

# Ultracentrifugation method for exosome isolation of HEK293 cells as a candidate model in-vitro therapy of breast cancer

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**Abstract.** Amelida RN, Mariya S, Zaelani BFD, Yuliana, Dayana H, Darusman HS. 2023. Ultracentrifugation method for exosome isolation of HEK293 cells as a candidate model in-vitro therapy of breast cancer. *Biodiversitas* 24: 5074-5079. Exosomes are secreted by all cells and found in all body fluids, providing the biological system. They have great potential in cancer immunotherapy and have different roles and targets. The biological function of an exosome relies on its bioactive activities, supported by lipids, proteins, and nucleic acids. Exosomes can be isolated by the ultracentrifugation method, widely used to produce exosomes from various sources and intended to pellet containing heterogeneous particles and the exosomes. In this study, we isolated exosomes derived from HEK293 cells using the ultracentrifugation method and applied directly in vitro to MCF-7 cells. The exosomes were added to the MCF-7 cells at various concentrations, 3.5 µg/mL, 7 µg/mL, and 14 µg/mL, evaluated using *P53* and *PCNA* markers. The study showed that the exosomes had been successfully isolated; the concentration obtained was 24.95 µg/mL and expressed exosome markers *CD9* and *CD81*. The MCF-7 cells were treated by exosome and demonstrated epithelial-like morphology. The cell population survived, but the confluency decreased at higher concentrations, as did viability. The marker of *P53* and *PCNA* expression resulted from the effect of exosome treatment. The results indicate that the exosome can potentially inhibit the growth of breast cancer cells in vitro.

**Keywords:** Exosome, HEK293, in-vitro models, MCF-7, ultracentrifugation

## INTRODUCTION

The highest percentage of cancer cases was breast cancer at 11.7%, with a mortality rate of 6.9%. The same thing happened in Indonesia data in 2020. There were 65,858 cases, the highest percentage of cancer cases, namely 16.9%, with a mortality rate of 9.6% (GLOBOCAN 2020). Cancer treatments commonly used today still have adverse effects; they can suppress normal cell growth or proliferation, cause toxicity and drug resistance, and cause long-term complications (Xu et al. 2020). This negative effect has led to a new treatment method of exosome-based cancer therapy or cancer immunotherapy that can be isolated from various cells (Shi et al. 2016). Cells communicate with each other primarily through chemical messengers in the form of extracellular vesicles (EVs). Many recent works have focused on EVs for therapeutic medicine development due to their unique structure, which means that they can be modified to contain specific proteins, genetic materials, and genetic lipids, including microRNA (miRNA), messenger RNA (mRNA), and genomic DNA (gDNA) from their progenitor cell, and other small non-coding RNAs (Bungulawa et al. 2018).

Exosomes are characterized as a single-membrane organelle with a diameter of about 40-150 nm secreted by various types of cells, including cancer cells (Mizutani et al. 2014). Exosomes can be produced from various body

fluids such as serum (Jung et al. 2020), saliva (Li et al. 2022), and urine (Pan et al. 2021). Exosomes also contain proteins and lipids that function as signaling molecules between cancer cells and surrounding cells (Li et al. 2019); they can also contain many biomolecules, including carbohydrates and nucleic acids. The protein composition within exosomes also marks the existence of disease pathologies, for example, cancer or inflammatory diseases; however, exosomes can contain several common proteins and those that participate in vesicle trafficking and formation (Schorey and Harding 2016).

The potential of exosomes in cancer immunotherapy is enormous, and exosomes can be the most effective cancer vaccine candidates and carriers of targeted antigens or drugs. Active ingredients in exosomes, such as MHC and costimulatory molecules, have been shown to contribute biologically to the anticancer immune response (Xu et al. 2020). The exosome can also be isolated from the cancer cells; the cancer-derived exosomes can be home to their parent tumors, and drug-loaded cancer exosomes can be used for targeted cancer therapies. Despite these promising results, using cancer-derived exosomes in cancer treatment should be done with awareness due to their described metastatic role in tumor progression, and exosomes derived from cancer patients might be a good substance in the fight against cancer (Li et al. 2019).

Moreover, various methods can be used to isolate Exosomes, including magnetic beads coated (Jiawei et al. 2022), differential centrifugation including the ultracentrifuge method (Coughlan et al. 2020), and commercialized exosomes isolation kit (Skottvoll et al. 2018). The ultracentrifugation method has been used to isolate exosomes from various sources, including cell cultures (Tran et al. 2019). The ultracentrifugation method is commonly used, but validation and optimization regarding the exosome isolation results must be validated. Yang et al. (2020) demonstrated that exosome samples isolated using ultracentrifugation often produce a low purity. That might be expected by several factors that influence the effectiveness of the ultracentrifugation method, including the centrifugal force used and the sample source. According to Skottvoll et al. (2018), the ultracentrifugation method has the same quality as the kit method in isolating exosomes from cell cultures. Application in microenvironments such as cell culture is very important to validate this.

Since the exosome can be produced from the cells of various sources, we isolated exosomes derived from HEK293 (ATCC CRL-1573) using the ultracentrifuge method. We applied directly in-vitro to MCF-7 cells at various concentrations. In this study, we highlight the application of exosomes as a candidate for in-vitro models on alternative therapies in breast cancer.

## MATERIALS AND METHODS

### HEK293 cell culture

HEK293 cells (ATCC CRL-1573) were grown in DMEM (Dulbecco's Modified Eagle's Medium) growth medium supplemented with 10% FBS (Fetal Bovine Serum), 100 units/L penicillin and 100 µg/mL streptomycin. The HEK293 cell densities were plated appropriately and incubated in a humidified atmosphere at 37°C, 5% CO<sub>2</sub>. Subculture was performed when the cell population reached 80% confluency (Mizutani et al. 2014).

### Exosome isolation

The supernatant from HEK293 cells was centrifuged at 750 x g for 10 minutes. The supernatant was transferred into a polyallomer tube for ultracentrifugation using a W32Ti rotor (L-80XP) at 110.000 x g for 70 minutes. Next, the supernatant was discarded, and the pellet was resuspended using 100 µL PBS and centrifuged at 1500 x g

for 90 minutes using a microcentrifuge (Bu et al. 2019). The molecular markers of exosomes *CD9* and *CD81* were validated using the Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) technique. The validated exosomes were applied in vitro to MCF-7 cells.

### MCF-7 treatment by exosomes

MCF-7 cells were cultured 5 x 10<sup>4</sup> per well on a 6-well TC plate in RPMI (Roswell Park Memorial Institute) growth medium supplemented with 10% FBS, 100 units/L penicillin, and 100 µg/mL streptomycin for 18-20 hours. Furthermore, exosomes isolated from HEK293 cells were added to MCF-7 cells with various concentrations, 3.5 µg/mL, 7 µg/mL, and 14 µg/mL. The cells were incubated at 37°C for 48 hours with 5% CO<sub>2</sub> (Faruqu et al. 2018). Cells were harvested and examined for proliferation markers *PCNA* and *P53* using the RT-qPCR.

### Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was extracted from cells using RNeasy kits (Qiagen, Hilden, Germany), while the reverse-transcribed by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) followed the manufacturer's instructions. Quantitative real-time (RT-qPCR) analysis was used to measure the expression of markers for Exosome (*CD9*, *CD81*) and proliferation (*P53*, *PCNA*). The study presented sequences of primers used in Table 1; the reactions were performed by SsoFast EvaGreen Supermix (BioRad, Hercules, CA) on the Icyler iQ5 (BioRad). Thermocycler conditions were 2 min at 98°C, followed by denaturation at 98°C on 40 cycles for 5 sec and annealing-elongation at the optimal primer annealing temperature (Table 1) for 10 sec. GAPDH was used as the internal calibrator gene; the  $\Delta\Delta Ct$  method was used to determine the relative expression (Rao et al. 2013).

### Data analysis

The cell morphology and cell viability percentage were analyzed descriptively. Data analysis was performed using the Saphiro test, and log transformation was carried out for data normalization. Subsequently, the data were returned to the original ones and presented as mean  $\pm$  SD. The analysis used the Kruskal test for non-parametric data and the Mann-Whitney U test honestly significant difference (HSD).

**Table 1.** Primers for RT-qPCR test against exosome and proliferation markers

Mark	Forwards (5'-3')	Reverse (5'-3')	Reference	Annealing temperature (°C)
<i>CD9</i>	TCTTGGTGATATTCGCCATT	TTCGAGTACGTCCTTCTTGG	Kumar et al. 2015	48
<i>CD81</i>	CTGTATCTGGAGCTGGGAGA	GAACGTCTCACATCCTTGG	Kumar et al. 2015	53
<i>P53</i>	AATCATCCATTGCTTGGGACG	TAGAGACGGCTCTTCTGCC	Darusman et al. 2022	55
<i>PCNA</i>	CTGTAGCGGCGTTGTTGC	TCGTTGATGAGGTCCTTG	www.origene.com	52
<i>GAPDH</i>	CGGATTGGTCGTATTGG	TCAAAGGTTGGAGGAGTGG	Tian et al. 2010	56

## RESULTS AND DISCUSSION

### HEK293 cell culture

HEK293 cells in this study were successfully cultured, as indicated by microscopic observations (Figure 1). Adherent cells attach to the substrate as a monolayer, fibroblast-like in morphology, with an elongated shape, and grow attached to the substrate.

### Exosome isolation by ultracentrifugation method

Supernatant from HEK293 was spined using ultracentrifugation and physically characterized by pellets that settled at the bottom of the ultracentrifuge tube; the resulting concentration was 24,95  $\mu\text{g/mL}$  derived from 32 mL supernatant (Figure 2). The supernatant pellet generated in this study was validated by exosome markers CD9 and CD81 (Figure 3).

### Exosome treatment on MCF-7 cells

The pellet, which contains exosomes derived from HEK293 cells, was added to MCF-7 cells with various concentrations, 3,5  $\mu\text{g/mL}$ , 7  $\mu\text{g/mL}$ , and 14  $\mu\text{g/mL}$ . The cell without additional exosome was performed as the negative control. The morphology and viability of MCF-7 cells were observed after 48 hours of incubation. The cell morphology showed epithelial-like, the cell population showed less confluency, and viability decreased along with increasing concentration after incubation 48 hours. The viability percentage was performed by calculating the live cells and dead cells using a hemocytometer, resulting in 73,61%, 62,75%, and 50 %, respectively, presented in Figure 5.

### Expression analysis of proliferation gene markers in MCF-7 cells

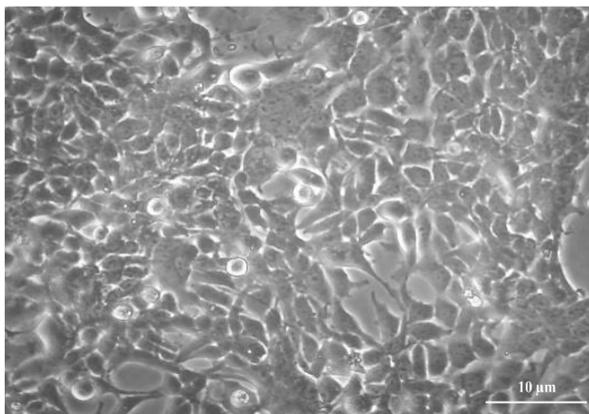
RT-qPCR performed apoptosis and proliferation potentiality of the exosome in this study on the expression of *P53* and *PCNA*. The results of this study showed that the *P53* and *PCNA* markers were expressed in all different treatments with different levels of expression (Figure 6 and Figure 7). The level of *P53* in the current study demonstrated

increased with each concentration, and the *PCNA* showed a lower expression than cell control, untreated cells without exosome.

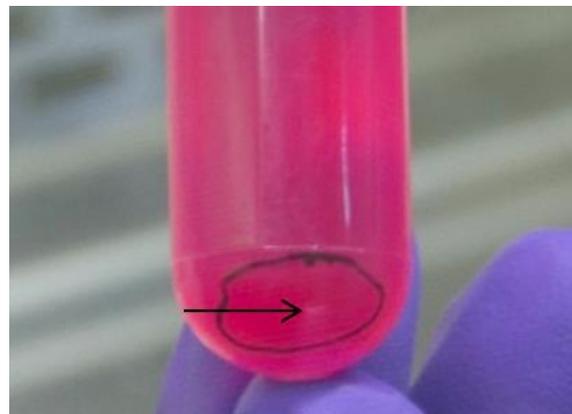
### Discussion

Research on exosomes in the field of cancer diagnosis and treatment is currently growing. The results of physiological and pathological studies have identified that exosomes are good candidates for treating and diagnosing disease, one of which is diagnosing cancer (Jafari et al. 2020). Exosomes, known as nanovesicles, play an important role in cell communication and transmission of molecules messenger, such as miRNAs and fragments of DNA molecules in the body. Exosomes have the potential as nanocarriers delivering siRNAs, miRNAs, and even chemotherapy drugs (Naseri et al. 2018). Exosomes are extracellular vesicles secreted by many cell types (Shen et al. 2018), including HEK293 cells. HEK293 cells are human embryonic kidney cells commonly used in biomedical research. HEK293 cells have high exosome productivity and can be used for biological production as therapeutic agents. The HEK293, also known as a cell, has no tumorigenic or toxic potential on other cells, whether in vitro or in vivo. However, the intrinsic effects of naïve HEK293-derived exosomes are still unclear; hence, in-depth studies are required to characterize HEK293-derived exosomes. (Kim et al. 2021).

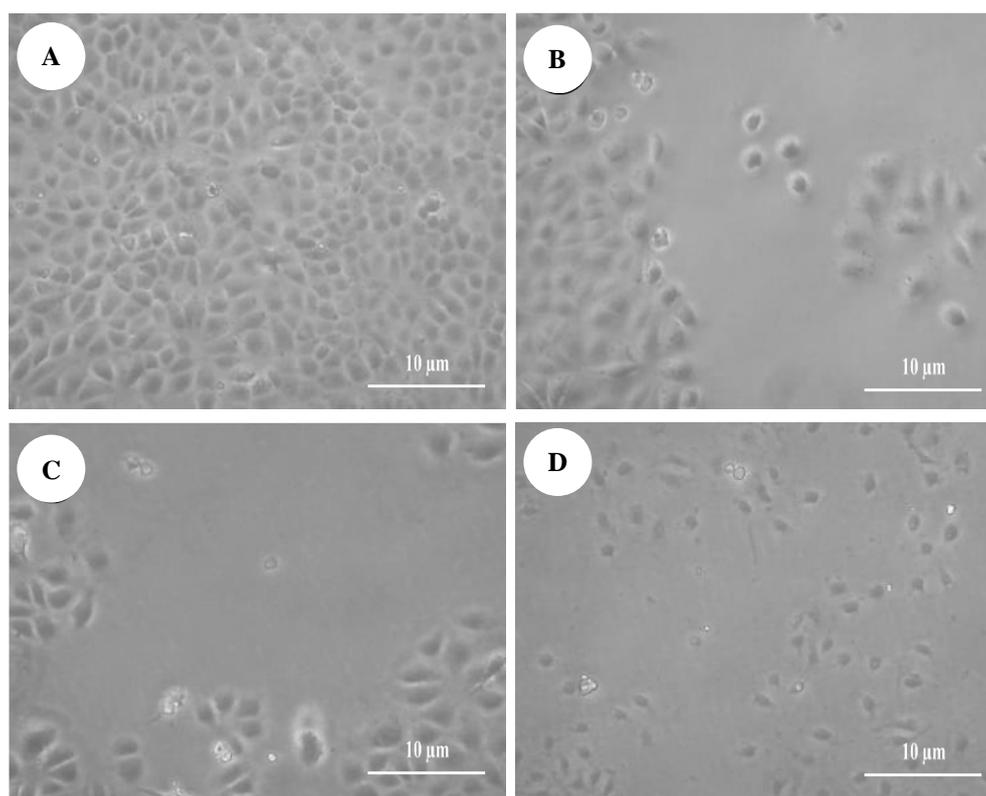
Exosomes are single-membrane organelles with a diameter of about 40-150 nm (Mizutani et al. 2014). Exosomes from HEK293 cells had a shape with spherical structure and were 107 nm in size (Faruqu et al. 2018). In the current study, we successfully isolated exosomes derived from HEK293 in a 24,95  $\mu\text{g/mL}$  concentration intended to contain heterogeneous particles. According to Faruqu et al. (2018), it takes an isolation protocol to achieve high exosome purity and yield like combining two methods. The supernatant is pre-cleared of dead cells and cellular debris by differential centrifugation. It is subjected to ultracentrifugation onto a sucrose cushion and then washed to collect the exosomes.



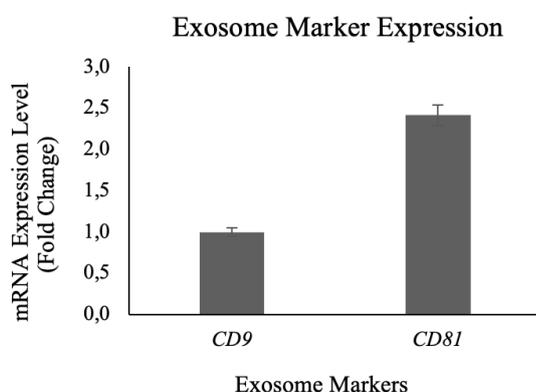
**Figure 1.** Morphology of HEK293 cells attached to the substrate with 70-80% confluency showed a homogeneous cell population with fibroblast-like morphology



**Figure 2.** The intended pellet contains heterogeneous particles, and the ultracentrifugation method produced the exosomes



**Figure 4.** Morphology of MCF-7 cells after exosome application. A. Negative control. B. 3,5 µg/mL. C. 7 µg/mL. D. 14 µg/mL



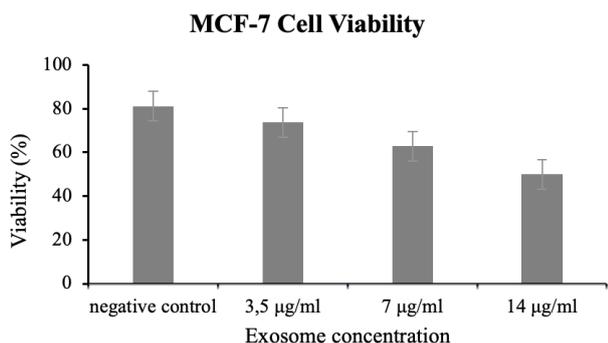
**Figure 3.** The exosome markers *CD9* and *CD81* were expressed derived from heterogeneous pellet

The potential of exosomes in clinical applications needs to be deepened; the success of exosome isolation depends on the method that influences the final results and downstream processes. According to Shtam et al. (2018), many methods for isolating exosomes must be compared and validated, including the ultracentrifugation method. The ultracentrifugation method is also used in purified exosomes derived from cell cultures. This method is the gold standard for exosome isolation, with a percentage of 80% compared to other methods.

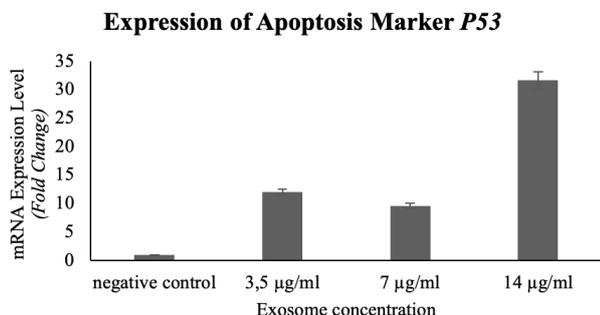
Exosome marker validation results in this study showed that the exosomes isolated from HEK293 cells

using the ultracentrifugation method were positive for CD9 and CD81 markers. The expression of the CD81 marker was demonstrated to be higher than the CD9 marker (Figure 3). That can be affected by several factors, including the selection of exosome isolation methods (Tauro et al. 2012), exosome sources (Mizutani et al. 2014), and exosome sample volume (Caradec et al. 2014). The exosomes in this study were applied in vitro to MCF-7 cells to investigate the direct effect of exosomes on the apoptosis and proliferation of MCF-7 cells.

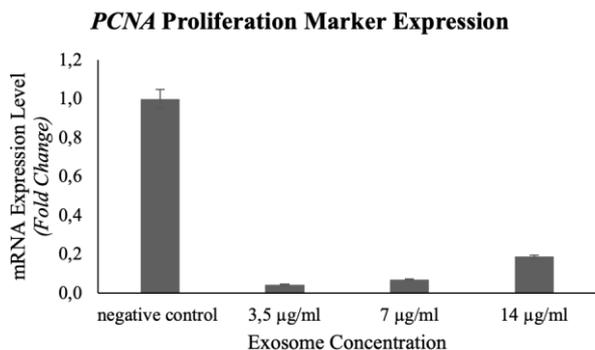
MCF-7 cells have been propagated as a commonly used breast cancer cell line for many years and are the world's most studied human breast cancer cell line. These cells were used in this study and showed epithelial-like morphology and more confluent after 48 hours of incubation without exosome treatment (Figure 4A). Treatment of exosome 14 µg/mL observed different morphology; the cells were found in smaller form and shrunk (Figure 4D). Cell viability verified the ability of a cell to survive during incubation for 48 hours. The percentage viability decreased along the concentrations of 3,5 µg/mL (73,61%), 7 µg/mL (62.75%), and 14 µg/mL (50 %) (Figure 5). Johnson et al. (2013) demonstrated that cell viability can be assessed from morphological changes or changes in the physiological state of cells. Changes in the viability percentage in MCF-7 cells could be due to apoptosis in these cells. According to Naseri et al. (2018), the viability of 4TI and TUBO cells treated with exosomes derived from mesenchymal stem cells was lower than that of the negative control without administering exosomes.



**Figure 5.** Percentage of the viability of MCF-7 cells after being treated by exosomes with various concentrations and incubation for 48 hours



**Figure 6.** As an apoptotic marker, the expression levels of the P53 marker increased with each concentration



**Figure 7.** The expression levels of the PCNA marker, as an apoptotic marker, showed lower than cell control with each concentration

P53 is a tumor suppressor that increases during DNA impairment. This gene also contributes to the G1 phase regulation of the cell cycle. It plays an important role in the cells by regulating the cell cycle process, DNA repair, apoptosis, and cell senescence (Liu et al. 2019). Figure 6 shows that exosomes derived from HEK293 cells can induce apoptosis in MCF-7 cells. The concentration of exosomes (14 µg/mL) resulted in a higher expression level of the P53 marker. According to Naseri et al. (2018),

exosomes have unique properties: they can be incorporated into target cells through culture. Exosomes can also enter target cells via endocytosis and membrane fusion pathways. The experiment done by Burdakov et al. (2018) demonstrated that the exosomes from cell lines with wild-type p53 like HEK293 suppress the growth of p53-negative cells of cancer lines. This indicates the possibility of tumor regression by exosomes that contain endogenous wild-type p53.

Proliferating Cell Nuclear Antigens (PCNA) is a marker of cell proliferation that influences cell growth (Wardhana 2019). The PCNA is an acidic nuclear protein related to DNA replication during the transition from the G1 phase to the S phase and is associated with tumor cell proliferation. PCNA is an indicator of cell proliferation; when the expression of PCNA increases, it also increases cell proliferation (Lu et al. 2017). Our findings showed that the expression level of PCNA markers in the exosome-treated cells was lower than the negative control, as in untreated cells (Figure 7). The experiment done by Lee et al. (2021) demonstrated that the tumor group treated with NK cell-derived exosomes showed reduced expression of PCNA compared to the control group. Other studies support our result; Karaoz et al. (2019) showed that WJ-MSC-derived exosomes reduce proliferation on cancer cell lines.

In this study, MCF-7 cells treated with exosomes showed apoptosis and inhibited cell proliferation at 3,5 µg/mL and 7 µg/mL concentrations, and a normal morphology was observed. These results indicate that the exosomes in this study have the potential to inhibit the proliferation of MCF-7 cells and can also be used for in vitro models in the development of breast cancer therapy.

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