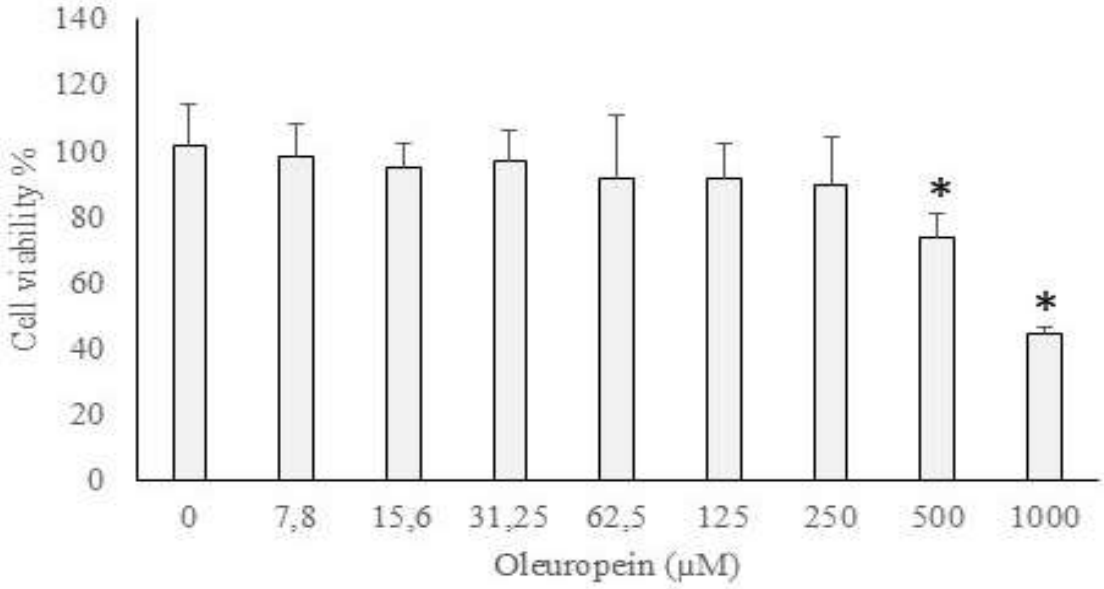

Figure 1. The cell toxicity of Ole-treated HepG2 cells was determined by MTT assay. Results were expressed as median ± interquartile range. * $p < 0.001$ versus zero concentration of Ole.

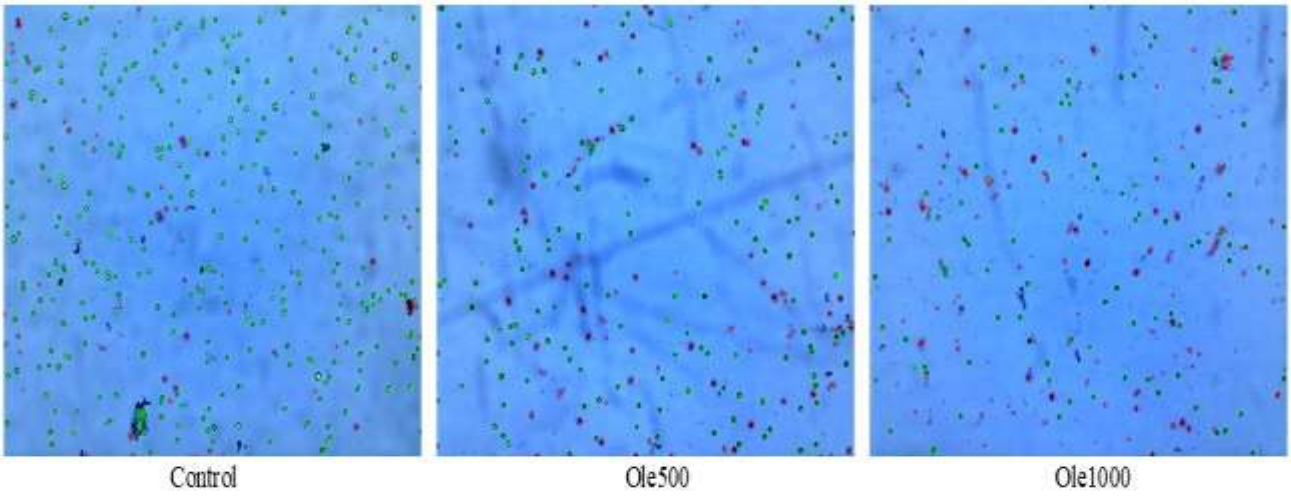

Figure 2. The cell viability of HepG2 cells treated with Ole500 or Ole1000 for 24 h. Viable cells were tagged with green, and dead cells were tagged with red.

first and last time points were represented in Figure 4. The gap fill (%) was 7.8, -0.4, and –10,8 and the migration rate (µm/h) was 1.1, 0.1, and -1, for control, Ole500, and Ole1000, respectively. With the increased concentration of Ole, the gap fill and migration rate values were reduced in a dose-dependent manner (Figure 4).

3.4. Proliferation capacity of HepG2 cells treated with Ole: A colony formation assay was performed to determine the proliferation capacity of HepG2 cells treated with Ole500 or Ole1000. The plate efficiency and survival fraction for Ole500 and Ole1000-treated HepG2 cells were zero because no colony was formed (Figure 5).

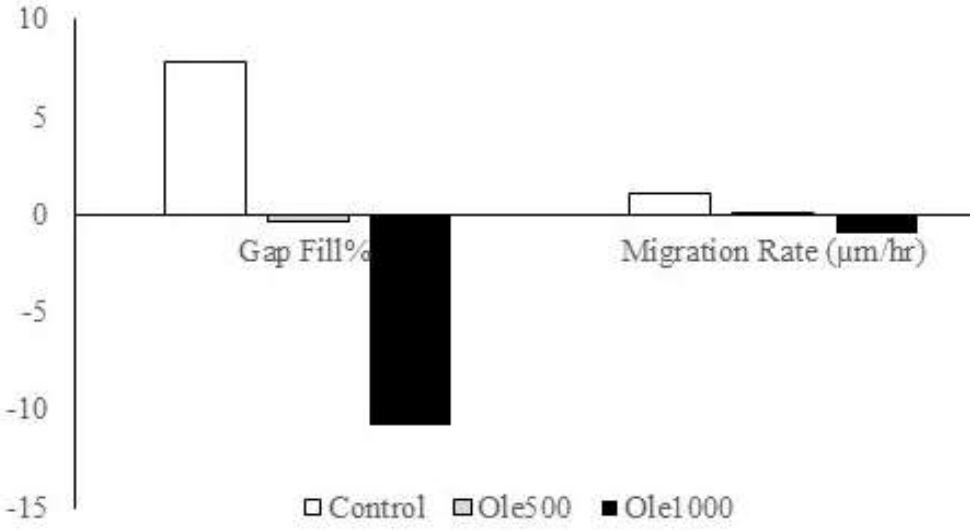

Figure 3. The migratory characteristics of HepG2 cells at the end of 24h treatment with Ole500 or Ole1000. After an initial scratch, the movement of HepG2 cells was observed under a cell imager. Gap fill (%) and the migration rate (µm/h) parameters of the cells were calculated by the software of the imager.

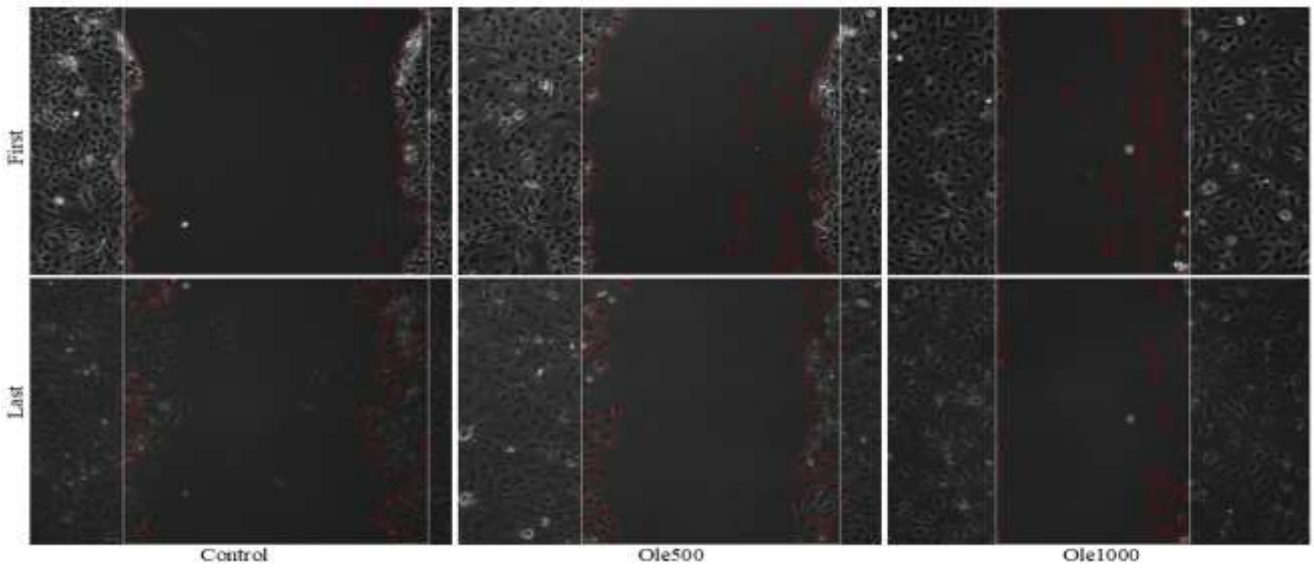

Figure 4. The migration of HepG2 cells was assessed using the wound healing assay. After making a scratch at the initial time point, cells were treated with Ole500 or Ole1000 for 24 hours. The scratch area was imaged at specified time intervals using a cell imager.

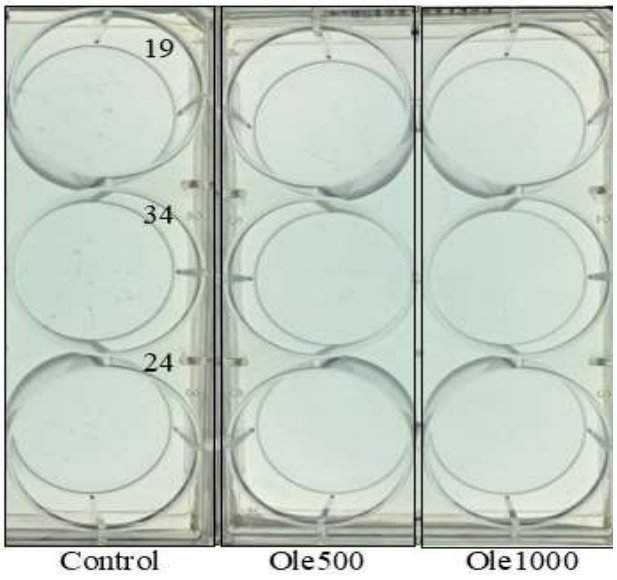

Figure 5. The proliferative capacity of Ole500 or Ole1000-treated HepG2 cells. In a triplicate experiment, there is no formed colony for Ole500 and Ole100. For control, the number of the formed colonies was 19, 34, and 24.

3.5. The effect of Ole treatment on IL-6 levels: The results of the effect of 24h Ole500 or Ole1000 treatment on IL-6 levels in HepG2 cells are shown in Figure 6. IL-6 levels were 308.88, 178.82, and 196.38 ng/L for control, Ole500, and Ole1000, respectively. Despite the reduction in IL-6 levels in Ole500- and Ole1000-treated cells, this difference didn't reach statistical significance.

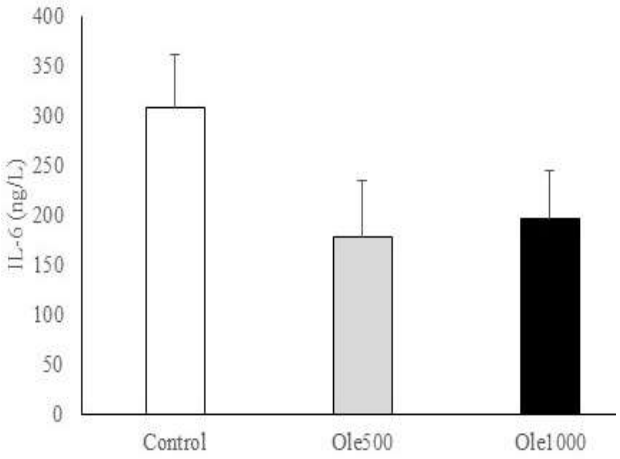

Figure 6. IL-6 levels in HepG2 cells treated with Ole500 or Ole1000 for 24 h were measured by ELISA. Measurements were repeated three times.

3.6. The effect of Ole treatment on STAT3 and pSTAT3 protein expressions in HepG2 cells: The effects of the 24-hour Ole500 or Ole1000 treatment on STAT3 and pSTAT3 protein levels in HepG2 cells are shown in Figure 7. STAT3 protein was downregulated and almost the same level in Ole500 (0.54-fold) and Ole1000-treated cells (0.54-fold) compared to the control. pSTAT3 protein level was downregulated in Ole500 (0.36-fold) and Ole1000-tretated cells (0.28). Downregulation in pSTAT3 levels in Ole1000-treated cells was statistically significant compared to the control.

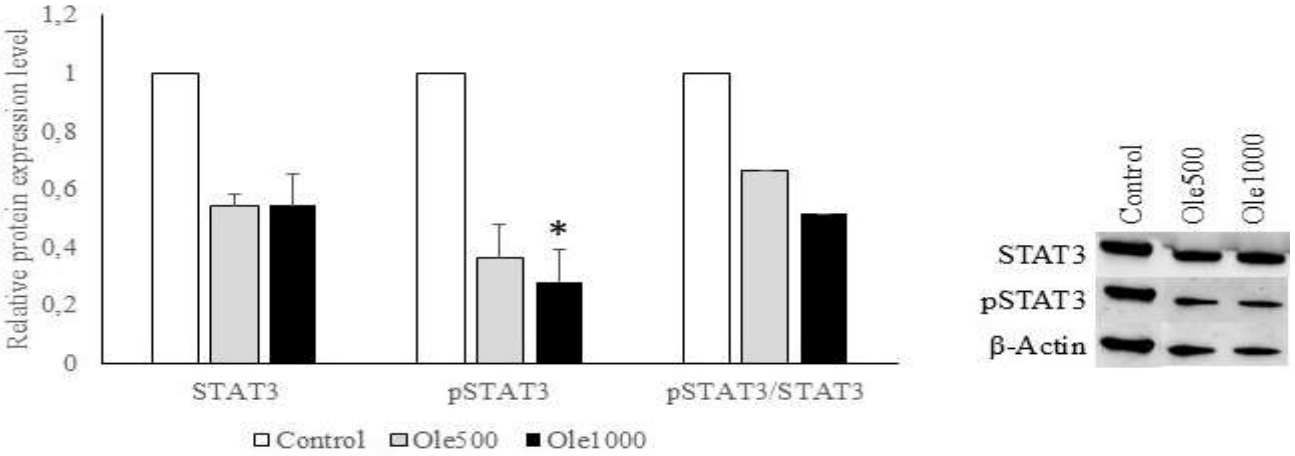

Figure 7. Protein expression of STAT3 and pSTAT3 in HepG2 cells treated with Ole500 or Ole1000 for 24 h was determined by Western blotting, and β-Actin was used as a housekeeping protein. Experiments were performed in duplicate. ** $p < 0.05$ versus control.*

4. Discussion

In this study, we aimed to investigate the influence of Ole on the viability, proliferation, migration properties, and IL6/STAT3 pathway of the HepG2 HCC cell line. Ole treatments caused a decrease in the viability, proliferation, and migration rates of HepG2 cells. Additionally, we observed a reduction in IL-6 level, STAT3, and pSTAT3 protein expressions in Ole-treated cells. Therefore, it can be inferred that Ole treatment diminished the proliferative and migratory properties of HepG2 cells by suppressing the IL6/STAT3 pathway.

With its diverse pharmacological benefits, Ole demonstrates potential in areas like anticancer, antioxidant, and anti-inflammatory. There is increasing evidence pointing to the anticancer effect of Ole in various cancers by affecting signaling pathways associated with proliferation, death, and migration of cells [15]. Yan et al. showed that Ole dose-dependently inhibited cell viability and promoted apoptosis by triggering the caspase pathway and inducing the expression level of proapoptotic proteins in HepG2 cells [16]. A study by Yamada et al. demonstrated that Ole has effectively suppressed the migration of Huh7 cells, which were pretreated with transforming growth factor α to induce migration, and this suppression was dose-dependent [17]. The obtained results of the cell viability with MTT assay and the number of viable cells with Trypan Blue were consistent, rate was 0.6 between Ole500 and Ole1000 treatments, indicating a dose-dependent effect of Ole between treatments. In addition to viability, the effect of the Ole500 and Ole1000 treatments on the migration rate of the cells was also dose-dependent.

HCC has emerged as a significant contributor to cancer-related death, making it crucial to investigate its underlying mechanisms. As a key characteristic of cancer, inflammation has substantial roles in both the development and progression mechanisms of HCC. A pleiotropic inflammatory cytokine, IL-6 is essential for the initiation and regulation of both inflammation and proliferation. Upon activation of the IL-6-driven pathways, cells acquire anti-apoptotic, proliferative, migratory, and invasive properties [18]. Although IL-6 levels in both the Ole-treated group declined to two-thirds of the control, no statistical difference was found. In contrast to the observed effect on the viability and migration rate of cells, there was no dose-dependent effect of Ole treatments on IL-6 levels. However, it's reasonable to conclude that mechanisms other than IL-6-promoted could contribute to the decrease in viability and migration rates of cells. In a cell-based study, Ole treatment has resulted in the dose-dependent increase of expression of cytosolic Cytochrome C, Caspase 9, and 3 proteins that reflect apoptosis [19].

Activation of major intracellular pathways, specifically STAT3 plays a key role in the inflammation that drives hepatocarcinogenesis, regulates cell survival, proliferation, and migration [20]. Beyond that, pSTAT3 has been identified as a cancer-promoting protein, its expression has been linked to clinical presentation and prognosis of HCC. In a study performed with 100 clinical HCC samples, the number of cases with positive expression of STAT3 and pSTAT3 was 72 and 58, respectively [21]. Another study with clinical samples, the positive rate of pSTAT3 in primary HCC and tumor cells of portal vein invasion and intrahepatic metastasis was 35.6%, 78.9%, and 75%, respectively. Higher levels of pSTAT3 were found to be strongly

linked to both shorter overall survival and disease-free survival, and pSTAT3 expression level was identified as an independent prognostic risk factor for disease-free survival [22]. While the downregulation of STAT3 expression was the same in both Ole treatments, the pSTAT3 expression in Ole1000-treated cells was suppressed more than in Ole500-treated cells. The suppression of STAT3 and pSTAT3 expression in both treatments reveals that Ole regulates the STAT3 mechanism in a slightly dose-dependent manner, specifically in the pSTAT3 level. Inhibition of STAT3 phosphorylation with a specific inhibitor caused to reduction in proliferation and migration of the liver cancer cells [23]. In our study, it was also found that the viability and migration rate of the cells treated with Ole1000, which had lower pSTAT3 levels, were reduced compared to Ole500-treated cells.

Given the severe impact of HCC on patient survival and the limitations of the current treatment, there is a pressing need to find new targets and therapeutic strategies [24]. Active compounds found in natural products play a crucial role in the discovery of novel treatment modalities thanks to a wide range of factors that target diverse steps of oncogenesis [15]. Furthermore, investigation of the antitumor activity of various naturally occurring compounds is very important for the adjunctive therapy of cancer. Results of the in vitro studies, including different types of human cells, show that Ole, even at high concentration, selectively kills cancer cells while having little or no effect on healthy cells [25].

The lack of more repeats for western blotting and using only one hepatocellular carcinoma cell line can be mentioned as a limitation of the current study.

4.1. Conclusion*:* Even though it didn't quite reach statistical significance, the treatment of HepG2 cells with Ole appeared to downregulate the IL-6 / STAT3 pathway, increase cell death, and cause a decrease in cell migration properties. According to the results of our study and the results of the previous studies performed with clinically HCC samples, we can conclude that Ole treatment regulates the aggressive nature of the HCC cells by downregulating IL-6 / STAT3 pathway, promoting cell death, and lowering cell migration in a dose-dependent manner.

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Conflict of Interest: *The authors declared no conflict of interest.*

Ethical Statement: *This study was carried out using a human cancer cell line, so there is no need for ethics committee approval.*

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