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Olive Leaf Extract Enhances Natural Killer Cell-Mediated Cytotoxicity against Colorectal Cancer Cells

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ABSTRACT

Background: Natural Killer (NK) cells are innate lymphoid cells that eliminate malignant cells via perforin/granzyme-mediated cytotoxicity. This study investigates whether Olive Leaf Extract (OLE), rich in oleuropein and hydroxytyrosol, can enhance NK-cell cytotoxicity against colorectal cancer (CRC) cells. Although OLE exhibits direct anticancer effects, its therapeutic utility is constrained by poor bioavailability, requiring supraphysiological concentrations for direct cytotoxicity.

Objective: To evaluate the effect of OLE on the cytotoxic activity of NK-92 cells against HT-29 colorectal cancer cells within a co-culture model.

Methods: Olive leaves obtained from Balıkesir, Türkiye were dried and extracted with 70% methanol. The effects of OLE on the viability of HT-29 cells and NK-92 cells were evaluated using MTT and ATP assays. Additionally, the cytotoxic activity of NK-92 cells against HT-29 cells was assessed in a direct co-culture system. Granzyme B and perforin levels were measured using ELISA kits.

Results: OLE inhibited the proliferation of HT-29 cells in a dose-dependent manner, with an IC_{50} values of 548 µg/mL. In NK-92 cells, low concentrations of OLE (100–200 µg/mL) promoted cell proliferation, whereas higher concentrations exerted cytotoxic effects. In co-culture experiments, NK-92-mediated cytotoxicity against HT-29 cells was significantly enhanced by the addition of OLE at non-toxic concentrations (100 and 200 µg/mL). This enhanced cytotoxicity was further supported by a significant increase in granzyme B and perforin levels following OLE treatment.

Conclusion: Our findings suggest that OLE can elicit a potent anticancer response via NK cells at lower, physiologically achievable doses. These results highlight a promising therapeutic strategy for CRC, leveraging OLE's immunomodulatory effects to enhance innate antitumor defenses.

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Keywords: Cancer Immunotherapy, Granzyme, Perforin, Phytochemicals

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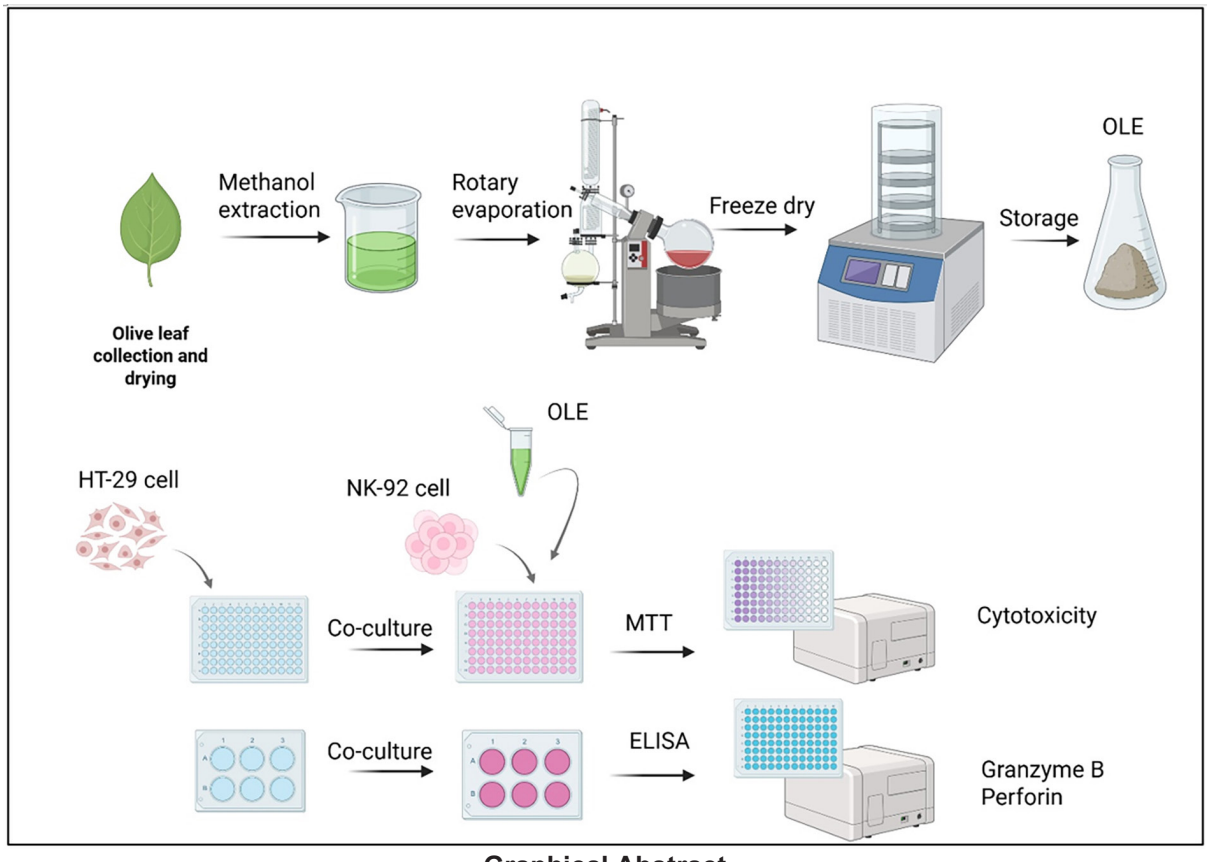

Graphical Abstract

INTRODUCTION

Colorectal cancer remains one of the leading causes of cancer-related mortality worldwide despite substantial advances in therapeutic strategies [1]. The immune system, particularly Natural Killer (NK) cells, plays a critical role in tumor immunosurveillance by recognizing and eliminating transformed cells [2, 3]. NK cells exert potent antitumor activity through the direct lysis of tumor cells via the release of cytotoxic granules containing perforin and granzymes [6, 8]. However, NK cell function is frequently impaired in patients with cancer, contributing to immune evasion and disease progression. This dysfunction has been associated with poor clinical outcomes, underscoring the need for effective immunomodulatory strategies to restore NK cell activity and enhance antitumor immunity [4, 7].

Phenolic compounds exhibit a broad spectrum of biological activities, including antioxidant, anti-inflammatory, anticancer, antidiabetic, and antihyperlipidemic effects [12]. In addition to these well-established properties, accumulating evidence highlights their significant immunomodulatory potential. Given the central role of immune-based therapies in cancer treatment, polyphenols have emerged as promising adjuncts to cancer immunotherapy due to their ability to modulate immune responses and enhance antitumor immunity [13].

Olive leaf extract (OLE), obtained from *Olea europaea L.,* is a rich source of bioactive phenolic compounds, particularly oleuropein [14, 15]. Epidemiological and experimental studies have associated olive-derived phytochemicals with a reduced risk of several malignancies, including colorectal, breast, and skin cancers [18]. OLE and its major constituents have demonstrated antiproliferative, pro-apoptotic, and antimetastatic effects activities across a range of cancer types, including colorectal, breast, liver, and ovarian cancers [19-22]. These antitumor effects are primarily attributed to

the modulation of oxidative stress, induction of apoptosis, and suppression of glycolytic metabolism [23, 24].

In addition to its direct anticancer effects, the immunomodulatory potential of OLE has been demonstrated in both *in vitro* and *ex vivo* experiments [5, 25, 26], as well as clinical trials [27]. For instance, Magrone et al.[5] reported that OLE significantly increased the absolute numbers of $CD8^+$ T cells and NK cells in cultured peripheral blood mononuclear cells while enhancing IFN γ production, indicating activation a of Th1-type immune response. Similarly, in a clinical trial conducted by Kocyigit et al. [27], consumption of an olive leaf infusion significantly increased peripheral NK cell counts and was accompanied by elevated levels of IL-2 and IFN-γ.

Although *in vitro* and *in vivo* studies have extensively demonstrated the antitumor, antiproliferative, and anti-invasive properties of OLE, its clinical application remains limited by its inherently low bioavailability, which consequently reduces its therapeutic efficacy against cancer [28].

The immunomodulatory activity of OLE, which has also been demonstrated in previous studies, may contribute to an antitumor effect even at low concentrations. However, despite growing evidence supporting both its anticancer and immunomodulatory properties, no study has comprehensively investigated the chemopreventive and antitumor effects of OLE through the modulation of immune components, particularly NK cells. Therefore, the present study aimed to evaluate the effect of OLE on the cytotoxic activity of NK-92 cells against HT-29 colorectal cancer cells under co-culture conditions.

MATERIALS AND METHODS

Cell Lines and Reagents

The human colorectal adenocarcinoma cell line HT-29 and Natural Killer cell line NK-92 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human Perforin and Human Granzyme-B levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (BT LAB, China) according to the manufacturer's instructions. HT-29 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA). NK-92 cells were maintained in Alpha Minimum Essential Medium (α-MEM; Sigma, Germany) supplemented with L-Glutamine and 1.0 g/L Glucose (WISENT, Canada), 100 U/mL recombinant IL-2, 12.5% horse serum, and 12.5% FBS. All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

Preparation of Olive Leaf Extract

Olive leaves were collected from the Balıkesir province in the Aegean region of Turkey. OLE was prepared according to the method described by Kocyigit et al [29]. Briefly, the leaves were air-dried and extracted with 70% methanol at a concentration of 100 mg/mL. The mixture was incubated for 48 h to facilitate extraction, followed by filtration through filter paper. The Solvent was then removed under reduced pressure using a rotary evaporator (Heidolph, Germany) at 45 °C until complete evaporation of methanol. The resulting crude extract was freeze-dried using a lyophilizer (Labconco, Kansas City, USA) and stored at −20 °C until further use. The extraction yield was calculated as 220 mg of extract per gram of dried olive leaf.

Determination of Total Phenolic and Flavonoid Contents

Total phenolic (TP) content was determined using the Folin-Ciocalteu assay according to Singleton et al. (1999) [30]. Briefly, OLE was dissolved in distilled water at a concentration of 5 mg/mL and further diluted appropriately for analysis. An aliquot of 40 µL of OLE sample was mixed with 200 µL of Folin-Ciocalteu reagent and incubated

at room temperature for 8 min. Subsequently, 30 µL of 0.7 mol/L sodium carbonate solution was added, and the reaction mixture was incubated at room temperature for 2 h in the dark. Absorbance was measured at 746 nm using a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Scientific). A calibration curve was generated using gallic acid (0–5 mg/mL), and results were expressed as mg gallic acid equivalents (GAE) per gram of OLE.

Total flavonoid (TF) content was determined using the aluminum chloride colorimetric method described by Meda et al [31]. Briefly, 192 µL of OLE dissolved in 80% ethanol was mixed with 4 µL of 1 M potassium acetate and 4 µL of 10% aluminum nitrate solution. The mixture was incubated for 40 min in the dark, at room temperature. Absorbance was measured at 415 nm using the spectrophotometer. A quercetin standard curve was used for quantification, and results were expressed as mg quercetin equivalents (QE) per gram of OLE.

Total Antioxidant Capacity Assay

Total Antioxidant Capacity (TAC) was determined using the automated colorimetric method described by Erel (2004) [32]. Briefly, 5 µL of OLE was mixed with 500 µL of freshly prepared $ABTS^+$ working solution and incubated at room temperature for 90 s. The decrease in absorbance resulting from $ABTS^+$ radical scavenging activity was measured at 734 nm using a spectrophotometer. Antioxidant capacity was calculated using Trolox calibration curve, and results were expressed as Trolox Equivalents (TE).

Cell Viability Assays

HT-29 Cell Viability (MTT Assay)

HT-29 cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay. Cells were seeded in 96-well plates at a density of 7×10^3 cells per well and allowed to adhere overnight. Cells were then treated with various concentrations of OLE (100–800 µg/mL) or 0.1% dimethyl sulfoxide (DMSO) as the vehicle control for 24 or 48 h. Following incubation, cells were incubated with 10% MTT solution for 4 hours at 37 °C. The resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 540 nm using a spectrophotometer. Cell viability was expressed as a percentage relative to the vehicle control which was defined as 100% viability. The half-maximal inhibitory concentration (IC_{50}) values were calculated from dose-response curves using appropriate software (Microsoft Excel).

NK-92 Cell Viability (ATP-Based Assay)

Since NK-92 cells grow in suspension, cell viability was evaluated using the ATP-based CellTiter-Glo Luminescent cell viability assay (Promega, USA), which is more suitable for non-adherent cells. NK-92 cells were seeded at a density of 5×10^3 cells per well in 96-well plates and treated with OLE at concentrations ranging from 100 to 800 µg/mL or 0.1% DMSO as the vehicle control for 24 h. After treatment, the luciferin and cell lysis solution (Cell-Titer-Glo reagent) were added according to manufacturer's instructions to induce cell lysis and stabilize the luminescent signal. Following a 10 min incubation at room temperature, luminescence was measured using a Multiplate Reader. Results were normalized to the control group.

Co-culture Experiments of NK-92 cells with HT-29 Cells

HT-29 cells in the logarithmic growth phase were detached using trypsin, centrifuged, and seeded in 96-well plates at a density of 2×10^3 cells per well. After 24 h of incubation to allow cell attachment, NK-92 cells were added at a density of 5×10^3 cells per well for direct co-culture, resulting in an effector-to-target ratio of 2.5:1. The co-culture medium consisted of a 1:1 mixture of DMEM and α-MEM to support the growth requirements of both cell types. OLE was added at non-cytotoxic concentrations for both cell lines (100 and 200 µg/mL), as

determined in preliminary viability assays. HT-29 cells cultured alone served as the target-only controls (basal viability), while NK-92 cells cultured alone were used as effector-only controls. Additionally, NK-92/HT-29 co-cultures without OLE served as baseline cytotoxicity control to evaluate the effect of NK cells in the absence of treatment. Following incubation, HT-29 cells viability in co-culture was assessed using the MTT assay as described above.

Analysis of Cytotoxic Effector Molecules in the Co-culture System

To quantify the release of the cytotoxic effector molecules perforin and granzyme, HT-29 cells were seeded in 6-well plates at a density of 2×10^4 cells/well and co-cultured with NK-92 cells (5×10^4 cells/well) corresponding to an effector-to-target ratio of 2.5:1. Co-cultures were treated with non-cytotoxic concentrations of OLE (100 and 200 µg/mL) as determined by the cell viability assays. Following incubation, culture supernatants were collected and centrifuged to remove cellular debris. The concentrations of human granzyme B and perforin released into the culture medium were quantified using commercially available ELISA kits (BT LAB) according to the manufacturer's instructions. Co-cultures without OLE treatment served as the negative control group, and perforin and granzyme B levels in OLE-treated co-cultures were compared with those of the untreated controls.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). All experiments were performed in triplicate. Statistical analysis was conducted using GraphPad Prism software (version 10.5.0; GraphPad Software, San Diego, CA, USA). Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by an appropriate post hoc multiple-comparison test when significant differences were detected. A p value <0.05 was considered statistically significant.

RESULTS

Total Flavonoid, Phenolic, and Antioxidant Capacity of OLE

The phytochemical characterization of OLE demonstrated a total phenolic (TP) content of 53.81 mg GAE per gram extract and a TF content of 18.53 mg quercetin equivalents (QE) per gram of extract. Additionally, the TAC determined using the $ABTS^{\bullet+}$ assay, was 0.331 mmol Trolox equivalents (TE) per gram extract.

Antiproliferative Effects of OLE on HT-29 Cells

OLE treatment significantly reduced HT-29 cell viability in a concentration- and time-dependent manner (Figure 1). The calculated IC_{50} values were 548 µg/mL after 24 hours and 474 µg/mL after 48 hours indicating increased cytotoxicity with prolonged exposure. Compared with the vehicle control, treatment with OLE at concentrations of 300 µg/mL resulted in a statistically significant reduction in cell viability ($p < 0.05$).

Effect of OLE on NK-92 Cell Viability

The effect of OLE on NK-92 cell viability was evaluated using the ATP-based CellTiter-Glo luminescent assay following 24 h of treatment with OLE at concentrations ranging from 100 to 800 µg/mL. As shown in Figure 2, treatment with OLE at concentrations of 100–200 µg/mL significantly increased the ATP-derived viability signal compared with the vehicle control, whereas concentrations of 500 µg/mL and above significantly reduced cell viability. The calculated IC_{50} value for OLE in NK-92 cells was 666.50 µg/mL.

Effect of OLE on the Cytotoxic Activity of NK-92 Cells

In the co-culture system, NK-92 cells exhibited intrinsic cytotoxicity against HT-29 cells. Treatment with OLE at non-

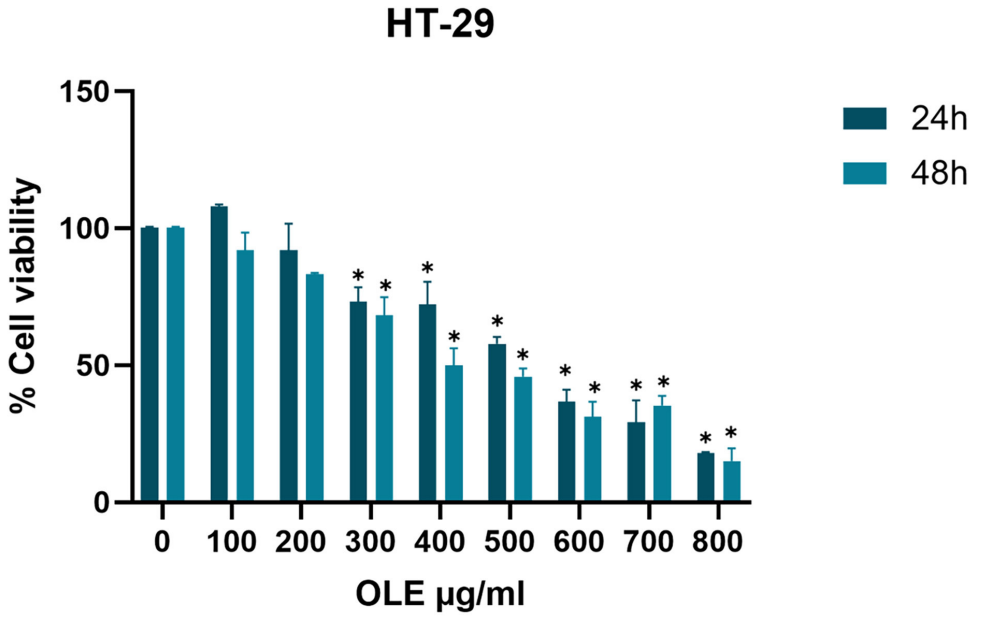

Figure 1. Effect of olive leaf extract (OLE) on the viability of HT-29 cells following 24 and 48 h of treatment, as determined by the MTT assay. Cells were treated with OLE at concentrations ranging from 100 to 800 µg/mL. The calculated IC_{50} values were 548 µg/mL after 24 hours and 474 µg/mL after 48 h of treatment, respectively. OLE exhibits significant cytotoxic activity at concentrations of 300 µg/mL and higher. Data are presented as the mean± SD of three independent experiments and expressed as mean ± SD. *p<0.05 versus vehicle control.

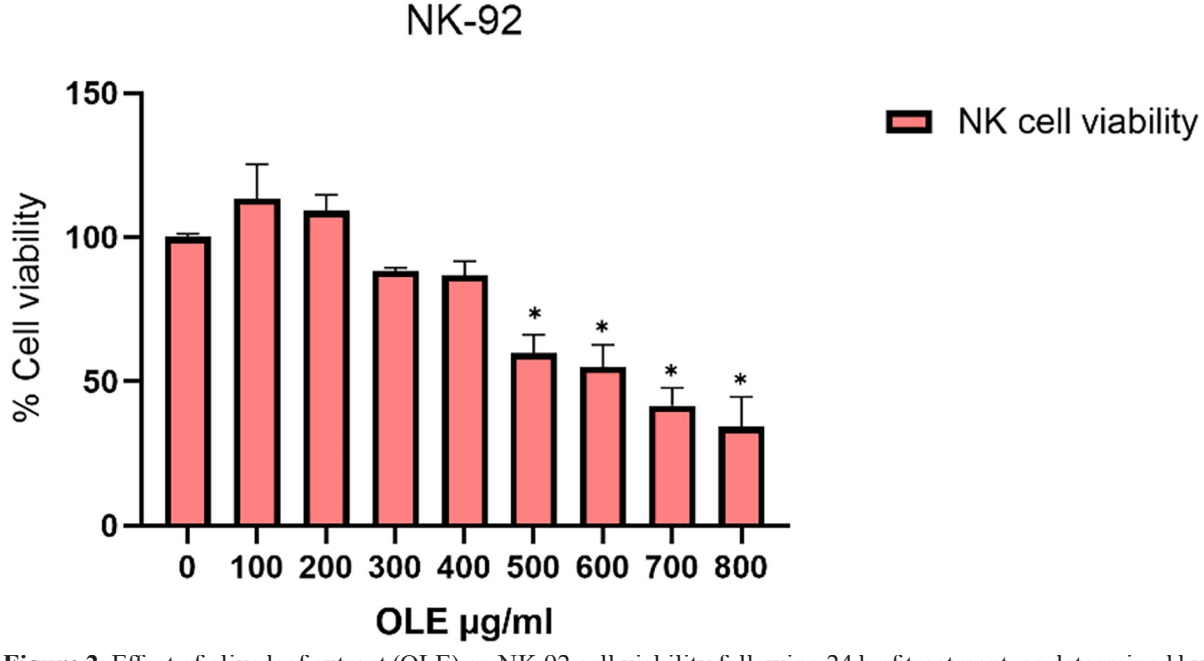

Figure 2. Effect of olive leaf extract (OLE) on NK-92 cell viability following 24 h of treatment, as determined by the ATP-based CellTiter-Glo luminescent cell viability assay. Cells were treated with at concentrations ranging from 100 to 800 µg/mL. The calculated IC_{50} value was 666.5 µg/mL. Data are presented as the mean ± SD of three independent experiments. *p<0.05 versus the vehicle control.

toxic concentrations (100 and 200 µg/mL) further enhanced NK-92-mediated cytotoxicity, resulting in a significantly greater reduction in HT-29 cell viability compared with the untreated co-culture control (Figure 3-B). Furthermore, the cytotoxic effect observed in the co-culture system was significantly greater than that produced by OLE treatment alone at the corresponding concentrations (Figure 3-A).

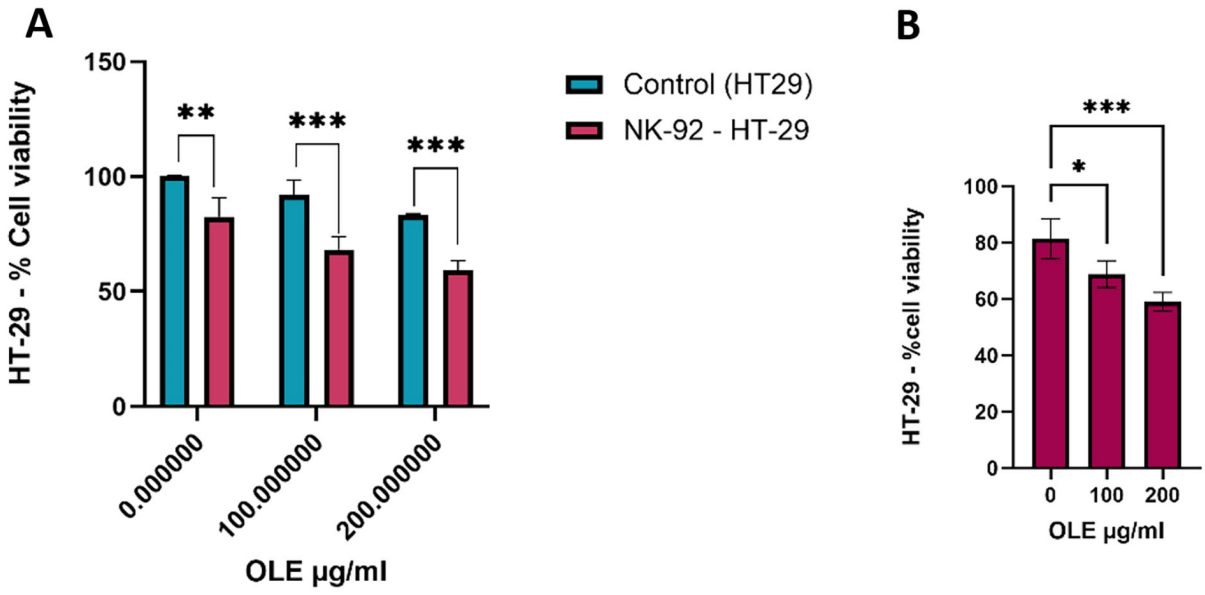

Figure 3. Effect of olive leaf extract (OLE) on NK-92-mediated cytotoxicity against HT-29 cells in a direct co-culture system. HT-29 cell were co-cultured with NK-92 cells at an effector-to-target ratio of 2.5:1 and treated with non-toxic concentrations of OLE (100 and 200 µg/mL) for 48 h. Cell viability was assessed using the MTT assay. **A.** Cytotoxicity of NK-92 in the presence of OLE compared with HT-29 cells treated with OLE alone at the corresponding concentrations. **B.** Cytotoxicity of NK-92 in the presence of OLE compared with the untreated NK-92/HT-29 co-culture. Data are presented as the mean± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

To investigate the potential mechanism underlying this enhanced cytotoxicity, the release of granzyme B and perforin into the culture supernatant was quantified by ELISA. Treatment with 200 µg/mL significantly increased the concentrations of both perforin and granzyme B compared with the untreated co-culture control (Figure 4A, B). These findings are consistent with enhanced activation of NK cell cytolytic effector functions following OLE treatment.

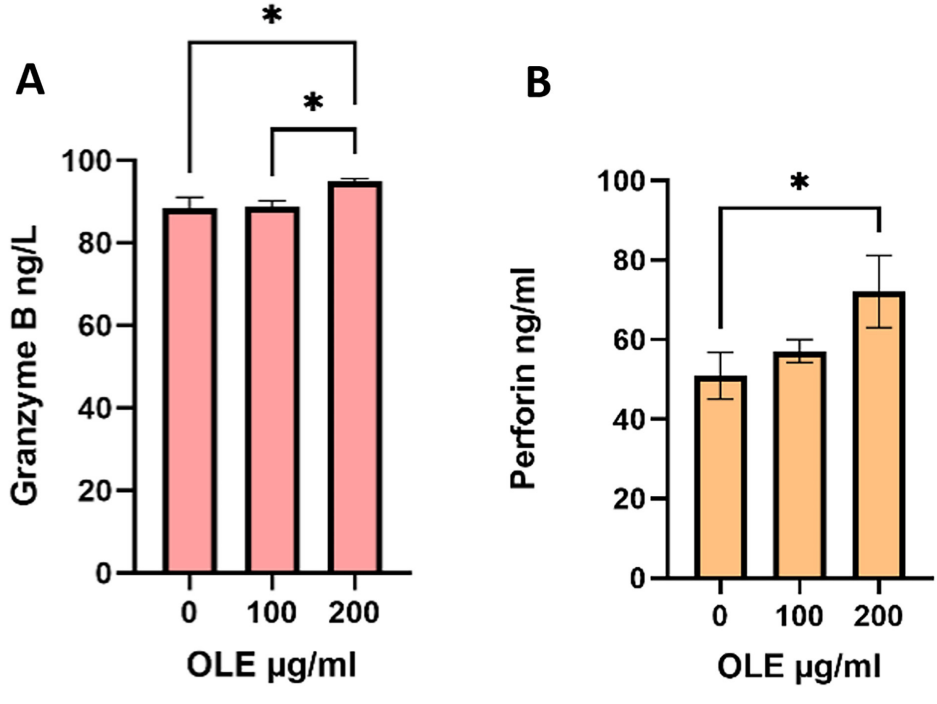

Figure 4. Effect of olive leaf extract (OLE) on the secretion of cytotoxic effector molecules in the NK-92/HT-29 co-culture system following 48 h of treatment with non-cytotoxic concentrations of OLE (100 and 200 µg/mL). **A.** Concentration of Granzyme B in culture supernatants **B.** Concentration of Perforin in culture supernatants. Data are presented as the mean ± SD of three independent experiments. *p<0.05.

DISCUSSION

The present study demonstrates that OLE enhances the cytotoxic activity of NK-92 cells against HT-29 colorectal cancer cells, particularly at non-toxic concentrations. Although OLE exerted direct cytotoxic effects on HT-29 cells, relatively high concentrations were required to achieve a 50% reduction in cell viability (IC_{50} > 470 µg/mL). In contrast, lower concentrations of OLE (100–200 µg/mL) enhanced NK-92-mediated cytotoxicity without adversely affecting the viability of either cell type. This enhanced cytotoxic response was accompanied by increased secretion of perforin and granzyme B, suggesting improved activation of NK cell cytolytic effector functions. These findings suggest that the antitumor effects of OLE in colorectal cancer may extend beyond its direct tumor cytotoxicity and include modulation of innate immune responses. Given the limited bioavailability of OLE reported in previous studies [28], enhancement of NK cell function at relatively low concentrations may represent an additional mechanism contributing to its potential therapeutic or chemopreventive effects in colorectal cancer

Consistent with previous *in vitro* studies demonstrating the pro-apoptotic and anti-proliferative effects of OLE in a variety of cancer cell lines, including cervical, gastric, prostate, and colorectal cancers, our findings confirmed that OLE reduced the viability of HT-29 colorectal cancer cells [19-21]. Several studies have attributed the anticancer activity of OLE to multiple mechanisms, including suppression of glycolytic metabolism [23] and increased mitochondrial reactive oxygen species (ROS) production, leading to oxidative stress and apoptosis [24]. Collectively, these observations provide mechanistic support for epidemiological evidence linking olive-derived products, particularly those consumed as part of the Mediterranean diet, with a reduced incidence of several cancer types [14, 35]. In the present study, we focused on the immunomodulatory effects of OLE, aiming to determine whether enhancement of NK cell function could contribute to its antitumor effect at concentrations substantially lower doses than those required for direct cytotoxicity. Our findings demonstrate that OLE enhanced NK-92-mediated cytotoxicity against HT-29 cells at non-cytotoxic concentrations, suggesting that modulation of innate immune responses may contribute to the anticancer activity of OLE even at relatively low concentrations.

Our analysis of the effect of OLE on NK-92 cell viability revealed a biphasic, dose-dependent response. The increase in ATP-derived viability observed at lower concentrations is of particular interest, as it is consistent with previous studies demonstrating the immunomodulatory properties of polyphenols. Flavonoids, which are abundant constituents of OLE, have been reported to enhance NK cell antitumor activity in vitro [5], while clinical studies have shown that OLE supplementation increases NK cell numbers [27]. In contrast, the reduction in NK-92 cell viability observed at higher concentration may be attributable to pro-oxidant effects of OLE that have been described in other cell types [24, 36]. This findings are consistent with the dose-dependent biological activity of OLE, whereby lower concentrations are associated with antioxidant, and immunomodulatory effects, whereas higher concentrations may promote oxidative stress and cytotoxicity.

Our co-culture experiments demonstrated that OLE significantly enhanced the cytotoxic activity of NK-92 cells against HT-29 colorectal cancer cells at non-cytotoxic concentrations. Mechanistically, our co-culture results demonstrate that OLE enhances NK cell-mediated cytotoxicity by upregulating key cytolytic effector molecules. The significant OLE-induced (200 µg/mL) elevation in extracellular perforin and granzyme B correlates directly with reduced HT-29 target cell viability in the co-culture system. Given that NK cells rely on these cytotoxic granules to induce target cell apoptosis [6, 37], our

data suggest that OLE sensitizes NK cells for more efficient tumor targets recognition and lysis. This finding extends the previous work of Magrone et al. [5], which focused on T-cell mediated responses, by demonstrating a direct enhancement of NK cell cytolytic machinery.

Thus, our findings reveal a novel mechanism of anticancer protection, distinct from OLE's documented ability to prevent free radical-induced DNA damage and influence hormone-related neoplasia [35]. Crucially, OLE exhibits this activity at non-toxic, low doses, a noteworthy observation given the generally low bioavailability of dietary phytochemicals.

Limitations

This study has several limitations inherent to *in vitro* experimental models. Although the use of established cell lines (NK-92 and HT-29) provides a controlled and reproducible system for investigating NK cell–tumor cell interactions, it does not fully recapitulate the complexity of the tumor microenvironment, including interactions with stromal cells, other immune cell populations, and immunosuppressive or cytokines. Furthermore, although OLE treatment was associated with increased extracellular concentrations of perforin and granzyme B and enhanced NK-92-mediated cytotoxicity, the intracellular signaling pathways through which OLE phenolics modulate NK cell effector functions were not investigated. Consequently, the molecular mechanisms underlying these immunomodulatory effects remain to be elucidated.

Another limitation of the present study is that the molecular composition of the olive leaf extract, including key phenolic constituents such as oleuropein, hydroxytyrosol, and verbascoside, was not directly characterized or quantified. Consequently, the observed biological effects cannot be attributed to specific individual compounds, and future studies incorporating comprehensive phytochemical profiling are warranted. Finally, in vivo studies are required to determine whether the low-concentration immunomodulatory effects demonstrated in vitro can be reproduced under physiological conditions and to establish the bioavailability and pharmacokinetic profiles of OLE and its bioactive metabolites.

CONCLUSIONS

In conclusion, this study demonstrates that OLE significantly enhances NK-92 cell cytotoxicity against HT-29 colorectal cancer cells at non-toxic concentrations. This enhanced anti-tumor activity was accompanied by increased extracellular concentrations of the cytolytic effector molecules perforin and granzyme B, suggesting that OLE promotes NK cell cytolytic effector function in addition to its direct cytotoxic effects on colorectal cancer cells. Notably, the ability of OLE to enhance NK cell-mediated antitumor activity at concentrations substantially lower than those required for direct cytotoxicity highlights its potential as an immunomodulatory adjuvant in cancer therapy. Collectively, these findings provide new evidence supporting the immunomodulatory properties of OLE and warrant further validation in physiologically relevant preclinical models to clarify its therapeutic potential and underlying mechanisms.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTION STATEMENT

GS and AK conceived and designed the study, analyzed the data, and drafted the manuscript. GS and AS performed experiments. All authors critically reviewed the manuscript and approved the final version.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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