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Isolation and characterization of Kumquat-derived exosome-like nanovesicles and their cytotoxic effects on HCT 116 colon cancer cells

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MAIN POINTS

- Uniform and reproducible KNVs were successfully isolated and purified using sucrose density gradient ultracentrifugation method.
- The NTA results showed that the purified KNVs appeared as homogeneous vesicles with an approximate diameter of 153.1±1.0 nm and a concentration of $6.67x10^{12}$ particles particles/mL.
- KNVs showed strong cytotoxic activity against HCT 116 cells in a concentration- and time-dependent manner

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ABSTRACT

Aim: We aimed to isolate and characterize Kumquat-derived exosome-like nanovesicles (KNVs) and evaluate their potential therapeutic effects on colon cancer.

Materials and Methods: KNVs were obtained by the ultracentrifugation method, and their purification was carried out by the sucrose density gradient ultracentrifugation method. The NTA method was used to measure the size distribution and particle concentration of KNVs. The BCA assay was utilized to determine the total protein concentrations of KNVs. MTT analysis was performed to examine the cytotoxic effects of KNVs against HCT 116 colon cancer cells.

Results: Uniform and reproducible KNVs were successfully isolated. High yield and pure KNVs were obtained in the 30% sucrose layer after sucrose density gradient ultracentrifugation. NTA results showed that KNVs sizes were 153.1±1.0 nm and particle concentrations were 6.67 × 10^{12} particles/mL. The total protein concentration of KNVs were determined as 1,79 μg/μL. Cell viability results revealed that KNVs showed strong cytotoxic activity against HCT 116 cells in a concentration- and time-dependent manner. Furthermore, at a concentration of 20 μg/mL KNVs, HCT 116 cells showed a 50% reduction in cell viability in 48 hours.

Conclusion: Consequently, our study shows that KNVs may hold promise as therapeutic candidates for the treatment of colon cancer in the future, and the research serves as a valuable resource for further research.

Keywords: Kumquat, Exosome-like nanovesicles, HCT 116, Colon cancer

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INTRODUCTION

Cancer stands out as one of the most significant health issues of the 21st century. Globally, one in every six deaths (16.8%) and one in every four deaths due to non-communicable diseases (22.8%) are attributed to cancer [1]. Colorectal cancer or colon cancer (CC) is the third most common type of cancer and the second leading cause of cancer-related deaths [2]. CC is a cancer characterized by a high mutation burden, resulting from the accumulation of somatic mutations, and it possesses a genetically complex structure. This cancer type typically progresses through a process known as the adenoma-carcinoma sequence and is closely associated with environmental and biological factors such as inflammation [3]. Standard treatment strategies for CC include surgical intervention, chemotherapy, and targeted therapies. While surgical intervention may be applied in early-stage patients, approximately 20% of patients are diagnosed with metastases, which often makes surgery unfeasible. Chemotherapy serves as the primary treatment for metastatic CC. Depending on RAS gene mutations and tumor location, adding targeted agents such as bevacizumab or cetuximab enhances the efficacy of chemotherapy. Despite these treatments, the prognosis of the

disease typically remains unfavorable [4]. Additionally, the chemotherapeutic drugs used in CC treatment often lead to severe side effects, including gastrointestinal and neurological toxicities, anemia, and dermatological issues [5,6]. These challenges emphasize the urgent need for the development of innovative treatment strategies to achieve more effective outcomes in CC therapy. Today, one of the most notable and promising approaches for treating cancer is the therapeutic use of exosomes [7].

Various cell types secrete exosomes, which are extracellular vesicles (EVs) with a size range of 30 to 150 nm. Upon their initial discovery, these vesicles were thought to be structures solely responsible for eliminating cellular waste [8]. However, it is now known that exosomes carry bioactive components such as proteins, RNA molecules and lipids, and play significant roles in various biological processes, including signal transduction, antigen presentation, and the regulation of immune responses [8-10]. These versatile functions of exosomes have enabled their exploration as a therapeutic tool in specific fields, such as CC treatment. Studies on the efficacy of mammalian exosomes in CC therapy have indicated that these exosomes may enhance treatment efficacy by targeting cancer cells [11-13]. However, the high risk of contamination during the isolation and purification processes of mammalian exosomes [14], as well as their potential to cause high levels of toxicity and immunogenicity [15,16], complicate their therapeutic use. Furthermore, it has been emphasized that the production of mammalian exosomes is limited, with variations in exosome production capacity between different cell types, and the amount of exosomes released from certain beneficial cells may be insufficient to achieve a clinical effect [17]. Additionally, the high costs, safety risks, and ethical issues associated with mammalian exosomes are among the main factors hindering their use in clinical applications. To overcome these limitations and risks, alternative sources of exosomes must be explored. Plant-derived exosome-like vesicles (PELNVs) are one of these alternatives. The ability of PELNVs' miRNAs to target mammalian genes and facilitate both intercellular and interspecies communication has drawn the attention of researchers [18]. PELNVs exhibit characteristics similar to mammalian exosomes in terms of molecular content [19] and offer significant advantages over mammalian exosomes when compared to synthetic carriers. These advantages include being non-toxic, having low immunogenicity, the ability to cross the blood-brain barrier, having good biocompatibility, and stability in the gastrointestinal tract [18, 19]. These features provide significant advantages over current drug delivery systems. A significant advantage of PELNVs, distinct from mammalian exosomes, is their possession of secondary metabolites. Flavonoids, anthocyanidins, and phenolic acids present in PELNVs endow them with strong antioxidant properties [20]. Flavonoids such as hesperidin and quercetin, abundantly found in the peel and fruit of citrus fruits, stand out not only for their antioxidant properties but also for their anti-inflammatory effects [21]. According to a study by Raimondo et al., lemon-derived EVs were found to suppress the ERK/NF-κB signaling pathways, which decreased the expression of pro-inflammatory cytokines (IL-6 and TNF-α) in macrophages [20]. Furthermore, it has been shown in the literature that PELNVs may inhibit tumor growth and induce cancer cells to undergo apoptosis. In a study conducted by Takakura et al., Citrus limon L.-derived nanovesicles were reported to exert inhibitory effects on the growth of three different CC cell lines carrying K-Ras activation mutations [22]. In another study, nanovesicles isolated from mandarin juice were loaded with siRNA molecules targeting the DDHD domain-containing protein 1 (DDHD1) gene with an efficiency of 13%, resulting in a 60% suppression of DDHD1 gene expression in SW480 cells. Consequently, cell viability in CC cells was reduced by 17-23% [23].

Fortunella margarita Swing, a member of the citrus family (Rutaceae), has recently attracted significant attention. In a recent study conducted by Vrca et al., the essential oils of kumquat were reported to exhibit cytotoxic effects on cancer cell lines (HeLa, HCT 116, U2OS) while demonstrating low toxicity in healthy cells. Additionally, antibacterial activity against S. aureus and E. coli, along with antioxidant properties, was highlighted [24]. Although the anticancer properties of kumquat have been documented in the literature, no data regarding the PELNVs of Kumquat have been reported. In this study, we aimed to isolate and characterize the PELNVs derived from the Kumquat fruits (KNVs) and to evaluate their potential therapeutic effects against the HCT 116 CC cell line by cytotoxicity assay *in vitro*.

MATERIALS AND METHODS

Plant material and isolation of KNVs

Fresh (Kumquat) fruits (1 kg) obtained from a private farmer in Antalya-Türkiye, were kept at -80 C until the isolation procedure. Before the KNVs isolation, kumquat fruits were carefully washed 3 times with sterile distilled water. Subsequently, the exocarp and mesocarp of the kumquat were peeled, the seeds were removed and the endocarp was taken. A blender was used to chop Kumquat endocarp in sterile phosphate buffered saline (PBS) solution at high speed for two minutes. Plant cell wall residues were then removed by filtering the homogenate through a 100 μm nylon mesh. The filtrate was subjected to centrifugation at 300 × g for 15 min, 2.000 × g for 30 min at 4 C to remove large particles and cellular debris and then underwent centrifugation at 20.000 g for 30 min at 4 C to eliminate microparticles. Subsequently, the supernatant was collected and ultracentrifuged at 100.000 × g for 3 h at 4 C using an ultracentrifuge (Optima XPN-100, Beckman Coulter, Brea CA, USA). The obtained pellets were resuspended in 5 mL sterile PBS and filtered at 0.22 μm pore filter. After isolation, KNVs were stored at −80 C. No more than 1 freeze–thaw cycle was used during experiments.

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KNVs purification using sucrose gradient centrifugation

In order to purify KNVs, their suspension was loaded on top of a discontinuously dispersed sucrose gradient solution (8%, 30%, 45%, and 60%, w/v) and then ultracentrifuged at 120.000 × g for 2 h at 4 C using an SW 32 rotor. KNVs were collected from the 30% sucrose layer and diluted with 30 mL PBS. Purified KNVs were ultracentrifuged at 100,000×g for 1 h at 4 C, and then the pellet was resuspended in 1 mL of sterile PBS. Purified KNVs were stored at −80 C until use.

Nanoparticle tracking analysis (NTA)

Quantification and size distributions of KNVs were determined using Nanoparticle Tracking Analysis (NTA) with a 488 nm laser and a high-sensitivity CMOS camera. The isolated KNVs were analyzed utilizing NanoSight NS300 (Malvern Instruments). The NTA method measured the size and concentration of particles in liquid suspension using light scattering and Brownian motion. The tracking algorithm utilized each particle's movement in Brownian motion to estimate the diffusion coefficient (Dt). Using the diffusion coefficient, the Stokes-Einstein equation calculated the particle diameter [25]. The samples were put into the device's vessel after being diluted with sterile-filtered PBS, and the size dispersion was determined using the NTA method. The test was carried out using the NTA 3.3.301 software, and the video recorded the nanoparticles at least five times at 60-second intervals.

Bicinchoninic acid assay (BCA)

The BCA protein assay kit (Thermo Fisher Scientific, 23225) was used to measure the total protein concentrations of KNVs. Briefly, 25 µl of standards were added to the 96-well plate at increasing concentrations. KNVs were diluted in ddH_2O and 25 µl of exosome mixture was added to each well. Solutions A and B in the kit were mixed at a ratio of 1:50 and 175 µl mixture was added to the standards and KNV samples. After that, the plate was incubated for half an hour at room temperature (RT). Multiskan GO spectrophotometer (Thermo Fisher Scientific) was used to make measurements at a wavelength of 562 nm. The standard curve graph was used to calculate the total protein concentrations of the obtained KNVs. Each experiment was performed in 3 replicates.

Cell culture

The human HCT 116 CC cell line was purchased from the American Type Culture Collection (ATCC, CCL-247) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biosera, USA). Medium was supplemented with 10% fetal bovine serum (FBS, Gibco),100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) and 2 mM L-Glutamine (Hyclone). The cells' morphology and growth were assessed every day under an inverted microscope. HCT 116 CC cells were cultivated at 37°C with 5% CO_2 in a humidified incubator and were routinely checked for mycoplasma contamination [26]. For the KNV treatment, DMEM was supplemented with 10% exosome-depleted FBS.

Cell viability assay

The cytotoxic effects of KNVs on HCT 116 cell line was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, AR1156, Boster) assay. Cells were seeded at $1x10^4$ cells/well density into 96-well cell culture plates. Following overnight incubation, HCT 116 CC cells were washed and treated with different concentrations of KNVs (0 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL) for 24 h or 48 h. Then, 100 µL of fresh culture media with 10 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Subsequently, the supernatants were discarded and the remaining formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO, P60-36720100, PAN-Biotech). MTT analysis was carried out three times in triplicate. Optical density (OD) values were measured at a wavelength of 570 nm using a Multiskan GO spectrophotometer, and the percentage of viable cells was calculated as: Type% % viable cells (OD treatments/OD untreated control) × 100.

Statistical analysis

The data were statistically compared by PRISM v7 (GraphPad, w) software. First, the Shapiro-Wilk test was used to determine the normalization of data. Two-way RM ANOVA and Sidak's multiple comparisons test were used for statistical analysis. Data are given as mean ± standart deviation (SD) and $p<0.05$ values considered statistically significant. Each analysis was carried out three times in triplicate.

RESULTS

Isolation of KNVs

KNVs were isolated from fresh Kumquat fruits (Figure 1A) using a differential centrifugation method. Large particles and debris were disposed of following a series of low-velocity centrifugation processes. Subsequently, the collected supernatants were ultracentrifuged at 100.000 × g. KNVs were obtained in the form of orange pellets found at the bottom of the ultracentrifuge tubes (Figure 1B). The KNV pellets were dissolved in sterile PBS and subsequently filtered with a 0.22 µm pore filter. KNVs were purified with sucrose density gradient ultracentrifugation. KNVs were obtained in the 30% sucrose layer (Figure 1C).

Characterization of KNVs

Characterization of KNVs, including size distribution and particle concentration, was performed using NTA via particle-by-particle inspection. As a result of NTA, it was determined that KNVs purified by the sucrose density gradient ultracentrifugation method appeared as homogeneous vesicles, had a diameter of approximately 153.1±1.0 nm, and had a concentration of 6.67×10^{12} particles/mL (Figure

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Table 1. Statistical analysis of MTT cell viability assay results. Data are given as mean±SD.

	0 μg/mL/Control	2.5 μg/mL	5 μg/mL	10 μg/mL	20 μg/mL	40 μg/mL
24 h	99.69±1.65	95.01±1.32 p=0.76	85.65±4.29* p<0.001	72.45±2.12* p<0.001	60.92±3.08* p<0.001	34.15±4.53* p<0.001
48 h	99.40±2.76	89.30±2.72* p<0.001	77.83±3.10* p<0.001	60.40±4.66* p<0.001	46.51±3.38* p<0.001	15.68±2.31* p<0.001

**p values <0.05 were considered significant compared with 0 μg/mL.*

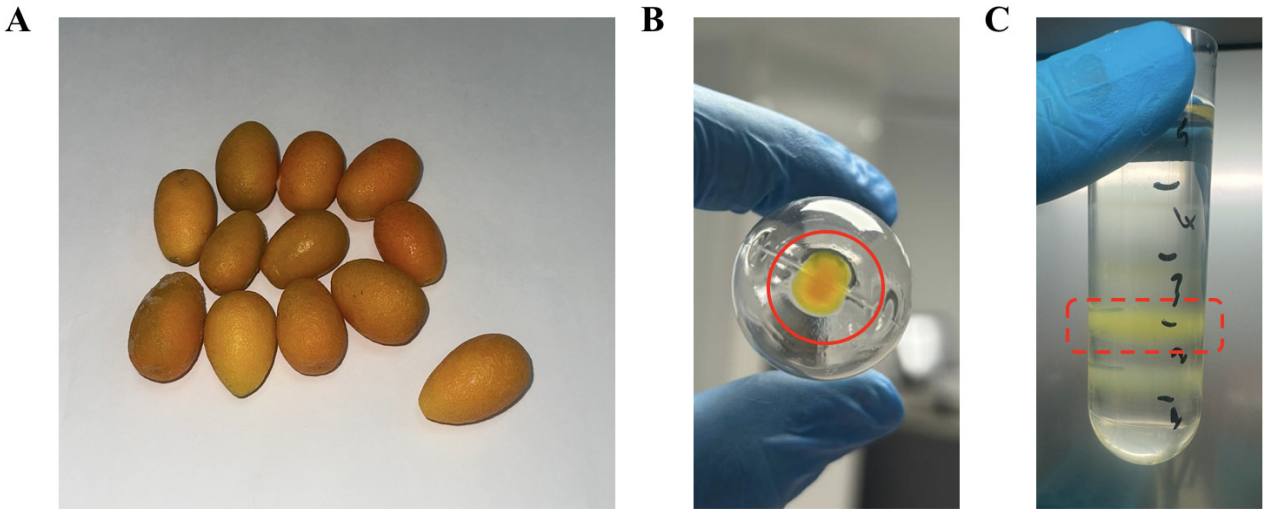

Figure 1. Isolation of KNVs. Kumquat fruits (A); KNVs pellet after 100.000 × g ultracentrifugation (B); Purified KNVs after sucrose gradient (8/30/45/60%, w/v) ultracentrifugation (C).

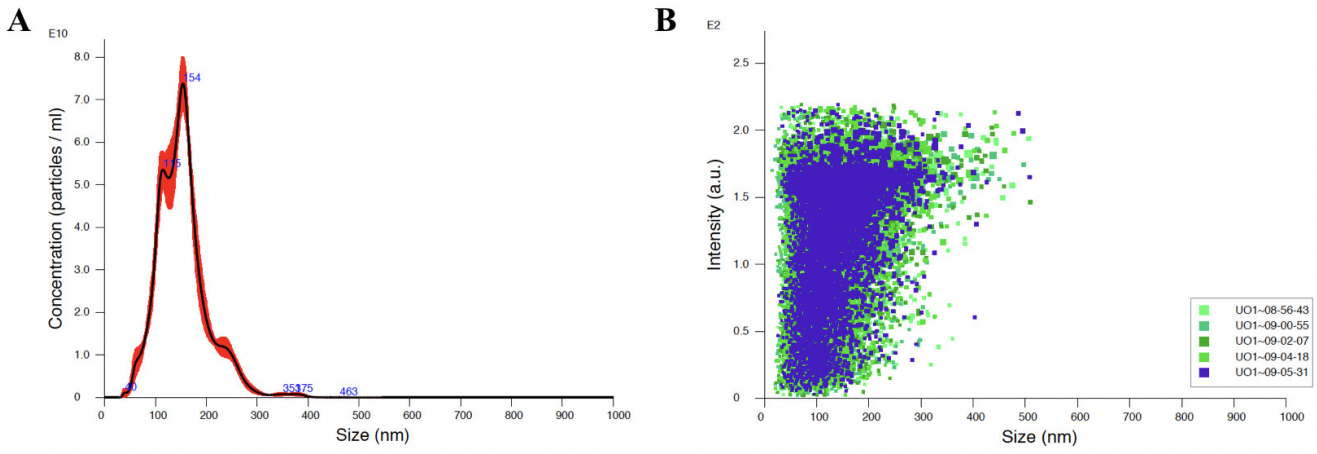

Figure 2. Characterization of KNVs. NTA results showing the size distribution (A) and intensity (B) of KNVs.

2A and 2B). The quantity of KNVs was estimated based on protein concentration using the BCA protein analysis kit. The total protein concentration of KNVs were determined as 1.79 μg/μL.

Cell viability results

We performed an MTT assay to detect the effects of KVNs on the viability and proliferation of HCT 116 cells. For this purpose, we treated HCT 116 CC cells with increasing concentrations of KNVs (2.5, 5, 10, 20, and 40 μg/mL) for durations of 24 and 48 hours, followed by the administration of MTT reagent. According to our findings, KNV's treatment caused concentration- and time-dependent cytotoxicity in HCT 116 cells. It was found that 40 μg/mL KNVs concentration reduced HCT 116 cell viability to 34% and 15%

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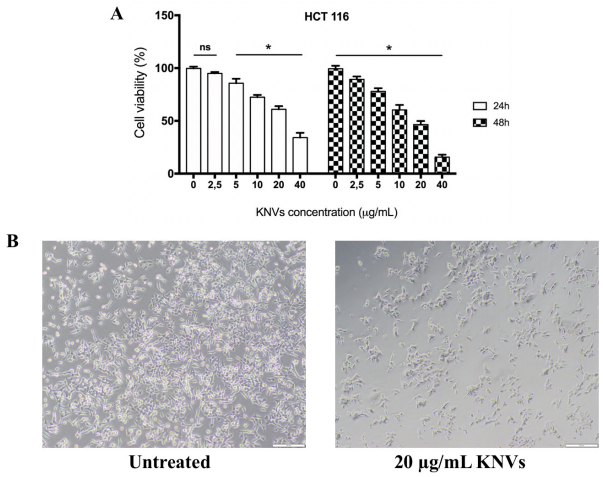

Figure 3. The effect of KNVs on the viability and proliferation of HCT 116 cells. The cell viability was detected using the MTT assay (A), Microscopic images of untreated and 20 µg/mL KNV treated groups (B). The data represent mean ± standard deviation (n = 3). * indicates statistical significance of differences in cell viability treated with KNVs compared to untreated cell viability ($p < 0.05$, Two-way RM ANOVA).

at 24 and 48 hours, respectively, while 20 µg/mL KNVs concentration reduced cell viability to 61% and 46% compared to the untreated group (Figure 3A, Table 1). Therefore, when HCT 116 cells were co-cultured for 48 hours with a concentration of 20 µg/mL KNVs, which caused approximately 50% cell viability, increased cytotoxic and cytopathic activity was observed in the cells under an inverted microscope (Figure 3B).

DISCUSSION

Exosomes are a subpopulation of EVs, which are nanoscale lipid bilayer particles secreted from living cells into the surrounding environment, playing critical roles in intercellular communication, tumor metastasis and signal transduction [27]. Exosomes derived from mammalian cells have recently gained widespread use in various biomedical fields, including tissue reconstruction, drug delivery, and diagnosis [28]. Despite the great therapeutic nanoplatform potential of these exosomes, some major problems limit their clinical applications, including low production efficiency, time-consuming and laborious manufacturing procedures, and difficulties in obtaining high-quality and homogeneous exosomes [28-30]. It's exciting to note that plant cells also release exosome-like vesicles, which are mass-produced, eco-friendly, economical, and biosafe nanoplatforms [28, 30, 31]. PELNVs are identical in morphology and functionality to their mammalian analogues, and their inability to be detected by the immune system prolongs their circulation time in the blood. Additionally, unlike their mammalian counterparts, they do not harbor zoonotic or human pathogens [32, 33].

Ultracentrifugation is the most widely used technique for purifying exosomes and has long been considered the gold standard for isolating exosomes of relatively homogeneous sizes [33, 34]. Recent studies on the biological activities of PELNVs have largely used the 30%–45% interphase of sucrose layers [33]. In our study, relatively homogeneous and stable exosome-like nanovesicles were successfully isolated from the 30% sucrose layer and used in the study.

NTA is a real-time imaging technique that can quickly detect the size and concentration of exosomes and is frequently used for exosome characterization [35]. The spectrum of the size of exosome-like nanovesicles obtained from plants varies between 50 and 500 nm among plant species and even within the same species [30, 36]. NTA results showed that the mean size of KNVs was 153.1±1.0 nm, and the nanoparticle concentration was measured as 6.67×10^{12} particles/mL. According to the BCA results of KNVs, the total protein concentration was determined as 1.79 µg/µL. When the total protein amounts obtained with BCA were compared with the particle concentrations measured with NTA, it was determined that purified and high-yield KNVs were obtained, and these amounts were sufficient for our study.

There is increasing evidence that reveals the regulatory roles of PELNVs in critical processes of the organisms, such as metabolism, inflammation, homeostasis, and tumorigenesis [28]. Significantly, these platforms have been used to treat a variety of inflammatory and malignant diseases because they contain versatile bioactive substances (polyphenols, functional proteins, and flavones). Over the last decade, a large number of natural and green nanovesicles have been successfully isolated from edible plants [28, 30]. PELNVs obtained from ginger, lemon, grapefruit, grape, and Chinese bamboo shoots are reported to have anticancer properties and have anti-proliferative effects both in vivo and in vitro. Tea flower-derived PELNVs have been reported to accumulate in breast tumors and their lung metastatic sites after intravenous injection or oral administration, inhibit the growth and spread of breast cancer, and regulate the gut microbiome [37]. You et al. reported that PELNVs derived from cabbage and red cabbage loaded with the chemotherapy drug doxorubicin (DOX) killed SW480 CC cells. They also showed that by loading miR-184 into cabbage PELNVs, nucleic acids can be efficiently transported by PELNVs [38]. Raimondo et al. reported that PELNVs derived from lemon juice inhibited Acetyl-CoA Carboxylase 1, thereby suppressing the growth of CC cells [39]. Furthermore, in another study, lemon exosomes were shown to increase the mRNA levels of the pro-apoptotic molecules Bad and Bax while decreasing the levels of the pro-survival molecules Survivin and Bcl-XL. It was also noted that lemon exosomes exhibited anticancer properties by inducing TRAIL-mediated apoptosis in various tumor cell lines [40]. With this study, we aimed to investigate, for the first time in literature, the cytotoxic effects of kumquat-derived nanovesicles on HCT 116 CC cell line. According to the MTT results, 20 µg/mL KNVs concentration was found to reduce HCT 116 cell viability to 61% and 46% at 24 and 48 hours, respectively. Researchers have shown in numerous

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studies that PELNVs trigger the anticancer mechanism, particularly by inducing apoptosis in tumor cells in vitro. The results of our cytotoxicity analysis are also consistent with the literature and suggest that KNVs may exert the cytotoxic effect most likely through apoptosis.

Plants have been used for thousands of years to treat many diseases due to their therapeutic properties. The fact that plants produce thousands of different secondary compounds with therapeutic properties in their leaves, roots, seeds, and flower buds is the reason why plants have been used for therapeutic purposes for so long. For this reason, PELNVs obtained from different plant species are being tested in cancer treatment, and the number of studies in this field is increasing day by day. In our study, uniform and reproducible KNVs were successfully isolated with high yield and purity by sucrose density gradient ultracentrifugation. Our preliminary results indicate that KNV treatment induces concentration- and time-dependent cytotoxicity in HCT 116 CC cells.

Limitations

There are limitations of our study. We used a single cell line in our research to test our KNVs. Using different colon cancer cell lines will be better representative of the groups and will hence provide more accurate results. Also, the inclusion of normal colon cells will reveal the level of safety and biocompatibility of KNVs.

CONCLUSION

In conclusion, in terms of therapeutic applications, we consider KNVs as a potential nanomedical drug with potential in CC treatment as a single agent or in combination with other drugs. Our study is a preliminary step for further detailed analyses and sheds light on the mechanism of action of KNVs and their biosafety and biodistribution analyses in both in vitro and in vivo models.

Ethics Committee Approval: It is a study that does not require ethics committee approval.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there are no financial or personal relationships that could be perceived as potential conflicts of interest influencing the research reported in this article.

Author Contributions: M.Ö: Conception, Design, Supervision, Materials, Analysis and/or Interpretation, Critical Review; H.B.Ö: Conception, Materials, Critical Review; B.Ş.H: Conception, Writing, Critical Review; H.Ö: Conception, Design, Materials, Data Collection and/or Processing, Literature Review, Writing, Critical Review; R.B.Ç. Data Collection and/or Processing, Analysis and/or Interpretation, Critical Review; S.G: Data Collection and/or Processing, Analysis and/or Interpretation, Critical Review.

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