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Research Article

The *in vitro* Potential Antitumor Effect of *S. typhimurium* Outer Membrane Vesicles on MCF-7 and Caco-2 Cell Lines Compared to Chemotherapies

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ABSTRACT

Salmonella Typhimurium (*S.* Typhimurium) is one of the most widely studied bacteria-mediated cancer therapies. OMVs is a much safer form of *Salmonella* that could be effective in small doses. This study was directed towards assessing the *in vitro S.* Typhimurium OMVs antitumor effect, alone or combined with chemotherapeutic drugs. The cytotoxic effect of *S.* Typhimurium OMVs and chemotherapeutic drugs on MCF-7 and Caco-2 cell lines at different doses was expressed as cell viability. Using neutral red uptake assay revealed that that *S.* Typhimurium OMVs were cytotoxic to cancer cell lines and decreased their viability more than chemotherapies. It was found that the IC50 value of *S.* Typhimurium OMVs which caused 50% growth inhibition was 16.7 µg/ml on MCF-7 and 14.4 µg/ml on Caco-2 cells. On the other hand, the IC50 of Paclitaxel on MCF-7 was 2.2 µM, while 5.3 µM was the IC50 of Doxorubicin on Caco-2 cells. Combining *S.* Typhimurium OMVs with chemotherapies exhibited a magnificent cytotoxic effect expressed by changes in the morphology and decrease in viability of MCF-7 and Caco-2 cells. These results were significantly different from untreated controls at P≤0.001. This may render *S.* Typhimurium OMVs as a promising novel antitumor monotherapy, just like the parent bacteria and even better or an adjuvant potentiating the antitumor efficiency of chemotherapies and decreasing their harmful side effects.

Key words: OMVs, *S.* Typhimurium, Cancer, Cytotoxicity, Chemotherapy

INTRODUCTION

Breast cancer ranks the cancer related death in women in developing countries (Bray *et al.*, 2018), while colorectal cancer is the forth cause of mortality among the world (Arnold *et al.*, 2017). Chemotherapy, surgery and radiotherapy are the most extensively used weapons against cancer nowadays (Arruebo *et al*., 2011). Despite being an essential option for treatment, separately used or accompanied by other treatment regimens, conventional chemotherapies suffer decreased aqueous solubility, decreased selectivity and increased multidrug resistance (Chidambaram *et al*., 2011). Defeating cancer using bacterial therapies dates back to the Nineteenth century, when William Coley tried to treat a female patient suffering from a neck tumor by cauterizing that tumor and placing her into a previously occupied bed with "erysipelas", a *Streptococcus pyogenes* infected patient, and in contrary to expectations, the woman was infected, while the tumor was regressed. Decades after, he advanced a mixture of inactivated bacteria known as (Coley toxin) (McCarthy, 2006). Since then the antitumor effect of various bacterial species as *Clostridium, Listeria*, genus *Bifidobacterium*, *Escherichia and Salmonella* had been proven, with *Salmonella* being the most extensively evaluated and explored bacterial genus as an anti- cancer therapeutic nowadays (Zheng and Min, 2016). *S.* Typhimurium known to infect mice and humans, achieved a lot of success as a promising therapeutic agent against cancer in preclinical trials (Hoffman and Zhao, 2014; Drees *et al*., 2015). Unfortunately, attenuated *S.* Typhimurium, examined in the first phase I human clinical trial for toxicity and anticancer potential, although confirmed its safety for patients with cancer, it showed major limitation for further clinical development (Toso *et al.*, 2002). Thence solving this obstacle was rendered as a major priority in developing bacteria that could be used for cancer treatment. Outer membrane vesicles (OMVs) are natural, metabolically inert, lipid bilayer nanospheres that pinches off the Gram negative bacterial outer

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membrane, and contains lipopolysaccharide (LPS), periplasmic and membrane-bound proteins, peptidoglycan, DNA, RNA, enzymes and toxins. These vesicles are about ~ 20 to 250 nm in diameter and play a great role in bacterial communication, transmission of virulence factors into the host cells as well as influencing the host defense (Schwechheimer and Kuehn, 2015; O'Donoghue and Krachler, 2016). *S*. Typhimurium OMVs assimilates their parent bacteria, being as potent stimulators of macrophages and dendritic cells, causing maturation of dendritic cells, stimulating the production of the proinflammatory TNFα, NO and IL-12 and contain antigens detected by B and $CD4^+$ T cells that are specific for *Salmonella* (Alaniz *et al*., 2007). The biological role of these vesicles and their potential exploitation as therapeutics proved them recently as promising vaccines and drug-delivery vehicles even for cancer therapies (Cai *et al.*, 2018). Unfortunately, the potential use of *S*. Typhimurium OMVs as an antitumor agent had not been widely examined yet. MCF-7 human breast adenocarcinoma cell line acts as a premium model representing breast cancer and is used for studying the *in vitro* mechanism of tumor response, besides being considered as an estrogens receptor (ER) positive control cell line (Dillon *et al*., 2010). Caco-2 cell line is derived mainly from colon adenocarcinoma of human and is widely used as an *in vitro* model in detecting of the intestinal drug permeability (Awortwe *et al*., 2014). Paclitaxel is a cytotoxic microtubule stabilizing drug used frequently in treating different types of cancers such as breast cancer (Zasadil *et al*., 2014). While doxorubicin, an antibiotic with broad spectrum activity, is used as a therapy for different cancer types, suffering from being cardiotoxic in high doses so preventing its use in some therapeutic cases (Sadeghi-Aliabadi *et al*., 2010). Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) uptake assay known as one of the frequently used *in vitro* cytotoxicity and cell viability assays, being used in cancer studies nowadays to detect either the toxicity of tested agent or the inhibition of tumor cells growth during drug development (Bacanli *et al*., 2017). It relies on the fact that living cells are able to uptake and concentrate this dye inside their lysosomes following nonionic diffusion (Repetto *et al*., 2008). Putting all these in our consideration we conducted this study with a hypothesis that *S*. Typhimurium OMVs may assimilate their parental bacteria being of tumoricidal effect so that it could be safer, cheaper and effective in targeting tumors. And whether *S*. Typhimurium OMVs could act as adjuvant capable of restoring the antitumor effect of conventional chemotherapeutic drugs that was decreased due to multidrug resistance. So we investigated for the first time the *in vitro* potential antitumor effect of *S*. Typhimurium OMVs on two highly malignant, drug resisting tumor cell lines, human breast adenocarcinoma (MCF-7) and human colorectal adenocarcinoma (Caco-2) cell lines, and compared their cytotoxic effect to that of paclitaxel and doxorubicin as two of the most widely used conventional chemotherapeutic drugs used in cancer treatment regimens and detected the half maximal inhibitory concentration (IC50) of *S*. Typhimurium OMVs compared to that of tested chemotherapies. We also studied the cytotoxic effect of both OMVs and chemotherapeutic drugs on the morphology and the viability of MCF-7 and Caco-2 cells, using neutral red uptake assay.

MATERIALS AND METHODS

Drugs and chemicals

Paclitaxel (Taxol)®; Ebewe Pharma (Austria), Doxorubicin; Ebewe Pharma (Austria). A stock solution for neutral red 4mg/ml was prepared by dissolving 40 mg neutral red dye (SERVA) in 10 ml PBS, this preparation was kept in the dark at 4ºC.

Bacterial strain

Salmonella Typhimurium ATCC 14028 was kindly provided by Ministry of Health Central Laboratories (Egypt).

Isolation of *S*. Typhimurium OMVs

S. Typhimurium OMVs were isolated following the protocol previously used by (Park *et al*., 2010) with some modifications. *S*. Typhimurium was grown on MacConkey's agar (Oxoid) and incubated aerobically at 37ºC for 24 h. One single colony was inoculated into 5 ml LB broth for 8h in incubator shaker at 200 rpm and 37ºC. In one-liter flask, 2.5 ml of previously prepared bacterial broth were added to 250 ml freshly prepared LB broth (1:100). The flask was incubated aerobically in incubator shaker at 37ºC and 200 rpm overnight. Culture was pulsed with 50 µg/ ml gentamycin in the last 30 minutes to increase the production of OMVs (Alaniz *et al*., 2007). Centrifugation twice at 6,000 xg at 4ºC for 20 min was used to remove bacterial sediment. Sterile Millipore membrane filters (Sigma) of 0.45 µm and 0.22 µm were used for filtrating the supernatant. OMVs were then pelleted by ultracentrifugation at 4°C for 3 h at 150,000 g (Beckman L7 Ultracentrifuge), washed and suspended in phosphate buffered saline, followed by filtration using a sterile Millipore membrane filter, 0.22 µm. The purified OMVs were checked for sterility by culturing onto blood agar, MacConkey's and Sabouraud dextrose agar (Oxoid) plates followed by incubation for 72 h at 37° C. The protein concentration of OMVs was determined using Qubit fluorometer (Invetrogen), then the vesicles were kept frozen at - 40 till being used.

OMVs characterization

Isolated *S.* Typhimurium OMVs were subjected to negative staining by 1% phosphotungstic acid (pH 7.0) and placed on 400-mesh grids. Images were obtained using a JEM 2100 transmission electron microscope (JEOL).

Cancer cell lines and cultures

Michigan cancer foundation -7 (MCF-7); cell line of human breast cancer and (Caco-2) human colorectal adenocarcinoma cell line, were originally obtained from the cell lines Bank of VACSERA (Cairo, Egypt). Both cell lines were primarily cultured in humidified air with CO2 5% at 37ºC in complete medium; DMEM (Dulbecco's Modified Eagle Medium) (Lonza), supplemented with 10% fetal bovine serum (LSP), 1% antibiotics; penicillin G potassium (SERVA) + Streptomycin (Sigma). All *in-vitro* studies were carried out in Cairo University Research Park, Faculty of Agriculture, Cell culture lab.

Determination of cell viability/cytotoxicity of *S.* Typhimurium OMVs and chemotherapies

The antitumor activity of the tested agents on both MCF-7 and Caco-2 cells were determined according to (Repetto *et al*., 2008) using neutral red uptake assay. Cultures of 1 x10^5 and 2 x10^4 cells of MCF-7 or Caco-2, respectively in complete media were seeded in 96-well microtiter plates and were incubated in humidified CO2 5% at 37°C so that they can grow. 24h later, the culture media were decanted from the microtiter plates and cells were refilled with serum free media treated with or without the tested agents. For MCF-7, the cells were treated either with *S.* Typhimurium OMVs at concentrations of 1, 5, 10 and 20µg/ml (Lee *et al.*, 2012) or Paclitaxel at concentrations of 0.5, 1, 2 µM (Luo *et al.*, 2010) in four replicates each. While for Caco-2, the cells were treated with or without *S.* Typhimurium OMVs at the same previous concentrations or Doxorubicin at doses of 0.1, 1 and 10 µM (Eid *et al*., 2012) in four replicates each. The plates were then incubated at appropriate conditions of temperature, CO2 and humidified atmosphere. Cells incubated in culture medium alone served as a control for cell viability (untreated wells). Cultures were examined 48hrs after incubation, under a phase-contrast inverted microscope (LEICA DMI 3000 B), and changes in morphology of the cells due to cytotoxic effects of the tested agents were recorded. Culture media then decanted and replaced by medium supplemented with neutral red (40µg/ml), followed by incubation at the appropriate culture conditions for 4 h. Neutral red medium was then removed, and the cells were washed with PBS and eluting buffer solution (mixture of acetic acid solution/ ethanol 50%, 1:99 v/v) was added to each well followed by shaking the plates for at least 10 min or until the neutral red has been fully extracted from the cells and a homogeneous solution is formed. Cell viability was detected by measuring the OD of neutral red extract at 540 nm in a microtiter plate reader spectrophotometer (BioTek, ELX808), against blank containing no cells as a reference.

Cell viability (%) = Mean tested OD / Control OD x 100%, the results were expressed as percentage of cell survival relative to the untreated control (100%).

Determination of the half maximal inhibitory concentration (IC50)

Curves of dose-response were drawn and IC50 values were determined graphically at that concentrations of *S.* Typhimurium OMVs, Paclitaxel and Doxorubicin which required to produce 50 % inhibition of MCF-7 and Caco-2 cells viability. Standard Graph was plotted by taking the concentration of the drug on X axis and relative cell viability on Y axis.

Assaying the cytotoxic effect of the combined treatment of *S.* Typhimurium OMVs with chemotherapies in relation to their sole treatments on MCF-7 and Caco-2 cell lines

In another two experiments, Cultures of 1 x10^5 and 2 x10^4 cells of MCF-7 or Caco-2, respectively in complete media were seeded in 96-well microtiter plates and were incubated in humidified 5% CO2 at 37°C for 24h. MCF-7 cells were inoculated with 10µg/ml OMVs (a chosen dose; lower than the IC50) and/ or 1µM paclitaxel (Luo *et al.*, 2010), while Caco-2 cells were inoculated with 10µg/ml OMVs (a chosen dose; lower than the IC50) and/or 1µM doxorubicin (Eid *et al*., 2012). The cells were incubated for 48h, at appropriate conditions of temperature, CO2 and humidified atmosphere, then examined under a phase-contrast inverted microscope for detection of the alterations in cells morphology then neutral red uptake assays were carried out as mentioned above to detect the cell viability (%).

Statistical analysis

Data are presented as means ± SD. Comparisons between different groups were carried out using one- way analysis of variance (ANOVA) followed by LDS correction test, using SPSS. $P<0.05$ was considered statistically significant.

RESULTS

Isolation and characterization of *S.* Typhimurium OMVs

S. Typhimurium ATCC 14028 wild type was found to be a low producer of OMVs even with pulsing the bacterial culture with gentamycin to increase the OMVs yield. By TEM analysis *S.* Typhimurium OMVs appeared as bilayered spherical nanovesicles of 20-200 nm diameter. The isolated OMVs appeared with variant densities which may be a result of differences in OMVs contents. The OMVs isolation examined by TEM completely lacked whole bacteria, bacterial debris and other structures like flagella, all the attempts were made to detect if there is any bacterial growth from the OMVs isolates, by being checked on blood agar, MacConkey′s and Sabouraud dextrose agar plates, and found no bacterial growth, confirming the purity of the OMV fraction. Protein concentration of the OMVs was detected and the concentrations of 1, 5, 10 and 20 µg/ml were prepared.

The cytotoxic effect of *S.* Typhimurium OMVs and chemotherapeutic drugs on MCF-7 and Caco-2 cell lines at different doses expressed as cell viability

The sensitivity of MCF-7 to *S.* Typhimurium OMVs or Paclitaxel at different doses as well as that of Caco-2 to *S.* Typhimurium OMVs or Doxorubicin at different doses were determined 48h after exposure, under phase-contrast inverted microscope. Variable changes in cellular morphology such as; growth inhibition, vacuolation, rounding, cellular detachment and clumping as a result of the cytotoxic effects of the tested agents were recorded (Figures 1 and 2) compared with the control negative cells which appeared as confluent sheets.

Generally, a significant dose dependent decrease in the cell viability percent was observed. The treatment with OMVs at concentrations of 10 and 20µg/ml, significantly decreased the cell viability percent of MCF-7 by 40%, 56% and that of Caco-2 cells by 39%, 66%, respectively, compared to untreated control cells. The treatment with Paclitaxel showed significant decrease in cell viability at concentrations of 1, 2µM reached 35% and 47% on MCF-7, while Doxorubicin decreased the cell viability percent by 32% and 72% µM at doses of 1, 10 µM on Caco-2, respectively. (Figures 3a and b).

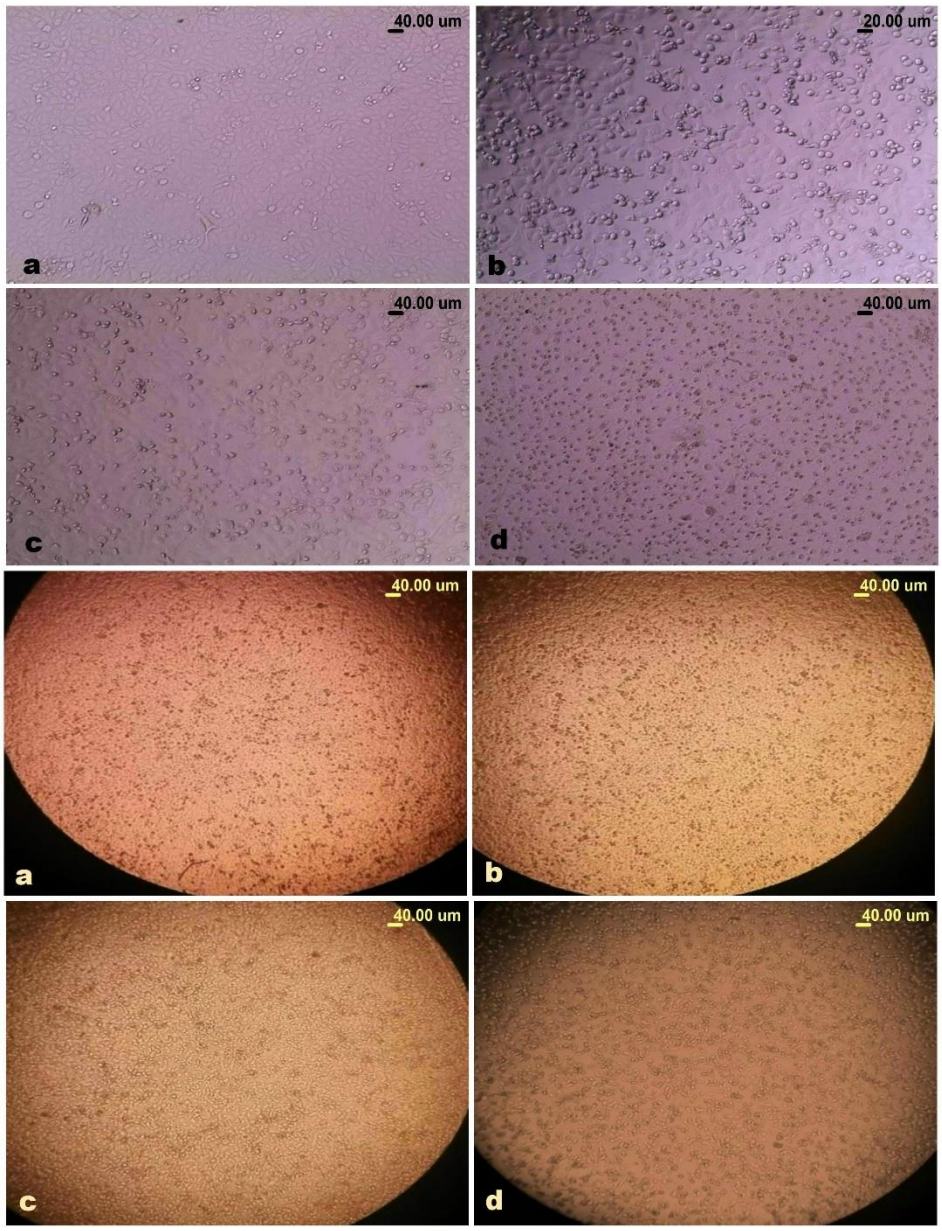

Fig. 1: Representative photos of MCF-7 showing the morphological changes and the cytotoxic effect of the tested compounds. (a) normal control, (b) *S.* Typhimurium OMVs (10µg/ml), (c) Paclitaxel (1µM) and (d) their combination which showing severe cellular rounding, clumping and detachment. (OMVs: Outer Membrane Vesicles).

Fig. 2: Representative photos of Caco-2 showing the morphological changes and the cytotoxic effect of the test compounds. (a) normal control, (b) *S.* Typhimurium OMVs (10µg/ml), (c) Doxorubicin (1µM) and (d) their combination which showing severe cellular rounding and detachment.

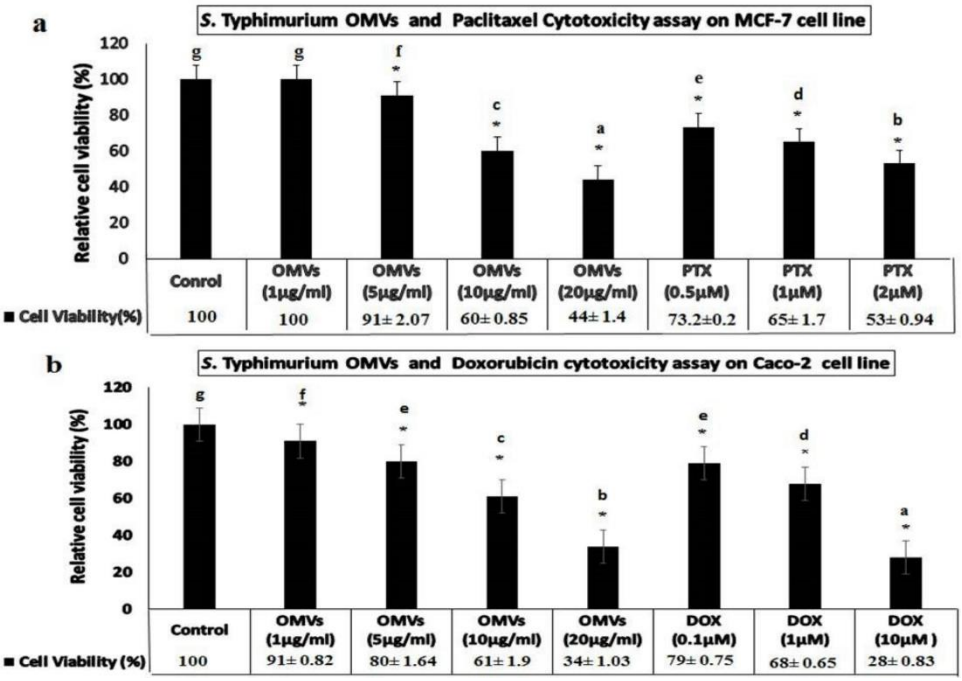

Fig. 3: (a) The cytotoxic effect (cell viability percent) of *S.* Typhimurium OMVs (1, 5, 10, 20 µg/ml) and Paclitaxel (0.5, 1 and 2 µM) on MCF-7 cells. **(b)** The cytotoxic effect (cell viability percent) of *S.* Typhimurium OMVs (1, 5, 10, 20 µg/ml) and Doxorubicin (0.1, 1 and 10 µM) on Caco-2 cells. Assessed by neutral red uptake assay, after 48 h incubation.
*results are significantly different from untreated controls at P≤0.001, different letters: results showed significant difference between treated groups at P≤0.001. Results are mean ± SD of 4 replicates, ANOVA followed by LSD test.

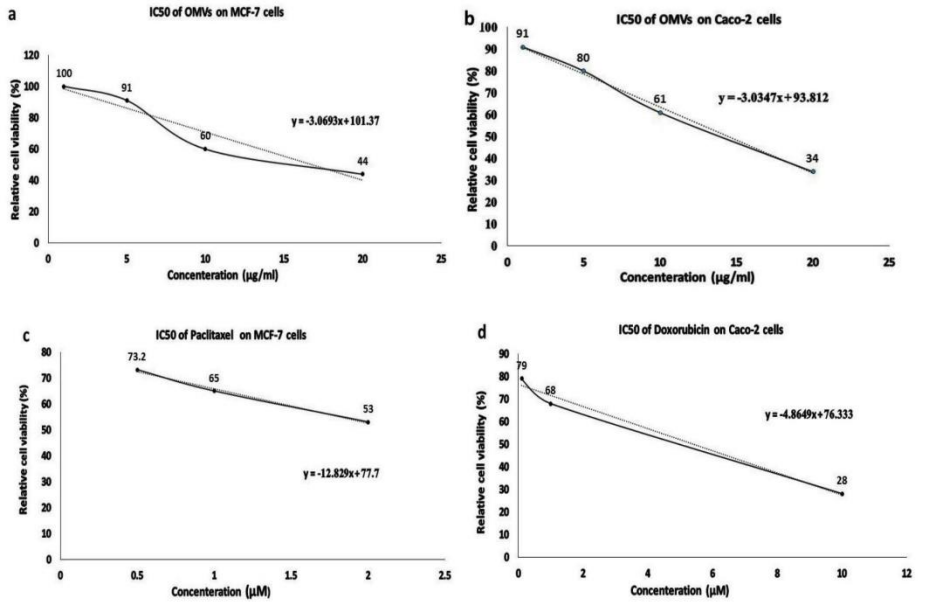

Fig. 4: (a) Sensitivity of MCF-7 cells to *S.* Typhimurium OMVs, cells were treated with increasing doses of OMVs (1, 5, 10, 20 µg/ml) for 48h, IC50 was shown to be 16.7 µg/ml. **(b)** Sensitivity of Caco-2 cells to *S.* Typhimurium OMVs. Cells were treated with increasing doses of OMVs (1, 5, 10, 20 µg/ml) for 48h, IC50 was shown to be 14.4 µg/ml. **(c)** Sensitivity of MCF-7 cells to Paclitaxel, cells were treated with increasing doses of Paclitaxel (0.5 ,1 and 2 µM) for 48h, IC50 was shown to be 2.2 µM. **(d)** Sensitivity of Caco-2 cells to Doxorubicin, cells were treated with increasing doses of Doxorubicin (0.1 ,1 and 10 µM) for 48h, IC50 was shown to be 5.3 µM. Mean ± SD were used to detect IC50 by neutral red uptake assay.

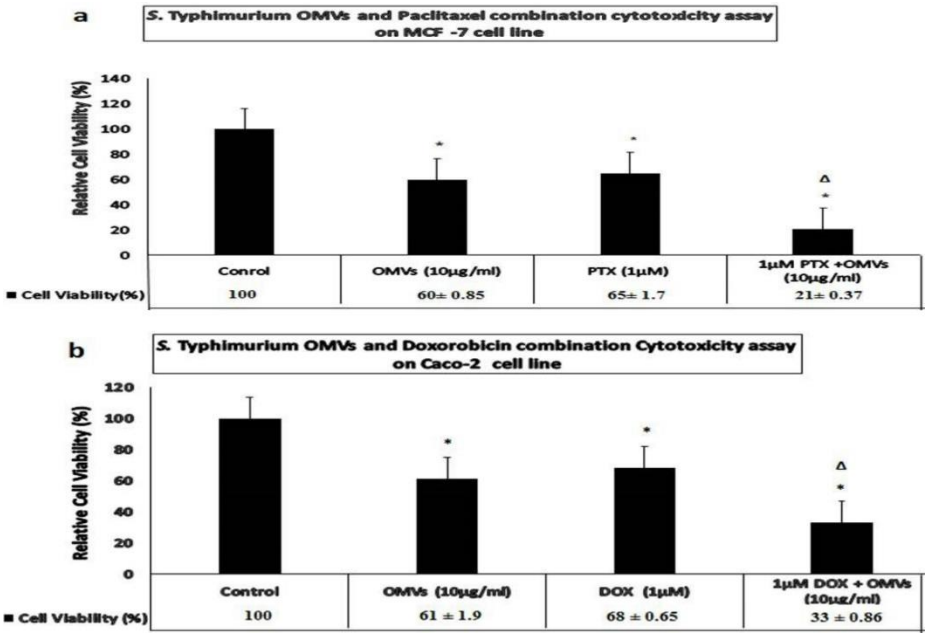

Fig. 5: (a) The cytotoxic effect (cell viability percent) of the combined treatment of *S.* Typhimurium OMVs at selected dose (10µg/ml) with paclitaxel at reference dose (1µM) compared to each single agent on MCF-7cells. (**b**) The cytotoxic effect (cell viability percent) of the combined treatment of *S.* Typhimurium OMVs at selected dose (10µg/ml) with Doxorubicin at reference dose (1µM) compared to each single agent on Caco-2 cells. Assessed by neutral red uptake assay, after 48 h incubation. * results are significantly different from untreated controls at $P \leq 0.001$, different letters: results showed significant difference between treated groups at $P \leq 0.001$. Results are mean ± SD of 4 replicates, ANOVA followed by LSD test.

The IC50 of *S.* Typhimurium OMVs, Paclitaxel and Doxorubicin on MCF-7 and Caco-2 cell lines

It was found that the IC50 value of *S.* Typhimurium OMVs which caused 50% growth inhibition was 16.7 µg/ml on MCF-7 and 14.4 µg/ml on Caco-2 cells. On the other hand, the IC50 of Paclitaxel on MCF-7 was 2.2 µM, while 5.3 µM was the IC50 of Doxorubicin on Caco-2 cells (Figures 4a-d).

The cytotoxic effect of the combination treatment of *S.* Typhimurium OMVs and each type of the chemotherapeutic drugs on MCF-7 and Caco-2 cell lines compared to their separate treatment

Generally, spectacular changes in cell morphology as presented by apoptosis, rounding and detachment as well as decreased cell viability of both MCF-7 and Caco-2 cell lines were detected 48h after exposure to the combined treatments of each of the chemotherapeutic drugs with the selected dose of the tested OMVs (10 µg/ml) compared with the sole used of Paclitaxel (1µM), Doxorubicin (1µM) or OMVs (10µg/ml). These combinations treatment showed a cell viability percent of 21% and 33% in MCF-7 and Caco-2 cell lines, respectively, compared to 65% of Paclitaxel on MCF-7 cells and 68% of Doxorubicin on Caco-2. On the other hand, OMVs showed 60% and 61% cell viability percent in MCF-7 and Caco-2 cell lines, respectively, (Figures 5a and b).

DISCUSSION

Cancer ranks as a main global cause of death, with colorectal and breast as major cancer types (Arnold *et al.*, 2017; Bray *et al.*, 2018). Although chemotherapies are widely used nowadays in treating most types of cancer, drug resistance persists as their major problem, resulting in increasing the efflux pump activity, decreasing drug absorption, activating the enzymes of detoxification, altering the drug targets and decreasing cell apoptosis (Shain and Dalton, 2001). And as all the researchers efforts recently are directed towards discovering novel

agents fixing this problem, we investigated for the first time in this study the potential effect of combining bacterial OMVs particularly those of *S.* Typhimurium (being metabolically inert, safer and used in small doses) with two widely used chemotherapeutic drugs: Paclitaxel or Doxorubicin on both MCF-7 and Caco-2 tumor cell lines.

In this study we found that *S.* Typhimurium ATCC 14028 wild type was a low producer of OMVs even with pulsing the bacterial culture with gentamycin to increase the OMVs yield. This agrees with (Lee *et al.*, 2009), who previously reported that OMVs from *msbB* mutant of *S.* Typhimurium, were greater in amount compared to those of wild type *S.* Typhimurium, similar results were detected by (Kim *et al*., 2017) on other Gram negative bacterial mutant OMVs. This alteration in vesiculation may indicate that OMVs formation is influenced by bacterial outer membrane structure.

It was found that *S.* Typhimurium OMVs appeared with TEM as spherical double layered vesicles of about 20-200 nm diameter, which assimilates previous finding by (Alaniz *et al.*, 2007; Lee *et al.*, 2012; Liu *et al*., 2017), who said that outer membrane vesicles are natural, lipid bilayered nanospheres and these vesicles are about ~ 20 to 250 nm in diameter.

The discovery of anticancer drugs usually begins with *in vitro* screens, which focuses mainly on cytotoxicity and was useful in detecting highly toxic agents. Then *in vivo* screening is used to evaluate the normal tissue damage by these agents. The neutral red uptake assay is one of the *in vitro* tissue culture colorimetric cell viability and cytotoxicity assays that depends on the selective ability of healthy living cells to concentrate this weakly basic vital stain within its lysosomes following nonionic diffusion. Cytotoxic agents alter the integrity of the exposed cells, decreasing neutral red uptake. Acidified ethanol solution is then used to extract the dye from the viable cells using an. This study is an indirect method that measures the cell viability through quantifying the intensity of the resulting color using a spectrophotometer (Repetto *et al*., 2008). The use of neutral red uptake assay was reported in various previous cytotoxicity and cell viability studies (Parker *et al*., 2010; Thay *et al*., 2014).

The doses of *S.* Typhimurium OMVs (1- 20 µg/ ml) examined in this study were more or less similar to those mentioned by (Ismail *et al*., 2003; Lee *et al*., 2012).

We found that *S.* Typhimurium OMVs particularly at doses of 10 - 20µg/ ml had significant cytotoxic effect detected by alteration in the cells morphology and decreased cell viability of the two examined cell lines by 40%, 56% for MCF-7 and by 39%, 66% for Caco-2 cells, respectively.

The detected cytotoxic effect of *S.* Typhimurium OMVs in this study may refer to the transition of its vesicular contents of proteins, lipopolysaccharides, peptidoglycans, virulence factors, enzymes as proteases to the cultured cells, which may cause damage, morphological and biological changes in these cells, leading to apoptosis. On the other hand, *S.* Typhimurium OMVs contains immunomodulatory compounds (PAMPs), capable of initiating innate immune response in the exposed cells, which in turn activates APC to produce pro-inflammatory mediators such as TNF-α, NO and IL-12, which may also cause apoptotic cell death. This agrees with the pro-inflammatory response reported by (Lee *et al*., 2012; Cai *et al.*, 2018) towards OMVs of Gram negative pathogens.

Our results also agree with previous studies, conducted to investigate the potential antitumor and cytotoxic effects of different bacterial outer membrane vesicles on different tumor cell lines, among which (Fábrega *et al*., 2016) who reported that microbiota OMVs downregulated TGF-β and MUC1(overexpressed in several cancer types) by 25 and 40%, respectively, suggesting that these strains of microbiota may help in tumor regression or may improve tumor treatment. Also, (Nho *et al*., 2015) found that OMVs of *Acinetobacter nosocomialis* were cytotoxic to the human laryngeal cancer cells at doses ≥ 20 µg/ml.

On the other hand our results disagree with other researchers who found that outer membrane vesicles of different bacteria had no cytotoxic effect as (Lee *et al*., 2012) who found that *K. pneumoniae* OMVs were non cytotoxic to Hep-2 and U937 cells at concentrations up to 50µg/ml, as no morphological changes, or growth inhibition were observed. Also Cañas *et al.* (2016) investigated the effect of OMVs of the commensal ECOR12 strains and the *E. coli* probiotic EcN on HT-29 cells and found that it did not affect cell viability but inhibited cell proliferation . Moreover, (Li *et al*., 2012) investigated the *in vitro* effect of *S*. Typhimurium bacteria on human hepatoma HepG2 cells and found that it showed a significant effect, suggesting that it may have an antitumor effect.

The IC50 value of *S*. Typhimurium OMVs which caused 50% growth inhibition was 16.7 µg/ml on MCF-7 and 14.4 µg/ml on Caco-2 cells. On the other hand, the IC50 of Paclitaxel on MCF-7 was 2.2 µM, while 5.3 µM was the IC50 of Doxorubicin on Caco-2 cell.

Paclitaxel is considered as a microtubule poison that arrests cells in mitosis, enhancing stabilization of microtubule and induces apoptosis. Paclitaxel-mediated cell death also may refer to chromosome missegregation on abnormal mitotic spindles without mitotic arrest (Zasadil *et al*., 2014). Our results in this study were supported by (Luo *et al*., 2010; Sivakumaran *et al*., 2018) who reported that the IC50 of Paclitaxel was in the range of 2.2 - 2.3µM on MCF-7 cell line.

On the other hand Doxorubicin is one of the mostly used therapeutics in the treatment of different solid and hematopoietic cancers, characterized by its intercalation between the strands of DNA and hydrolyzing them (Lentacker *et al*., 2010). Our results were similar to those by (Li *et al*., 2018; Zhou and Wink, 2018), who reported IC50 of Doxorubicin to be 4.97 and 5.40 ± 1.27 µM on Caco-2 cell lines, respectively.

In this study we found that the cytotoxic effect of *S.* Typhimurium OMVs was higher than that of either Paclitaxel or Doxorubicin on MCF-7 and Caco-2 cell lines. As *S.* Typhimurium OMVs showed 60% and 61% viable cells percent in MCF-7 and Caco-2 cell lines respectively, while Paclitaxel and Doxorubicin showed viable cells percent of 65% and 68% in MCF-7 and Caco-2 cell lines respectively.

On the other hand, combining bacterial OMVs particularly those of *S.* Typhimurium with two widely

used chemotherapeutic drugs showed a magnificent effect on the tumor cells viability exceeding that of *S.* Typhimurium OMVs or of Paclitaxel or Doxorubicin alone on both MCF-7 and Caco-2 tumor cell lines indicating that *S.* Typhimurium OMVs combination with chemotherapies may act as a promising novel therapeutic strategy against tumor.

These results assimilated other researchers findings who found that combination of the parent *Salmonella enterica* strains with different chemotherapeutic drugs resulted in synergistic effect on tumor regression *in vitro* as (Chang *et al*., 2013; Yang *et al.*, 2018).

Conclusions

In the current study, *S.* Typhimurium OMVs showed a promising *in vitro* antitumor effect, suggesting that *S.* Typhimurium OMVs could be a novel promising candidate in cancer therapy, just like their parent bacteria and even better, and could act as a promising adjuvant as its combination with chemotherapies may be a promising method of cancer treatment, with better effect than their both sole effect. This combination could potentiate the effect of chemotherapeutic drugs, averting the drug resistance problem and decreasing its harmful effects to normal cells by decreasing the dose of the chemotherapeutic drugs. Further investigation is in need to detect *the in vivo* antitumor effect of *S.* Typhimurium OMVs and its safety on living lab animals.

Author contributions

All authors contributed to the reagents/materials/ analysis tools, collected the material, analyzed the data and wrote and revised the manuscript.

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