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Coffee bean-derived Extracellular Vesicles exhibit anti-cancer effects on neuroblastoma cells

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Abstract

While most studies on the anti-carcinogenic effects of coffee have focused on coffee extracts or individual compounds such as caffeine and chlorogenic acid, the therapeutic potential of coffee-derived Extracellular Vesicles (EVs) remains largely unexplored. Plant-Derived EVs have recently emerged as natural nanocarriers rich in bioactive molecules and have shown promise as innovative cancer therapeutics due to their high biocompatibility and low toxicity. This study investigated the anti-cancer effects of EVs isolated from unroasted and roasted *Coffea arabica* beans on SH-SY5Y neuroblastoma cells. Coffee EVs were characterized by nanoparticle tracking analysis and TET8 protein expression, confirming their identity and stability. Treatment with both unroasted and roasted coffee EVs significantly reduced neuroblastoma cell viability, as demonstrated by MTT assays. Further analysis using DAPI staining and flow cytometry found that both types of EVs induced apoptosis, with roasted coffee EVs exhibiting a stronger pro-apoptotic effect, particularly in promoting late-stage apoptosis. This is the first study to comparatively evaluate the anti-cancer effects of EVs derived from unroasted and roasted coffee beans. The findings provide evidence that coffee-derived EVs can induce apoptosis in neuroblastoma cancer cells and suggest that roasting enhances their therapeutic properties, potentially through the enrichment of secondary metabolites and melanoidins. The results highlight the potential of coffee EVs not only as natural anti-cancer agents but also as biocompatible delivery vehicles for combinatorial cancer therapies. Further investigation that elucidates the underlying molecular mechanisms and measures their effects across other cancer cells is needed to realize this potential.

Keywords

Coffee, Exosomes, Extracellular Vesicles, Plant derived Extracellular Vesicles, Cancer, Neuroblastoma, Cell viability, Apoptosis, Anticancer

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1. Introduction

1.1 Plant-Derived Extracellular Vesicles

Coffee consumption is ubiquitous, and the age of coffee drinking has been declining worldwide. Drinking coffee has often been discouraged especially for young people, due to its association with adverse effects, mainly attributed to caffeine. Despite this "bad reputation" related to coffee, a growing body of evidence demonstrates that coffee's health benefits offset its potential negative effects. Findings from epidemiological studies and clinical research indicate that coffee consumption is significantly associated with a reduced risk and an inverse correlation for developing different chronic diseases, neurodegenerative diseases as well as many types of cancers (1-5). Among these diseases, cancer accounts for one in six deaths (16.8%) and one in four deaths (22.8%) from noncommunicable diseases (NCDs) worldwide (6). Therefore, cancer represents one of the most serious public health and economic problems of the twenty-first century. Although chemotherapy remains a widely used conventional approach in cancer treatment, its considerable side effects highlight the urgent need to develop novel alternatives for cancer therapy. There is a growing interest in harnessing less side effect-based natural resources to produce innovative therapeutic formulations as alternatives to conventional pharmaceuticals (7,8). Accordingly, the inverse correlation identified between coffee consumption and cancer development makes coffee a promising candidate for further investigation of its therapeutic effects against cancer. Supporting these cohort studies reporting that coffee consumption reduces the risk of many types of cancer, coffee extracts have demonstrated notable anti-cancer effects (9,10).

While different plant extracts have been identified to exhibit anti-cancer effects over many years, in the current decade Plant-Derived Extracellular Vesicles (PDEVs) have been highlighted for cancer therapeutic development (11). PDEVs are secreted by all plant cells, and contain lipids, RNA, proteins, active molecules, and many other biological components. PDEVs have the potential to infiltrate mammalian cells and regulate biological activities, and their internal cargoes are considered promising therapeutic agents due to their low immunogenicity, high intracellular intake, and low toxicity (12). Many different PDEVs from fruits, nuts and leaves such as Arabidopsis, blueberry, coconut, ginger and grape fruits have been reported to be effective in a range of chronic diseases, including colitis and other gut diseases, as well as cancers such as liver and colon cancer. While recent preclinical studies and clinical trials with curcumin, aloe and ginger support that the utilization of PDEVs could be an innovative approach to cancer treatment (13-17), research on coffee-derived Extracellular Vesicles (EVs) has so far been limited.

Studies on the anti-cancer effects of coffee have been mostly limited to its extract or its purified chemical constituents. Only two studies have investigated the therapeutic effects of coffee-derived EVs: Esmekaya et al. (18) reported possible neuroprotective effects of coffee EVs in Aβ (1-42)-toxicity-induced mouse hippocampal neuronal cells (HT-22), while Kantarcioglu et al. (19) demonstrated the therapeutic effects of coffee EVs on chronic liver diseases using human hepatic fibrosis stellate cells and HEP40

hepatocellular carcinoma cells, finding that coffee EVs may have anti-cancer effects.

While coffee's potential therapeutic effects were extensively studied via extracts or its individual bioactive compounds, coffee-bean derived exosomes were employed in this study as a novel approach since PDEVs contain higher concentrations of bioactive compounds compared to the extracts and thus have significant potential as cancer therapeutics (20). Additionally, neuroblastoma cancer cells were used in this study because these cells exhibit unlimited proliferation capacity, an important characteristic of cancer cells, as well as retaining the ability to differentiate into neuronal cell types on treatment with various agents. This unique capacity of neuroblastoma cells to both proliferate and differentiate makes them an ideal *in vitro* model for studies of cancer and neurodegeneration (21).

Neuroblastoma cancer cells were previously used in the investigation of the effects of coffee constituents, such as caffeine and chlorogenic acid, on the modulation of chemotherapeutic agent cytotoxicity, and on the induction of apoptosis (22, 23). They were also used in studies investigating the protective effects of coffee compounds against neurodegenerative disease models such as Parkinson's disease and Alzheimer's disease (24-26). Additionally, green and roasted coffee extracts and their main components, 5-O-caffeoylquinic acid and melanoidins, were also investigated for their anti-amyloidogenic properties in neuroblastoma cells (27).

While coffee is mostly consumed in roasted form, several studies have found that different levels of coffee roasting have distinct effects on various cancer cells (28). Based on the understanding that cellular responses may differ depending on how the coffee is processed, this study comparatively investigated the effects of EVs obtained from both unroasted (green) and roasted beans of *Coffea arabica*, one of the most widely consumed coffee types (29).

1.2 Summary

The effects of exosomes from unroasted and roasted beans on the survival of SH-SY5Y neuroblastoma cells were analyzed using the MTT (3-(4,5-diMethylThiazol-2-yl)-2,5-diphenylTetrazolium Bromide) cell viability assay. After confirming that both types of coffee EVs effectively reduced the viability of SH-SY5Y neuroblastoma cells, their capacity to induce apoptosis was analyzed via immunostaining with nuclear DAPI staining and flow cytometry analysis. The results demonstrated that treatment with both unroasted and roasted coffee EVs reduced cell viability and triggered apoptosis in SH-SY5Y neuroblastoma cancer cells. Roasted coffee EVs were more effective in inducing apoptosis than unroasted coffee EVs.

This study provides evidence that coffee EVs exhibit anti-cancer effects in neuroblastoma cancer cells. Further research into the molecular mechanisms underlying these effects could clarify the differences in apoptotic responses between unroasted and roasted coffee EVs. Additionally, examining their effects on other cancer cell types could help confirm the broader anti-cancer potential of coffee EVs.

2. Materials and Methods

2.1. Isolation and characterization of coffee bean-derived Extracellular Vesicles

Unroasted and roasted coffee Arabica beans (Arifoglu, Istanbul, Turkey) were milled and incubated in 1X Dulbecco's Phosphate-Buffered Saline (DPBS) (extracted in a two-fold volume of DPBS (liquid: solid ratio, ml/g) (Gibco Life Technologies, Carlsbad, CA, USA) for 1 hour at 4°C. The mixtures were filtered through gauze and glass filters to remove large debris and sequentially centrifuged at 300 x g for 10 minutes, followed by 3000 x g for 15 minutes at 4°C. Larger particles were removed by centrifugation at 10000 x g for 15 minutes at 4°C. Following centrifugation, the supernatants were filtered through a 0.45 μm pore size filter (Millipore, Darmstadt, Germany). Following filtration, a ~ 250 mL volume of suspension was obtained and processed further. Coffee EVs were purified and concentrated with the help of a Tangential Flow Filtration-based device for Extracellular Vesicles-Small (TFF-EVs-S) (Hansa Biomed Life Sciences, Tallinn, Estonia) (Figure 1). TFF was performed by a peristaltic pump Masterflex L/S, model 7535-04 (Avantor Inc., Radnor, PA, USA), operating with a flow velocity of 80 ml/minute. The retentate, containing EVs, was washed 3 times with 50 ml 1X PBS. Finally, the retentate was recovered in 1X PBS buffer in a final volume of 25 ml. Filtrates were collected as EV-depleted fractions and concentrated using the same method, in this case with a different filter, TFF-Easy (Hansa Biomed Life Sciences). The initial amount of filtrate was ~ 400 ml and was concentrated to 25 ml.

The particle size and surface zeta potential of the coffee EVs in aqueous solution were determined by nanoparticle tracking analysis (NTA) performed with the ZetaView PMX-120 NTA instrument (Particle Metrix, Munich, Germany) as described in Fortunato et al. (30). Briefly, the instrument was calibrated prior to the measurement with 100 nm polystyrene beads. Samples were diluted in 1X PBS to obtain 50 - 200 particles/frame count and analyzed in all 11 positions of the cell. The measurement conditions were set as follows: Camera sensitivity 85, shutter onespeed 100, high video quality (capturing 60 frames) at 30 frames/s (1 cycle), minimal area 10, maximal area 1000, and brightness 25. The number of EV particles/ml was calculated by the software. The ZetaView PMX-120 measures particle concentration and size by tracking the Brownian motion of individual particles in video frames, calculating their diffusion coefficients, and converting these into diameters using the Stokes–Einstein equation. Concentration is determined by counting detected particles within the instrument's calibrated viewing volume and adjusting for dilution, while size distribution comes from aggregating all calculated diameters into a histogram. The EVs samples were taken within the entire distribution, ~ in the range of 50-200 nm.

To further characterize the coffee EVs, the presence of TET8 (Tetraspanin 8) protein - a typical plant EV marker - expression was analyzed in the samples using ELISA. Briefly, coffee EVs were bound to high-binding plates (Hansa Biomed Life Sciences) by overnight incubation at 37 °C in a humid chamber. The next day, the unbound substrate was washed with washing buffer (0.5% Tween containing PBS; PBS-T), and wells were blocked with a solution of 3% bovine

serum albumin (BSA; Capricorn Scientific, Ebsdorfergrund, DE-HE, Germany) in 1X PBS for 2 hours at room temperature. After the wells were washed with 1X PBS-T, wells were incubated with 100 µL of 1:1000 diluted rabbit anti-TET8 primary antibody (Cat#HY1490S, PhytoAB, San Jose, CA, USA) for 2 hours at room temperature. Subsequently, the wells were rinsed with 1X PBS for 3 times. Next, the wells were incubated with 100 µL of 1:5000 diluted anti-rabbit HRP-conjugated secondary antibody (Cat#1662408, Bio-Rad Laboratories, Hercules, CA, USA) for 1 hour at room temperature, and after washing with 1X PBS-T three times, 100 µL TMB (3,3',5,5'-tetramethylbenzidine) ELISA Substrate (Cat#ab171527, Abcam, Cambridge, UK) was added to each well. After 20 minutes incubation, 100 µL sulphuric acid (H_2SO_4; Sigma-Aldrich, St. Louis, Missouri, USA) was added to each well as a stopping solution, and measurements were performed using a Varioskan LUX Multimode Microplate Reader (Thermo Fischer, Waltham, MA, USA) at 450 nm. PBS was used as a reference, and results reported the average of the expression fold to background (OD450 samples/OD450 blank). The experiment was performed in duplicate, and a Student's t-test was used for comparing expression among the two groups.

2.2. Cell culture

SH-SY5Y neuroblastoma cancer cells (RRID:CVCL_0019) were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies) supplied with 10% fetal bovine serum (FBS; Capricorn Scientific, Ebsdorfergrund, Germany) and 1% penicillin-streptomycin (Capricorn Scientific). Cell confluency was assessed visually using an inverted phase-contrast microscope (Olympus CK40; Shinjuku, Tokyo, Japan). When the percentage of the surface area covered by cells reached ~ 70% confluency, they were passaged using 2.5% trypsin (Gibco Life Technologies).

2.3. MTT cell viability assay

The cytotoxic effects of coffee EVs on SH-SY5Y neuroblastoma cancer cells were analyzed by seeding cells into 96-well plates at $30x10^3$ cells/well density in DMEM containing 10% FBS. After overnight incubation for adherence, cells were treated with different concentrations of unroasted or roasted coffee EVs (0 -$10x10^8$ particles/µl) for 24 hours. In addition, the cells were treated with EV-depleted fractions as controls. Since there were no particles to account for in the EV-depleted fractions, the volumes to be added were set equal to the volume used for the highest concentration ($8x10^8$) of coffee EVs. Subsequently, 10 µl of MTT ((3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; Millipore) reagent (5 mg/ml prepared with 1X PBS) was added to each well and incubated at 37°C for 4 hours. Then, formazan salts were dissolved in 200 µl DMSO (dimethyl sulfoxide), and absorbance measurements were obtained at 570 nm and 655 nm (reference filter) using a BIO-RAD Benchmark Plus microplate reader (Bio-Rad Laboratories).

2.4. DAPI staining of nuclei

SH-SY5Y neuroblastoma cancer cells were seeded on the 0.01% poly-L-lysine (Sigma-Aldrich) coated coverslips in 24-well plates at the density of $150x10^3$ cells/coverslips. After the cells were incubated overnight, they were treated with unroasted or roasted coffee EVs for 24 hours at a dose of $4x10^8$ particles/µl. Next, cells were fixed for 15 minutes using

4% paraformaldehyde and washed with 1X PBS. Then, cells were incubated with DAPI (at a 1:1000 dilution in PBS) (Invitrogen, Carlsbad, CA, USA), which was applied to the cells for 10 minutes at room temperature. After washing with 1X PBS, ProLong™ Diamond Antifade Mounting Medium (Invitrogen) was added on the slides, and coverslips were placed onto the mounting medium. A TCS SP2 SE Confocal Microscope (Leica, Germany) was used to observe prepared coverslips with a 63X oil immersion objective. All images were captured in a single focal plane with the Leica Application Suite X (LAS X) Version 3.5.1 software.

2.5. 7-AAD and Annexin-V apoptosis detection assay

Flow cytometry analysis was performed after 7-AAD (7-Aminoactinomycin D) and Annexin-V dual-staining to determine if unroasted or roasted coffee EVs induced apoptosis in SH-SY5Y neuroblastoma cancer cells. Cells were seeded into the 24-well plates at $5x10^4$ cells/well density and incubated overnight. Then, the cells were treated with unroasted or roasted coffee EVs at a density of $6x10^8$ particles/µl. After 24 hours, cells were trypsinized and centrifuged at 370 x g for 5 minutes. The cell pellets were then washed twice with 200 µl cold FACS (fluorescence-activated cell sorting) buffer (2% FBS in 1X PBS) and stained sequentially with 7-AAD (Cat# IM3422; Beckman Coulter, Brea, California, USA) and/or Alexa Fluor 647-conjugated Annexin-V (Cat#640912, BioLegend, San Diego, California, USA). For the 7-AAD staining, cell pellets were dissolved in 50 µl 7-AAD staining solution (5 µl 7-AAD and 45 µl FACS buffer) and incubated in the dark at 4℃ for 15 minutes. After incubation, 150 µl of FACS buffer was added to each tube, and cells were centrifuged at 370 x g for 5 minutes. Subsequently, cells were stained with 100 µl Annexin-V solution (1 µl Alexa Fluor 647-conjugated Annexin-V and 99 µl Annexin-V binding buffer) and incubated in the dark at 4℃ for 20 minutes. After centrifugation at 370 x g for 5 minutes, cell pellets were resuspended in 200 µl Annexin-V binding buffer (Cat#422201; BioLegend). Unstained cells were used as negative controls, while cells stained only with 7-AAD or Annexin-V were used as positive controls. Finally, cells were analyzed using flow cytometry (BD Accuri C6, Franklin Lakes, NJ, USA), and the percentage of apoptotic cells was computed utilizing the FlowJo Version 10 software. The colored dots in flow cytometry pseudocolor graphs are generated by the FlowJo software and they illustrate the relative density of cells with blue and green representing the areas of low cell density, red and orange representing the areas of high cell density, while yellow representing the areas of medium cell density.

2.6. Statistical analysis

GraphPad Prism 8.0.2 software (GraphPad Software Inc., CA, USA) was used for the statistical analyses. Two-way ANOVA was utilized for multiple group comparisons in the MTT assay, and one-way ANOVA was employed for the flow cytometry analysis. Error bars in the graphs were generated using ± standard deviations (SD). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization of coffee Extracellular Vesicles

To investigate the effects of coffee on cancer cells, EVs were isolated from the beans of

unroasted or roasted *Coffea arabica* using the protocol explained in section 2.1 and as summarized in Figure 1.

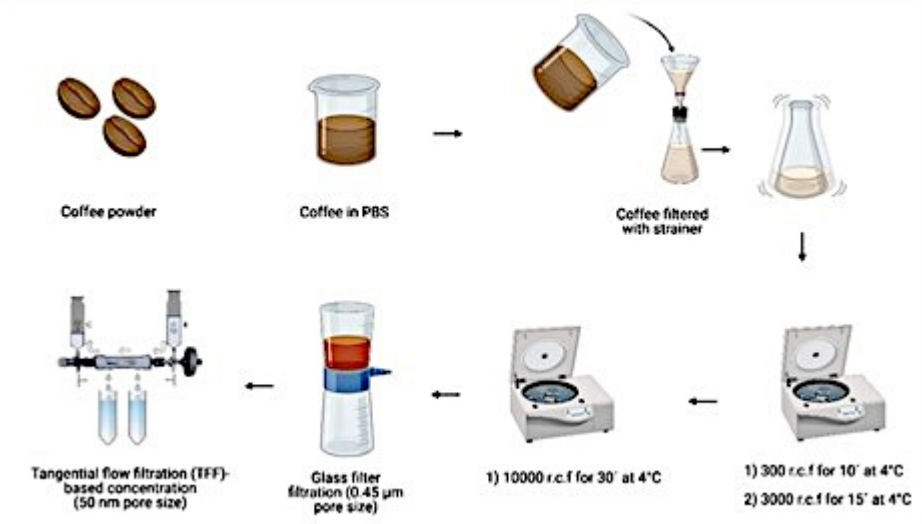

Figure 1. Schematic representation of EV isolation from coffee beans. EVs were extracted from beans of *Coffea arabica* by ultracentrifugation technique and purified using the TFF-EVs-S filters.

After isolation, the particle size distribution and the surface zeta potential of the EVs were analyzed to verify their biological functionality. The results showed that the size of the coffee EVs was in the range of 50 - 200 nm for unroasted beans (mean size 128.1 nm) and 50 -150 nm for roasted beans (mean size 102.3 nm) EVs (Figure 2A).

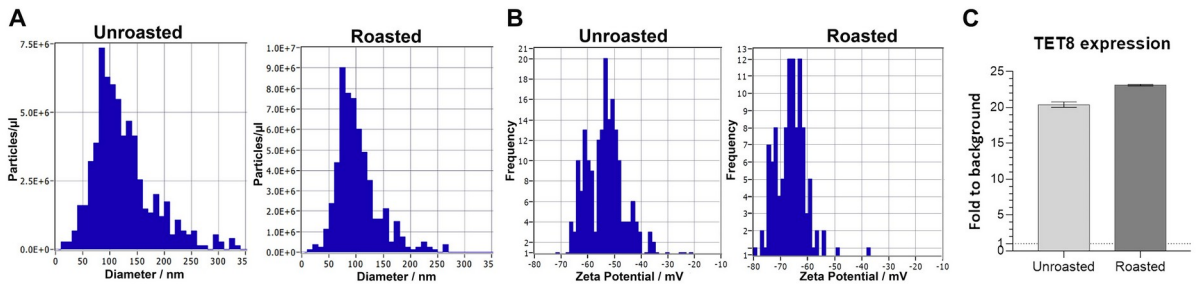

Figure 2. Characterization of unroasted and roasted coffee EVs. **(A)** Particle size and charge **(B)** distribution were analyzed with the NTA ZetaView PMX-120 NTA instrument, **(C)** TET8 expression, EV marker of plant cells, was analyzed by ELISA.

The zeta potential, an indicator of colloidal stability, was measured at approximately -60 mV for the unroasted and -65 mV for the roasted EVs (in 0.1% PBS diluent), indicating that EVs stable to coagulation were isolated (Figure 2B). Moreover, expression of TET8, a common marker of plant EVs, was also detected in the samples (Figure 2C), confirming the identity of isolated particles as being EVs from coffee beans. These findings demonstrated that the particles isolated from

both types of coffee beans were indeed plant-derived EVs (PDEVs).

3.2 Treatment with coffee EVs reduced viability of SH-SY5Y neuroblastoma cancer cells

Possible changes in the viability of SH-SY5Y neuroblastoma cancer cells upon treatment with both types of coffee EVs at a range of doses (0 to $8x10^8$ particles/µl) were analyzed by MTT assay. The results showed that treatment with $4x10^8$ particles/µl doses of each type of coffee EVs significantly reduced the viability of SH-SY5Y cells (Figure 3). Furthermore, while increasing doses of unroasted coffee EVs did not result in a significant additional decrease in cell viability compared to the $4x10^8$ particles/µl dose, a dose-dependent reduction in cell viability was observed with roasted coffee EVs in the range of 4-$8x10^8$ particles/µl (Figure 3).

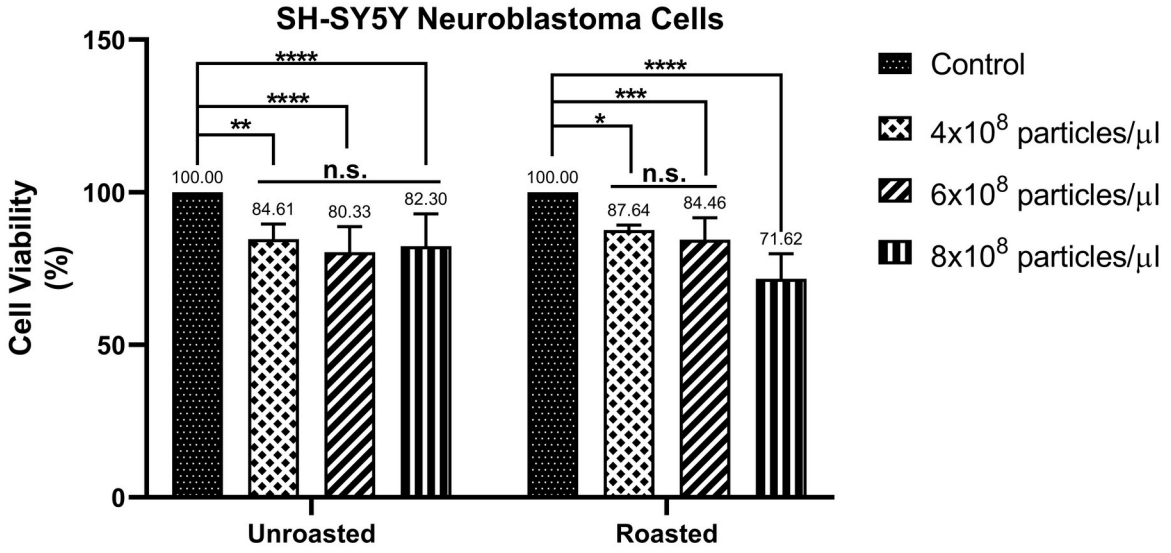

Figure 3. Investigation of the effects of coffee EVs on the viability of SH-SY5Y neuroblastoma cells. SH-SY5Y cells were seeded into a 96-well plate at $30x10^3$ cells/well density, and treated with unroasted or roasted coffee EVs at doses ranging from 0 to $8x10^8$ particles/µl. 24 hours after treatment, an MTT viability test was performed to determine the percentage viability of the cells compared to the control condition. Experiments were conducted in three biological replicates each containing five independent technical replicates. Statistical analysis was performed using a two- way ANOVA test. Data are presented as mean ± SD (Standard Deviation). n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.3. Treatment with coffee EVs triggered apoptosis in SH-SY5Y neuroblastoma cancer cells

Since the lowest dose at which both types of coffee EVs reduced the viability of SH-SY5Y cells was $4x10^8$ particles/µl, the apoptotic effects of both types of coffee EVs were investigated at this dose. For this purpose, DAPI staining was used to evaluate the nuclear morphology of SH-SY5Y cells, and nuclear shrinkage, a hallmark of apoptosis, was observed following treatment with both unroasted and roasted coffee EVs (Figure 4A). In addition, the stages of apoptosis were examined by flow cytometry using 7-AAD and Annexin-V dual staining. The results showed that treatment with both types of coffee EVs significantly increased the

percentage of both early and late apoptotic SH-SY5Y cells (Figure 4B). Furthermore, while no significant difference was observed between the two EV types in the induction of early apoptosis, roasted coffee EVs induced late apoptosis to a greater extent than the unroasted coffee EVs (Figure 4B). These findings demonstrated that treatment with both unroasted and roasted coffee EVs induced apoptosis in SH-SY5Y neuroblastoma cells; however, roasted coffee EVs appeared to promote cell death more strongly in both phases, particularly in the late apoptotic phase.

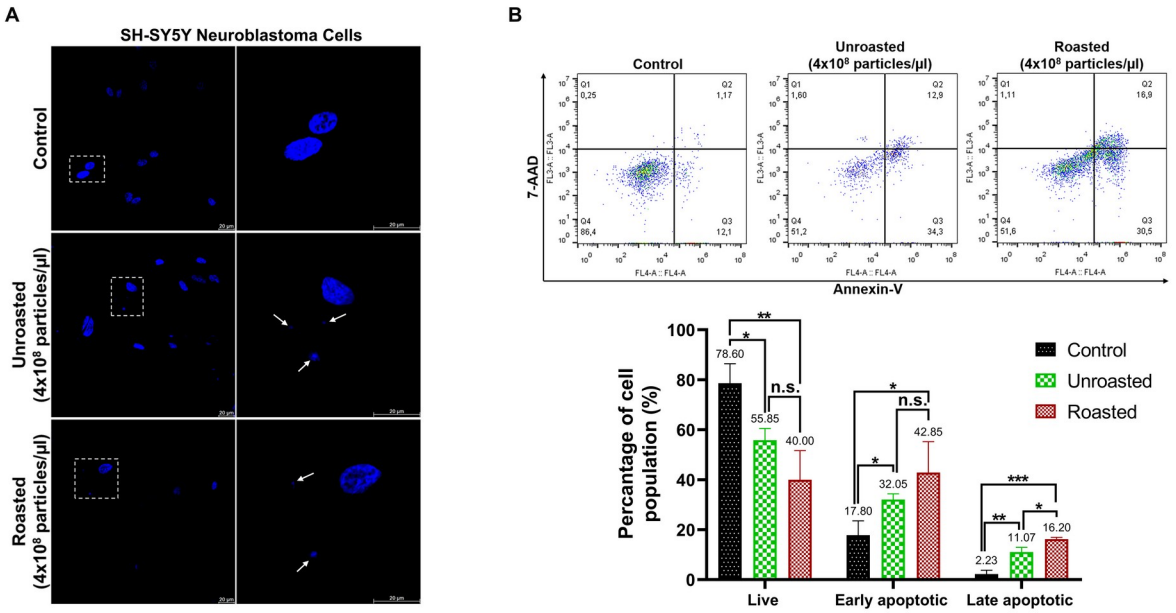

Figure 4. Investigation of the effect of coffee EV treatments in SH-SY5Y cells. **(A)** DAPI staining analysis of the effects of coffee EVs in SH-SY5Y cells. Cells were treated with unroasted and roasted coffee EVs at a dose of $4x10^8$ particles/µl for 24 hours, and nuclear morphology was analyzed using DAPI staining. Cells were visualized by TCS SP2 SE Confocal Microscope (Leica, Germany) using a 63X oil immersion. The right panel for each section shows higher magnification views of the boxed areas indicated in the left panel, and the arrows indicate apoptotic cells (scale bar: 20 µm). **(B)** Flow cytometry analysis to investigate the effects of coffee EVs on apoptosis in SH-SY5Y cells. Cells were treated with unroasted and roasted coffee EVs at a dose of $4x10^8$ particles/µl for 24 hours, and stained with 7-AAD and Annexin-V. Representative histograms obtained from flow cytometry analysis are shown on the upper panel, with the Q1, Q2, Q3, and Q4 quadrants representing cells in necrosis, late apoptosis, early apoptosis, and live cells, respectively. Experiments were performed in two biological replicates, each containing two independent technical replicates. Quantification of the percentage of live, early apoptotic, and late apoptotic cells is shown as a bar graph in the lower panel. Statistical analysis was performed by a one-way ANOVA test. Data are presented as mean ± SD (Standard deviation). n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4. Discussion

Coffee is one of the world's most widely consumed beverages ; consequently, the relationship between coffee consumption and cancer risk has been one of the most extensively explored topics. Several epidemiological studies have found a negative correlation between coffee consumption and cancer risk (31-33). Although coffee consumption is known to reduce the incidence of various types of cancer, research on the effectiveness of

coffee itself in the prevention or treatment of cancer remains quite limited. Instead, studies have mostly focused on the benefits of specific compounds found in coffee such as caffeic acid and caffeine, both of which are abundant in coffee (34, 35).

To date, most studies have evaluated individual active constituents of coffee to investigate its effects on cancer and neurodegenerative diseases. While these studies are informative, they have only examined isolated chemical components rather than the complete biological content. In contrast, EVs carry diverse biological components resembling their parent cells and plant-derived EVs (PDEVs) are more effective, stable, and targeted toward delivering the beneficial compounds than plant extracts traditionally used. Active ingredients in PDEVs are present at significantly higher levels than in extract forms, and they are biocompatible, because they are natural lipid-bound vesicles, similar to the structure of the cell membrane (36, 37).

Therefore, in this study, to examine the anti-cancer effects of coffee components, EVs containing biomolecules such as proteins, lipids, and RNA and other bioactive compounds (38), derived from *Coffea arabica* beans were investigated in SH-SY5Y neuroblastoma cancer cells. Since previous studies have shown that cellular responses may differ depending on how coffee is processed (28), EVs obtained from both unroasted and roasted coffee beans were used to analyze their effects on cancer cells.

First, the effects of unroasted and roasted coffee EVs on the viability of SH-SY5Y neuroblastoma cancer cells were investigated. It was found that both types of EVs, at a dose of $4x10^8$ particles/µl, reduced cell viability (Figure 3) and produced apoptotic bodies (Figure 4A) in SH-SY5Y neuroblastoma cancer cells. Furthermore, flow cytometry analysis found that treatment with both types of EVs induced early and late apoptosis (Figure 4B). However, roasted coffee EVs were more effective at inducing late apoptosis (Figure 4B, lower panel). Additionally, the higher number of cells in the Q2 and Q3 quadrants (representing late and early apoptosis, respectively) in both the unroasted and roasted EV panels compared to the corresponding quadrants of the control panel (Figure 4B) indicated that coffee EVs showed greater apoptotic effects than the EV-depleted fraction, which would contain the soluble compounds of coffee.

Overall, these findings revealed that both types of coffee EVs induced apoptosis in SH-SY5Y neuroblastoma cancer cells, and that EVs derived from roasted coffee beans were more effective in inducing apoptosis within a shorter period of time. This suggests that specific components which were either formed or increased during the roasting of coffee beans could enhance the effectiveness of EVs. For instance, particular phenolic acids, such as caffeic acid found in coffee, are known to have a variety of biological effects, including pro-apoptotic properties (39). While some secondary metabolites of coffee are known to be enriched during roasting (40), recent evidence indicates that secondary metabolites can also encapsulated in plant Extracellular Vesicles. Several metabolites have already been reported in PDEVs from important plant sources (41-43). Therefore, it is plausible that roasting not only enhances secondary metabolite levels but also promotes their encapsulation in coffee EVs, since PDEVs are known to contain higher

concentrations of bioactive compounds compared to their extract counterparts (20). In addition, melanoidins, which are formed as a result of the roasting process, have been shown to suppress the activity of several matrix metalloproteinases (MMP-1, MMP-2, and MMP-9) (44, 45). Since these MMP enzymes play active roles in tumor growth and metastasis (46), it is reasonable to predict that EVs obtained from roasted beans may lead to increased tumor cell death.

Furthermore, while treatment with roasted coffee EVs was found to be more effective, administration of unroasted coffee EVs also resulted in apoptotic cell death in SH-SY5Y neuroblastoma cancer cells. Since biological components, such as protein and RNA are present in EVs derived from unroasted coffee, the molecular mechanism of apoptotic cell death observed in cancer cells following their administration could be attributable to the influence of these components, as shown in a study investigating the anti-cancer effect of plant-derived miRNAs on breast cancer (47). In addition, a study on the impact of roasted coffee exosomes on hepatocellular carcinoma found that miRNAs in roasted coffee play an active role in preventing cell proliferation (19). Given the findings in this study, it is possible to attribute the apoptotic cell death observed in cancer cells with roasted coffee EVs not only to the increased phenolic acids but also to the cumulative effect of the continued activity of biological components that remain during roasting and are naturally present in unroasted coffee EVs.

Although chemotherapy is an important treatment modality for cancer malignancies, it is limited by significant toxicity, side effects and susceptibility to numerous drug interactions. While the interacting effects with medications are well known, there is limited evidence on the interaction with commonly consumed food and natural products. Among chemical components of coffee, caffeine, the most abundant compound, has been shown to modulate the cytotoxicity of chemotherapeutic agents such as doxorubicin, gemcitabine, and paclitaxel in (23). PDEVs exhibit anti-cancer effects not only via the substances they contain but also because most PDEVs are edible and could potentially cross intestinal barriers finding their way into the blood, allowing them to be used as carriers in combinatorial approaches to deliver specific anti-cancer drugs without added toxicity or side effects. PDEVs have the potential for large-scale production, and due to their intrinsic characteristics and ability to carry other compounds, such as drugs or small RNA (sRNA) molecules, they offer significant potential for exploring the mechanisms of action in disease treatment, making them invaluable for both basic and clinical research (48).

Plant-based bioactive compounds, such as polyphenols, flavonoids, and alkaloids, found in plant extracts are limited by poor oral bioavailability, rapid degradation in the gastrointestinal tract, and low intestinal permeability, resulting in inadequate systemic concentrations for therapeutic effects. In contrast, Plant-Derived Extracellular Vesicles present a novel solution, acting as biocompatible, stable, and non-immunogenic carriers that encapsulate these molecules, shield them from harsh digestive environments and enzymatic degradation, and enhance their targeted cellular uptake (49). Thus, PDEVs may hold significant advantages over traditional plant extracts.

Currently, clinical trials are ongoing with EVs derived from curcumin, aloe and ginger plants for cancer (16, 17). Based on the literature indicating the protective effects of coffee consumption against cancer and reported anti-cancer effects of coffee in this study, coffee EVs could be promising candidates for clinical trials in cancer treatment.

5. Conclusion

Extracellular Vesicles derived from both unroasted and roasted coffee beans induced apoptosis in SH-SY5Y neuroblastoma cells. Roasting of coffee beans increased the apoptosis-inducing effects of derived EVs. As the first study to comparatively investigate the apoptotic effects of EVs derived from unroasted and roasted coffee beans, these results suggest that coffee EVs have anti-cancer properties and the potential to be used as both natural therapeutic agents as well as vehicles alongside conventional chemotherapeutics in combinatorial treatments. Therefore, it is important to conduct further studies to elucidate the molecular mechanisms of action of these natural biocompatible components on cancer cells.

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