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Article

CYTOTOXIC IMPACT OF OUTER MEMBRANE NANOVESICLES (OMVS) ON OVARIAN CANCER

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ABSTRACT

This study focused on extracting the outer membrane nanovesicles (OMVs) from *Escherichia coli* BE2 (*EC*- OMVs) by ultracentrifugation, and the yield was 2.3mg/ml. This was followed by purification with gel filtration chromatography using Sephadex G-150, which was 2mg/ml. The morphology and size of purified *EC*-OMVs were confirmed by transmission electron microscopy (TEM) at 40-200 nm. The nature of functional groups in the vesicle vesicle was determined by Fourier transforms infrared spectroscopy (FT-IR) analysis. The antitumor activity of *EC*-OMVs was conducted in vitro by MTT assay in human ovarian (OV33) cancer cell line at 24,48 and 96hrs. The cytotoxicity test showed high susceptibility to the vesicles in ovarian compared to normal cells with IC50 of 7.169 µg/ml at 96hrs of incubation. The current strategy is an effective and simple method to obtain *EC*-OMVs.

Keywords: ovarian cancer, lung cancer, antitumor, breast cancer, nanovesicle, MTT assay.

INTRODUCTION

Gram-negative bacteria possess two membranes surrounding the cell, the outer membrane (OM) and the inner membrane (IM), in addition to a peptidoglycan network (PG) and the periplasmic space between them. These membranes comprise phospholipids and proteins, but only OM containing lipopolysaccharide (LPS). There are Small protrusions can form from the OM and separate from it, leading to the production of extracellular vesicles, which called outer membrane vesicles [3,11,13]. OMVs range in size between 20 and 300 nm in diameter, and they are composed of a single lipid bilayer, which consists of phospholipids, LPS, and several outer membrane proteins (OMPs). There are several suggestions about the mechanism of OMV formation since the force that led to the formation of OMV was long unknown [5,7,22]. The OMV formation was long believed to be a stress response from bacteria cells (14). OMVs are acellular bacterial structures

that act as immunostimulatory agents that many reporters recognize as delivery vehicles and candidate vaccines.

Furthermore, OMVs are mainly produced and released from bacterial cells during host infection and in culture, where they play a transporter role for cargo such as DNA, RNA, autolysins, virulence factors and toxins [8,4]. OMVs help the bacterial cells to survive in environmental conditions, bacterial intercellular communication and modification of host-pathogen interactions [3,8,12,18,20]. For instance, OMVs, which are produced by several species of bacteria, can modify the response of host immunity via immune cell activation and cytokine secretion promotion via transporting cytotoxic factors that lead to induction of apoptosis after internalization into cells of the host and via releasing substances that disrupt surrounding tissues. OMVs are considered delivery vehicles due to their topology of a lipid bilayer, which is perfect for carrying therapeutics to specific cells in host [4,17]. OMVs are included in preparations of vaccines because of their immunogenicity and ability to present antigens without risk caused by active bacterial cells [4]. However, the disadvantage of these applications is the low productivity of vesicles that can be extracted from supernatants of in vitro culture.

MATERIALS AND METHODS

Isolation and screening for OMVs producing E. coli isolate

Samples collection

Two hundred clinical samples were collected from different patients with different pathogenic conditions (Urinary tract infection, wound and vaginal infection) from Al-Yarmouk and Medical City Hospitals in Baghdad from November to February 2022. All samples were diagnosed for the isolation of E. coli using biochemical tests according to Bergey's manual [19].

Screening for antibiotic-resistant E. coli strains

One hindered strain of E. coli was obtained, and they were screened for antibiotic susceptibility, which was performed by using a modified Kirby-Bauer’s disk diffusion technique (19), according to guidelines of the Clinical and Laboratory Standards Institute’s (CLSI) (7,31). Which was achieved by using antibiotic disks: (Piperacillin Tazobactram (PIT) 100\10mcg; Ertrapenem (ETP) 10mcg; Trimethoprime Sulfomethoxazole (SXT) 1.25\23.75; Nitrofurantion (F) 100mg; Gentamicin CN 10ug; Ciprofloxacin (CIP) 10mg; ceftazidime (CFZ) 10mg; Ceftriaxone (CRO) 10mg; Meropenem MEM 10mg; and Amoxicillin/clavuianic acid (AMC) 20/10mg). All the antibiotics utilized in this work were obtained from the Mast Group.Ltd, England. The isolates were categorized as resistant (R) or sensitive (S), which was used as an indicator by the standards based on the CLSI (2020)

Identification of E. coli by using the VITEK2 system

To reveal the isolate of bacteria, the VITEK2 system device was utilized. In MacConkey agar dishes, the isolates of bacteria were grown. Suspensions of bacterial cells were utilized in 0.45% sterilized NaCl solution, identical to the 0.5 MacFarland standard. A densitometer has been utilized to adjust the turbidity of the suspension of bacterial cells. The VITEK2 system was loaded by hand with twelve cards and a suspension of bacterial cells. A bacterial suspension was covered and incubated for 6 h to fill each test card. The cards were recited every 15 min during the kinetic fluorescence measurement. The compact system software VITEK 2 initially analyzed the data and then mechanically reported its impact [27].

Bacterial growth conditions

Eight strains of E. coli (highly antibiotic-resistant) were initially grown on Luria-Bertani broth (LB) agar at 37ºC. The strains were pre-inoculated overnight in 50 ml of LB medium containing flasks (of size 250ml) at 37ºC and 250rpm with agitation. All eight strains of E. coli were incubated in one liter (4 flasks each) of LB broth and repeated to all strains with 0.1 (OD_{640} nm), 1.5 (OD_{640} nm) for 5/6hrs at 37ºC and 250rpm [20,27].

Extraction of bacterial OMVs

The samples were cultured for 36 hours (decelerating growth phase), after which they underwent 45-minute centrifugation at 7,000 rpm and 4 °C. Using phosphate-buffered saline (PBS) twice, the extracted pellets were cleaned. After that, it was centrifuged for 15 minutes at 10,000 rpm. Following that, the extracted pellets (4 ml/g pellet) were dissolved in a sodium chloride buffer and repeatedly mixed by pipetting to create a homogeneous suspension. The combination was centrifuged at 10,000 g for 15 minutes. The recovered pellets were then treated with 0.1 M Tris-HCl and pH 8; 10 mM EDTA (for six times the weight of the pellets), and the mixture was shaken to form a homogeneous mixture. Following that, the suspension was sonicated for 5 minutes in cold water. Afterward, 300 l of the combination (5% sodium deoxycholate (W/V), 10 mM EDTA pH 7.5, 0.1M Tris-HCl) was added to 5 ml of the sonicated suspension and then mixed. After 10 minutes, the sonicated suspension was centrifuged at 10,000rpm for 15 min. In a new tube, the supernatant was collected and treated with 200µl of (5% sodium deoxycholate (W/V), 10 mM EDTA pH 7.5, 0.1M Tris-HCl) and incubated for 10 min at RT. The peller was harvested for supernatant using a centrifuge at 50,000rpm for 2h at 4°C. The recovered pellet dissolved in 2ml of 3% sucrose and filtered using a 0.2 µm millipore filter to extract OMVs containing fluid, then stored at 4 °C [25].

Purification of bacterial OMVs

For purification of EC-OMVs, the dialysis sample was loaded into a column of Sephadex G-150 gel filtration and pre-equilibrated with PBS buffer (6.8 M, pH 7). The protein was eluted using the same buffer at a 5ml/min flow rate. The fraction collector was used to collect fractions at 4ºC. This technique was applied for protein activity at 280 nm. The active fractions were dialyzed against the 6.8M PBS buffer at pH 7 and then concentrated [8,9,20].

Characterization of the extracted OMVs

Transmission electron microscopy (TEM)

Samples were diluted and lodged in a formvar carbon-coated copper grid (EMS). After that, a solution of 10µL of 2% phosphotungstic acid was added to the sample. After 1 minute, the remaining liquid was removed via filter paper and dried by air. Images of TEM were taken on an EM10C (Zeiss, Germany) instrument [25].

Fourier Transform Infrared Spectrophotometer (FTIR)

The EC-OMVs were characterized by using the FTIR technique (Magna-IR 750, Nicolet Instrument, USA). The most common region utilized for analyzing the data is 400-4000 cm-1, as all the interest molecules have characteristic absorbance frequencies and primary molecular vibrations in this region. Small amounts of EC-OMVs crude were subjected to IR spectra measurement in the frequency range of 400 and 4000 cm-1. The material was prepared by dispersing the samples on an aluminum slide. After that, a transparent disc was formed by pressing the mixture. The disk was held in the instrument beam for spectroscopic examination. An FTIR spectrum was measured at room temperature [2].

Protein assay

In EC-OMVs, the total protein concentration was identified by the Bradford method and using bovine serum albumin as the standard [25].

Anticancer activity of OMVs on cancer cell lines

Cell culture

OV33 (ovarian cancer) and HdFn (human dermal fibroblast) human cell lines were collected from the American Type Stock Culture (ATCC) (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) containing 100 U/mL of streptomycin, 100 U/mL penicillin and 10% fetal bovine serum were utilized to maintain these cell lines. It was decided to maintain all cell lines at 37°C in a humidified environment with 5% CO_2 (Thermo Fisher Scientific, Waltham, MA, USA). At passages below 10, cell uptake, proliferation, and colony formation studies were conducted.

MTT assay

The cytotoxic effect of EC-OMVs was done using an MTT ready-to-use kit (Intron Biotech, Korea). Tumor cells ($1x10^4$ – $1x10^6$ cells/mL) were cultured in microtiter plates (containing 96 wells), where each well contained 200 mL of culture media. After that, the plate was sealed with sterile parafilm, shaken carefully and incubated with 5% CO_2 under 37°C for 24, 48 and 96 hrs. After each time of incubation, the medium was removed, and two-fold serial dilutions of the purified EC-OMVs (*6.25*, *12.5*,*25* and *50* mg/ml) were added to the wells, then the plates were incubated with 5% CO_2 under 37°C for (24,48, 96 hrs). After that, MTT (10 ml) 10 solution was placed in each well, and the plates were placed in an incubator with 5% CO_2 under 37°C for 4h. ELISA reader was used to determine the absorbance at a 575 nm wavelength. The statistical analysis of optical density data was performed to calculate the concentration of the compound, which needed to cause a 50% reduction in the cell viability for each cell line [21].

Statistical Analysis

A one-way analysis of variance ANOVA (Duncan) was performed to test whether group variance was significant. Statistical significance was defined as $p \leq 0.05$. Data were expressed as mean± standard deviation, and statistical significances were carried out using Graph Pad Prism version 6.

RESULTS

Screening for antibiotic-resistant E. coli strains

One hundred strains of E. coli were tested for different antibiotic-resistant types. Eight strains showed resistance to most of the tested antibiotics, as mentioned in Figure 1. The majority of isolates (88%) were resistant to Trimethoprim-sulfamethoxazole, and 74 % of isolates were resistant to ceftazidime, while for the Ceftriaxone, the resistant isolates were about 71.4 %. In addition, they showed approximately intermediate resistance for Nitrofurantoin, Gentamicin, Amoxicillin Clavulanic and Ciprofloxacin with resistance rates of 66.7%, 57.1%, 45.2% and 40.5%, respectively. However, they had low resistance rates for Meropenem (38.1%), Piperacillin Tazobactam (21.4%) and Ertapenem (7.1%). Many studies reported that OMVs are a bacterial defense against antibiotics and antimicrobials. For example, OMVs, isolated from β-lactam-resistant E. coli, have a vital role in the growth of susceptible bacterial cells by degrading β-lactam antibiotics before they can kill these bacteria [1, 11,12, 30].

Antibiotic	Antibiotic Sensitivity frequency (%) result		Probability
	Resistant	Sensitive	
Ertapenem	3(7.1)	39(92.9)	$p>0.001$
Nitrofurantoin	28(66.7)	14(33.3)	$p> 0.05$
Trimethoprim-sulfamethoxazole	37(88.1)	5(11.9)	$p>0.001$
Ceftazidime	31(73.8)	11(26.2)	$p>0.001$
Piperacillin/tazobactam	9(21.4)	33(78.6)	$p>0.001$
Gentamicin	24(57.1)	18(42.9)	$p> 0.05$
Meropenem	16(38.1)	26(61.9)	$p> 0.05$
Ciprofloxacin	17(40.5)	25(59.5)	$p> 0.05$
Ceftriaxone	30(71.4)	12(28.6)	$p>0.001$
Amoxicillin clavulanate	19(45.2)	23(54.8)	$p> 0.05$

Table 1. Antibiotic sensitivity of screening isolates of E. coli bacteria

Proof of identity Using VITEK2 System

VITEK2 system is a highly automated, novel technique for speed detection of species of gram-negative bacteria. The result showed that our 8 (antibiotics resistant) isolates were 100% E. coli.

Extraction of OMVs and protein assay

E. coli BE2 strain isolated from wound infection showed the highest protein concentration after ultracentrifugation for OMVs extraction with 2.3mg/ml. In contrast, the other strains showed lower yields of protein assay (data not shown). It is important to select a simple and highly efficient strategy for OMV extraction. Recently, the most effective method selected for extraction of OMVs is sequential ultracentrifugation above 100,000rpm. However, this strategy is only sometimes available. Hence, a non-high-speed centrifugation as an alternative procedure is necessary.

Purification of bacterial OMVs.

Fast and consistent purification of big biomolecules, such as large polysaccharides, proteoglycans, DNA, and tiny particles, is possible with Sephadex G-150 gel filtration media (e.g., viruses and membrane-bound vesicles). From (5000–150000) Dalton, it broke off. The gel filtration technology's superfine characteristic gives the procedure a high exclusion limit. The stable and inert matrix is created by covalently bonding allyl dextran with N, N'-methylene bisacrylamide. This treatment is specifically designed to assist the recovery of highly biopolymers. The enhanced mechanical strength of the

resultant beads allowed for high flow rates and quick separations [20]. Figure 1 from our data illustrates the emergence of a single peak (red line) of the Ec-OMVs derivate protein following elution by PBS buffer (6.8M, pH 7). Moreover, the second peak (blue line) for protein estimation by the Bradford method, which gave 2mg/ml of total protein

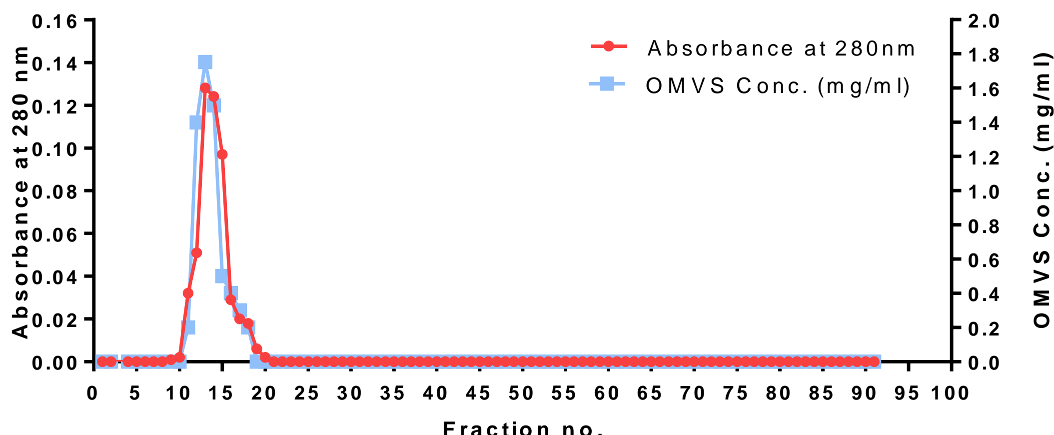

Figure 2. Gel filtration chromatography of OMVs on Sephadex G-150. Total protein was monitored at 280 nm. The separation of OMVs from a 2 mg/mL sample of the OMVs feedstock and 6.8M PBS at pH 7 of the preparation buffer.

Purifications stage	Volume (ml)	Protein Conc. (mg/ml)	Total protein (mg)	Recovery %
crude	15	2.3	34.5	100
Gel Filtration by Sephadex G-150	15	1.753	26.295	76.2%

Table 1. Summary of the purification steps of OMVs by a local isolate of E.Coli BE2

Transmission electron microscopy

To evaluate EC-OMVs morphology, the sample was negatively stained and tested with Transmission electron microscopy (TEM). The following (Figure 3) shows that these OMVs were nano-spherical in shape with nano-size ranging from 40 to 200 nm in diameter, which agrees with those reported in [4]. Our findings agreed with those studies that utilized high-speed ultracentrifugation, and our findings suggest the current method is an effective and appropriate strategy for extraction of OMVs from the local isolate E. coli in order to produce OMV-based vaccines and antitumor agents

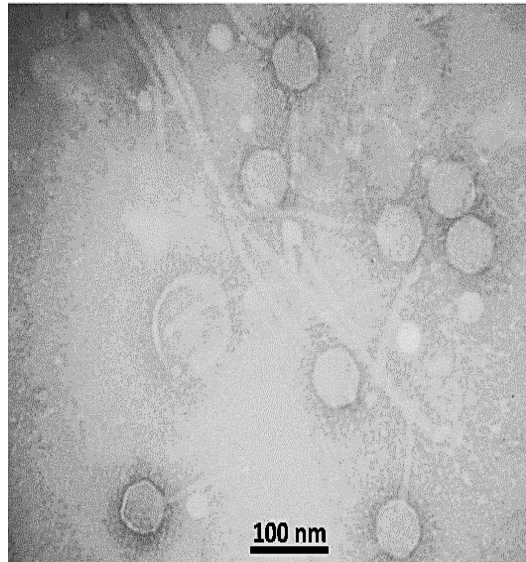

Figure 3. Characterization of the extracted EC-OMVs. By electron microscopy, the image of negatively stained OMVs

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR technique is a great tool for the structural analysis of Ec-OMVs. It is a non-destructive method for the polysaccharides structural analysis, which works by determining specific chemical groups on the internal reflection element surface. FTIR can get simultaneous information collection on the structural and chemical features of adherent bacteria within a minute. FT-IR spectroscopy can effectively provide data on a variety of biomolecules that are present in a biological process. These biomolecules include proteins, lipids, carbohydrates, and nucleic acids. Mid-infrared spectroscopy in the range of 2.5–25 µm (4000–400 cm-1) is the most common type of spectroscopy performed for biological purposes [28]. Figure 4: A and B display the FT-IR spectra of OMVs before and after purification produced from E. coli, which were resistant to several antibiotics. Specific bands were discovered after doing an FT-IR investigation on OMVs. In particular, each of the OMVs curves displayed the amide I absorption band, found at a wavelength of 1637 cm-1 and was related to the C=O stretching form of the peptide bond.

Additionally, Spectra around 3300 indicate the presence of (O-H) and (N-H) groups [5]. On the other side, every one of the OMVs curves displayed the amide II absorption band, found at a wavelength of 1452 cm-1 and was firstly attributable to the N-H vibrations of the peptide groups. Moreover, a band is evaluated at a frequency of 1421 cm-1 due to the symmetrical and asymmetrical vibration of COO [29]. Furthermore, bending in the region around 1400 cm-1 is associated with both lipids and proteins; hence, distinct contributions may affect the region in 1421 [23]. In addition, the absorbance of the ester groups of cholesterol triglycerides and phospholipids was discovered at 1738 cm-1, and acyl group vibrations were observed at 2930 and 2852 cm-1. One of the bands in the period at 986–992 cm-1 (986 cm-1) was related to the ribose phosphate main chain, while the band at 966 cm-1 resulted from the bending vibrations of the Backbone of DNA [28]. The spectra of nucleic acids can be broken down into four distinct spectral regions, which are as follows: the region with a wavelength of 1780–1550 cm-1 for in-plane vibrations of double bonds of the bases; the region with a wavelength of 1550–1270 cm-1 for deformation vibrations of the bases coupled with the sugar vibrations; the region with a wavelength of 1270–1000 cm-1 for vibrations of PO2. Signals in the spectral region of 800-1200 cm–1 are essential for the structural characterization of polysaccharides. The vesicle and bacterial

ranges in 1027, 1105 cm-1 and 1152 cm-1 peaks related to sugar and phosphate vibration. An additional zone between 1550 and 1080 cm-1 is attributed to waves from proteins in Escherichia coli. When comparing the FT-IR patterns of purified OMVs and the non-purified OMVs, we could see the identical profile in multiple regions of the spectra at 3381,2092, 1452, 1340, 1207, 1136, 1051,923, 835, 580, 549 cm-1.

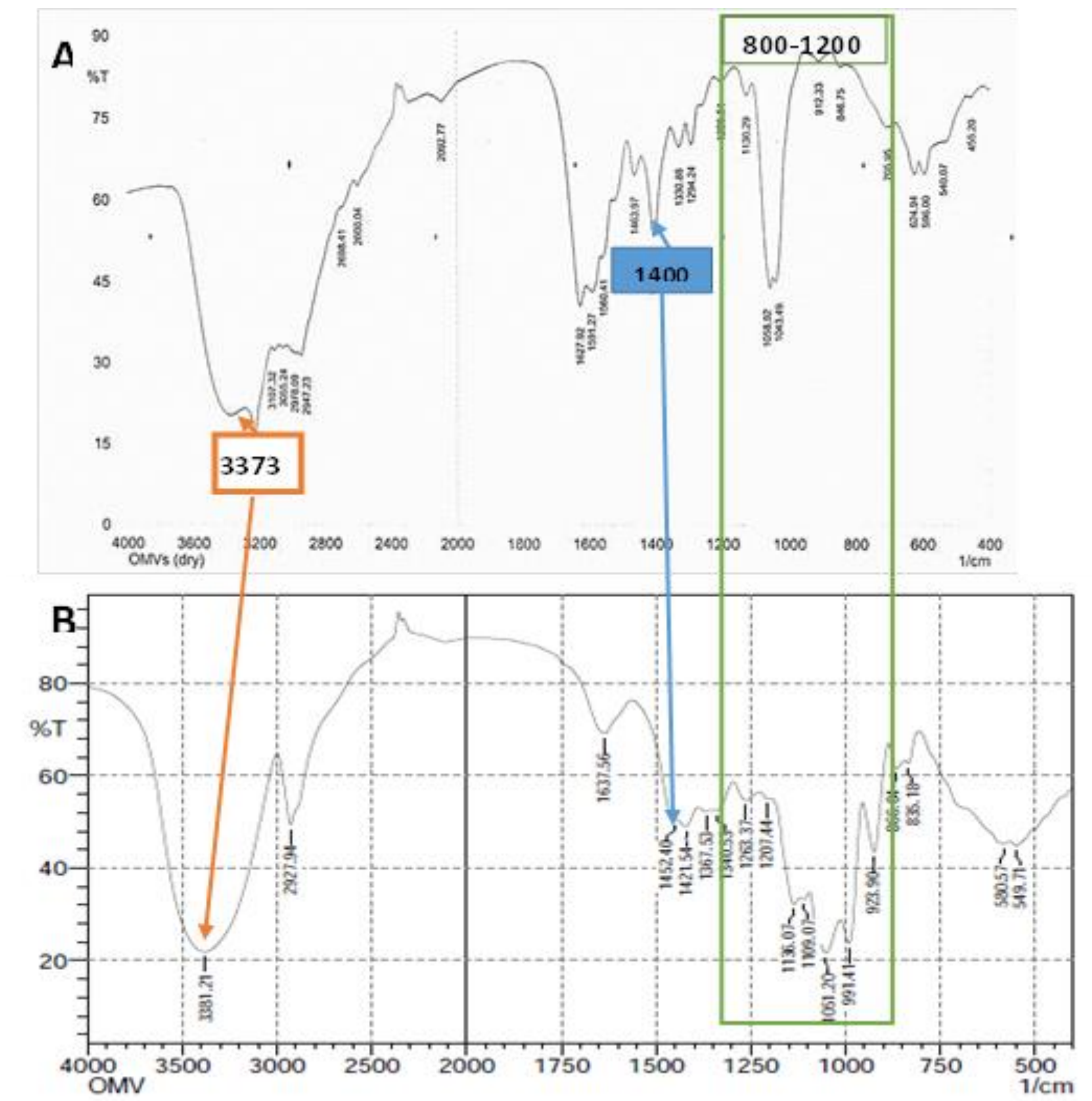

Figure 4. Fourier transforms infrared spectroscopy measurements of outer membrane vesicles (EC-OMVs) extracted from E. coli. A: FTIR analysis before EC-OMVs purification and B: FTIR analysis after EC-OMVs purification

The cytotoxic effect of EC-OMVs on cancer cell line

The sensitivity of the OV33 cell line after three different exposure times (24, 48, 96 hrs.) to EC-OMVs at different doses (6.25, 12.5, 25, and 50 µg /ml) was examined via MTT assay. A considerable dose-dependent reduction in the viability percentage of the cell line cancer was noted, especially upon treatment with EC-OMVs at 25 and 50 µg /ml. EC-OMVs 25µg /ml reduced the viability of cell % of OV33 by 43.7%, while the HdFn decreased its viability by only 12.8% in 24 hours of treatment exposure. In 48h of EC-OMVs incubation, the toxicity effect increased with the viability percent of OV33 by 50.66%, and the normal

cell viability was 23.7 %. On the other hand, EC-OMVs at 96 hrs of incubation dramatically decreased the cell viability % of OV33 by 60% compared to the positive control, which was 25.6% (Figure .5). In 50μg/ml decreased the viability in three incubation times. It was for OV33, 54.5 %, and 27.7% for the HdFn cell line in 24h of treatment. In 48 hours, the effect of EC- OMV on OV33 HdFn was 57.5%, and 29.2 %, respectively. The cytotoxic effect increased in 96hrs for OV33, 66.1%, and normal cell lines was 36.27%.

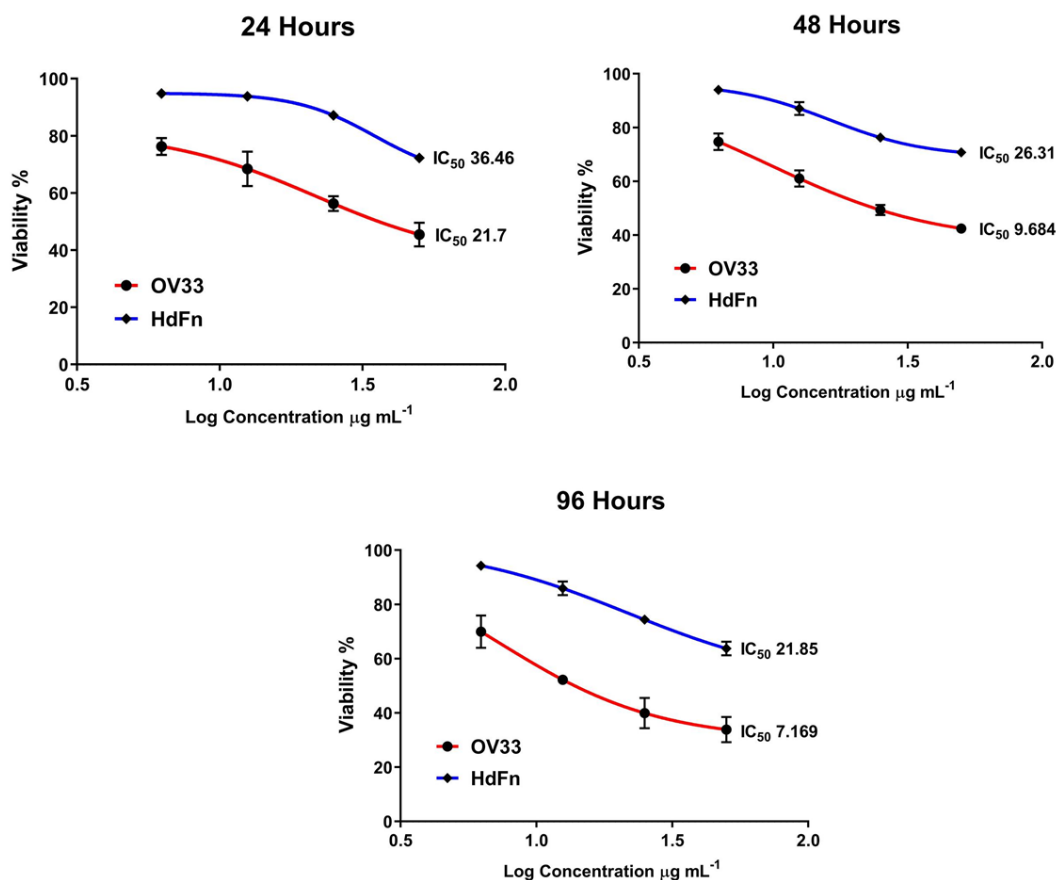

Figure 5. In vitro MTT assay used for detection of the cytotoxic effect of different doses (0.5, 1, 1.5, 2.0 μg/ml) of EC-OMVs after 24,48, 96 hrs incubation

DISCUSSION

The present study used a new strategy for OMV extraction from a local isolate of E. coli BE2. This strategy uses low-speed ultracentrifugation and good productivity of EC-OMVs, using several centrifugations up to 50,000rpm [25].

Indeed, the non-purified OMVs revealed many bands and stretching modes in the range of 3373-2947 cm-1 and 1500-1200 cm-1 that were not present in purified OMVs, which related to the absorption of the lipid acyl chain [22,26,29].

The IC50 value of EC-OMVs that caused 50% growth inhibition of OV33 and the control (HdFn) at 24hrs were 21.7 and 36.46 μg/ml, respectively. The IC50 for 48 hours of EC OMVs exposure were 9.684 and 26.31 μg/ml for OV33 and HdFn, respectively. At 96hrs of incubation, the IC50 concentrations for OV33 and HdFn were 7.169 and 21.85 μg/ml, respectively. Enhancing novel antitumors primarily begins with evaluating their antitumor activity against cell lines of

malignant. Bacterial OMVs possess a special structure as lipid bilayered nanospheres (20-200 nm in diameter), consisting of many enzymes, virulence factors, nucleic acids, phospholipids, LPS and proteins [21,10].

MTT assay is a speed in vitro colorimetric test that many scientists utilize to reveal the cytotoxicity and viability of cells. It was utilized in this study and revealed a considerable dose depending on the decrease in the percent of the viability of OV33 cells upon utilizing several doses of EC-OMVs, in particular, at 25 and 50 μg/ml for 24, 48 and 96h. The result indicated the transition of OMVs contents (virulence factors, enzymes, peptidoglycans lipopolysaccharides and proteins) into the cultured cells, which may cause their disruption of biological and morphological modification, resulting in apoptosis [10].

CONCLUSIONS

Our findings prove the probable antitumor activity of EC-OMVs that might be utilized as a safe antitumor immunotherapy for many cancer types.

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