INTRODUCTION
Cancer occurs with an uncontrolled proliferation and differentiation of the normal functions of cells. The disease causes irreversible functional abnormalities in tissues and organs of the body. Statistical reports have shown that cancer remains one of the most serious public health problems with its high global mortality rates. In addition, it is estimated that cancer will be ranked highest among those diseases that result in death in the next five years. Breast cancer has been reported as ranked second highest among those cancer cases with high mortality and morbidity (1). Breast cancer occurs in the mammary tissues of woman and men and this problem has become more intense in the last few years (2). Moreover, it is well document-
ed that breast cancer is the primary cause of death in women-sufferers of cancer with a high mortality rate worldwide (3). The main issue underlying breast cancer-caused death is the metastasis of the cancer cells to the other organs or the whole body because of delayed diagnosis or inappropriate treatment approaches (4). Therapy approaches for breast cancer mainly include surgery, radiation and chemotherapy (3). Metal-based compounds such as cisplatin, ruthenium and gold are widely used in cancer treatments due to their growth inhibition efficacies. However, they have several limitations such as severe side effects, narrow bioavailability and difficult administration. Furthermore, development of resistance to current chemotherapeutic agents is frequently reported (5). Based on the above mentioned limitations, investigations...
into finding novel agents for cancer therapy have increased over the last decades. Today, metal-based agents are one of the most investigated compounds because of their anti-cancer potencies. Vanadium and its various compounds are the focus of cancer research into using metal-based agents. Ovary cancer, testicular cancer, basophilic leukemia, lymphomas, nasopharyngeal tumors, bone tumors, and neuroblastomas, were investigated as cancer types against which the anti-cancer efficacies of vanadium and its compounds were demonstrated (5,6). Vanadium as a micronutrient as well as vanadium compounds were determined to be effective in killing cells of a variety of human cancers (7). Vanadium enters the human body mostly via the daily consumption of food such as black pepper, parsley, mushroom etc. and is found in two oxidation states (+4 and +5). The oxidation state of +4 is called vanadyl cation and diffuses into the cell through the cell membrane or uses anion channels. Vanadium also exists in extracellular fluids in the form of metavanadate (+5 oxidation state). Also, human blood contains vanadium in the range of 0.42 and 0.08 µg/L (8). Furthermore, vanadium and its compounds were found to accumulate in cancerous cells/tissues more than normal cells (9). Accordingly, it was shown that the accumulation of vanadium and vanadium compounds in the heterochromatin sides of the nuclei temporarily suppresses mitosis and leads to a reversible inhibition of cell cycle at late S and G2 phases (10).

The first anti-cancer research using vanadium salts dates from 1965. Thereafter, a variety of vanadium salts were investigated in several malign cell lines such as B and T cell lymphoma, hepatoma, osteosarcoma and testis, uterus, lung, kidney, nasopharynx and esophagus carcinoma cells (10,11). In addition, the salt vanadyl sulphate (+4), was found to have high anti-cancer activity under lymphoma, neuroblastoma, T cell, basophilic and erytroleukemia, liver, over, testis, esophagus and bone tumor cell lines (6,12,13). Also, the cytotoxic effect of vanadyl sulphate was reported to be lower in normal cells than in the cancer cells (14). Moreover, a greater accumulation of vanadium in cancerous breast tissues than in normal tissues has been well documented (9). The most important property of vanadium compounds to be investigated in terms of cancer research is that they have the potential to be an ideal/near ideal agent for cancer treatment in terms of inhibiting cell growth, causing cytotoxicity, stimulating cell death (apoptosis/necrosis), decreasing/inhibiting metastasis as well as reducing resistance development in cancer cells (15). In addition, their antiproliferative and proapoptotic activities vary among cell types exposed to them (12). Despite all of the above given properties, the anti-cancer activities of vanadyl sulphate on human breast cancer cells MCF-7 are still poorly investigated. Based on this knowledge, herein it is aimed to explore the cytotoxic, antiproliferative and proapoptotic potencies of vanadyl sulphate on human breast cancer cells MCF-7 along with vanadyl sulphate-derived morphological and ultrastructural changes in the cells.

**MATERIALS AND METHODS**

**Materials**

MCF-7 (ATCC® HTB-22™) cells were purchased from the American Type Culture Collection (Manassas, USA). Vanadium (IV)-oxid sulphate pentahydrate pure (VOSO₄) obtained from (riedel-de Haen cat: 14229 Lot: s29267-275, CA) was used as a test agent. Dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide) (M2003), Dulbecco’s Phosphate Buffered Saline (PBS) were purchased from Sigma-Aldrich (St. Louis, USA), Roswell Park Memorial Institute medium (RPMI-1640) was obtained from Gibco (Grand Island, USA), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Merck Schuchardt (Darmstadt, Germany). Osmium tetraoxide, gluteraldehyde, araldite, propilene oxide, uranyl acetate, lead citrate were from Electron Microscopy Science (USA). Cell Cycle DNA test plus reagent Kit and Annexin V apoptosis detection kit were from BD, Pharmingen (USA) and phalloidin, Anti-E cadherin were obtained from Thermo Scientific (USA). Fluo-3, ATP, Anti-cyclin B1 and Anti-cyclin D1 were purchased from Santa Cruz (CA, USA).

**Methods**

**Cell Culture**

Breast cancer cells MCF-7 (ATCC® HTB-22™) were cultured in RPMI 1640 medium (Gibco, USA) supplemented with penicillin-streptomycin (1%), fetal bovine serum (10%) at 37°C in a humidified atmosphere with CO₂ (5%). Passage 8 cells with the confluency of 85% were used as test cells in all experimentations.

**MTT Colorimetric Assay**

For the cell viability assessment, a MTT assay was used. In short, a stock solution of vanadyl sulphate was prepared in distilled water. MCF-7 cells were plated at a concentration of 1 ×10⁵ cells per well into 96-well plates. Concentrations ranging from 20-170µM were exposed to the cells and incubated for 24 hours under incubator conditions of 37 °C in a humidified atmosphere with 5% CO₂ in air. After incubation, the media were removed and MTT (20 µL in 200 µL fresh medium/per well) was added to the cells and re-incubated for 2 hours under the same incubation conditions. Following, incubation media from each well were replaced with 200 µL DMSO and plates were read on an ELISA reader at a wavelength of 570 nm (n=3). Viability percentages and the IC₅₀ value were determined from the absorbances from the ELISA reader by using the TRAP Version 1-22 programme of the United States Environmental Protection Agency (EPA).

**Analyses of the Morphological Changes**

For detecting any morphological changes in the MCF-7 cells caused by vanadyl sulphate, the confocal microscopy method was used. In this method, MCF-7 cells were plated on coverslips in 6-well plates at (3x10⁵/well) and exposed to IC₅₀ dose of vanadyl sulphate for 24 hours at 37 °C for 24 h. A plate of cells was kept untreated as control cells. Following the incubation period, cells were double stained with phalloidin and acridine orange at room temperature. Stained cells were imaged under a
confocal microscope Leica TCS-SP5 II supplemented with Leica Confocal Software Version 2.00 (16).

**Semi-quantitative Measurement of Intracellular Calcium Level**

The confocal microscopy technique was used for detecting any changes in intracellular calcium level. In this respect, MCF-7 cells were seeded onto circular coverslips (3x10⁵) and incubated for 24 hours at 37°C, 5% CO₂, incubator conditions with the IC₅₀ value of vanadyl sulphate. Untreated MCF-7 cells were grown under the same conditions as control cells. After incubation, all the cell groups were washed in PBS and incubated with fluo-3 dye solution containing pluronic acid for one hour under the same incubation conditions. Following this, coverslips were re-washed, placed on a sample holder and analysed after adding ATP solution under a confocal microscope Leica TCS-SP5 II supplemented with Leica Confocal Software Version 2.00 (17).

**Analysing the Ultrastructural Changes**

For ultrastructural analyses vanadyl sulphate (IC₅₀ value) treated MCF-7 cells were fixed in glutaraldehyde and post fixed in osmium tetroxide (2%). Following fixation, the cells were dehydrated in graded ethanol then embedded in Epon 812 epoxy. After polymerisation for 48 hours at 60°C, samples of thin sections were prepared at a maximum thickness of 100 nm. The sections were placed under copper grids and were stained with lead citrate and uranyl acetate, respectively. Stained samples were imaged under a TEM (FEI Tecnai BioT-WIN, The Nederlands) (18).

**Analysing the Cell Death Mode**

The flow cytometry technique was used for detecting the cell death mode caused by vanadyl sulphate on MCF-7 cells. For this process, the MCF-7 cells were plated in 6-well plates at a density of 5x10⁵ cells/well. After this, cells were exposed to IC₅₀ value vanadyl sulphate for 24 hours at 37°C, in a 5% CO₂ incubator. At the end of incubation period, the cells were harvested by trypsinization, washed (2xPBS) and the cell count in 1 mL of medium was adjusted to 1x10⁶ cells. For the staining process, 5 µL of annexin and 5 µL of PI were added to a facs tube, then 100 µL of cell suspension was added to the tubes containing the fluorescent dyes. Samples were incubated in the dark at room temperature for 15 minutes. After the incubation period the samples were read on a flow cytometer (BD FACSCalibur™, USA) according to the user manuals processes laid out in the Annexin V-FITC apoptosis detection kit (BD, Pharmingen, USA). All steps were done in triplicate.

**Labelling the Cyclins and E-cadherin Proteins by Immunohistochemical Analyses**

The vanadyl sulphate treated and untreated MCF-7 cells were incubated in the above mentioned incubator conditions. After the incubation period, the cells were fixed in glutaraldehyde (4%), placed on slides using a CytoSpin 3 (Thermo Scientific Shandon) device and were allowed to air dry. The dried samples were washed in distilled water and kept in PBS containing tween 20 for 3 minutes at room temperature. Then, all the samples were exposed to hydrogen peroxide (3%) for 10 minutes in a humidified staining chamber at room temperature. After this step, the samples were washed again for 3 minutes at room temperature in PBS (with tween 20) and kept in the staining chamber with ultra V block solution for 5 minutes under the same conditions. After that, the samples were incubated with the primary antibodies (Anti-cyclin B₁, Anti-cyclin D₁ and Anti-E-cadherin) for 1 hour in dilutions as indicated in the user manuals of Santa Cruz and Thermo Scientific, respectively. Then, an amplifier was added to the samples and treated with secondary antibodies for 30 minutes at room temperature. At the end of incubation time, AEC chromogene and haematoxylin retrieval were performed and samples were washed with distilled water and mounted with coverslips for imaging under a light microscope (Leica DM6000 B).

**Statistical Analysis**

For detecting the IC₅₀ value and confidence intervals of 95%, TRAP Version 1-22 software of the United States Environmental Protection Agency (EPA) was used. The results were evaluated by using one way anova for multiple comparisons and Tukey post-test of Graphpad Prism 6.0 for Windows. The data showed as Mean±SDs and p<0.05 was taken as significant.

**RESULTS**

**MTT Cytotoxicity Assay Findings**

The viability percentages of the MCF-7 cells exposed to different concentrations of vanadyl sulphate for 24 hours decreased according to dose (Figure 1). The half maximal inhibition concentration (IC₅₀) of vanadyl sulphate for 24 hours on the MCF-7 cells was detected to be 85µM (82,29-91,01 µM with confidence interval of 95%). A statistically significant decrease (p<0.05) was detected at the IC₅₀ concentration applied to the cells.

**Confocal Microscopic Findings**

Intensive alterations were detected on the confocal microscopic evaluation of the MCF-7 cells treated with IC₅₀ concentration of vanadyl sulphate for 24 hours when compared to the
untreated MCF-7 cells (Figures 2a and 2b). The fusiform shape of the untreated cells (Figure 2a) changed to a shrunken, circular shape in the treated cells (Figure 2b). The compact nuclei and cytoskeleton of the untreated cells were perforated, disintegrated as well as fragmented with the application of the vanadyl sulphate. In addition to the chromatin condensation that was shown in almost all cells exposed to the agent, a pyknotic nucleus formation was detected and the micronuclei of the treated cells were raised in number (Figure 2b).

Semiquantitative Measurement Evaluation Results of Basal Calcium Levels
The basal calcium level of untreated MCF-7 cells was detected to be 9.85 whereas this level in vanadyl sulphate treated cells increased to 13.47 (p<0.05) (Figure 3). This was a semiquantitative evaluation of basal calcium levels of cells. The emission of fluo-3 dye was taken for evaluation thus the result is given without a unit. Each examination was done in triplicate. The standard deviations for the replicates of each group of cells were slightly increased (Figure 3). This was attributed to the property of MCF-7 cells that were grown on cell culture flasks as multilayers of different thicknesses. Thus the emission of fluo-3 dye might be detected at different levels with confocal microscopy.

Transmission Electron Microscopy Findings
Ultrastructural changes derived from vanadyl sulphate being applied to MCF-7 cells were evaluated under a TEM. All the alterations detected in the TEM images of vanadyl sulphate treated cells were compared with the TEM images of the untreated MCF-7 cells. The cell membrane, cytoskeleton, membranous organelles, nuclear membrane, nucleolus and the chromatin of the untreated MCF-7 cells were compact, without disintegration or fragmentations (Figure 4a). In contrast with the control cells, the cellular membrane of vanadyl sulphate treated cells was found to undulate and make blebs (Figure 4b). The cytoskeleton of treated cells lost their integrity and the formation of autophagosomes, secondary lysosomes and lipid droplets as well as vacuolization were detected (Figures 4c, d, and e). The membranous organelles of vanadyl sulphate treated MCF-7 cells were lacerated (Figure 4e) of which the most affected organel was found to be the mitochondrion. Mitochondria were found disintegrated with swollen compartments and clear loss of cristae (Figures 4f and g) that might lead to their dysfunction.

Flow Cytometry Findings
In the annexin-V/PI analyses findings the apoptosis triggering activity of vanadyl sulphate was underlined. 100% of untreated MCF-7 cells stained with annexin-V/PI were found to be alive (Figure 5a). The population of MCF-7 cells exposed to the half maximal inhibition concentration of vanadyl sulphate for 24 hours resulted in 51.7% total apoptotic cells of which 9.9% were early apoptotic and 41.8% late apoptotic cells. The percentage
of necrotic cells in this group was 4.3% and 44% of the treated cells were live cells (Figure 5b).

**Immunohistochemical Results**
The semiquantitative evaluation results of cyclin B1 and D1 and E-cadherin are shown in Table 1. The average staining score of the untreated MCF-7 cells was 0.66. The scoring method for vanadyl sulphate treated MCF-7 cells was made in comparison to untreated cells. The cyclin B1 staining average score decreased to 0.33 in the treated cells. In contrast, the cyclin D1 average increased threefold to 1.66 in the same group of cells. The E-cadherin staining score of vanadyl sulphate treated MCF-7 cells was raised to 2, i.e. almost three times bigger than that of control cells (Table 1).
The stainings of cyclins (B1, D1) and E-cadherin of MCF-7 control cells are shown in Figures 6a, b and c, respectively. In Figures 6d, 6e and 6f the stainings of cyclin B1, cyclin D1 and E-cadherin for vanadyl sulphate treated MCF-7 cells are given. These images were used in the scoring method whose results are given in Table 1. Images were given to show the staining type of the cells.

### DISCUSSION

Cancer treatment with metal based chemotherapeutics has become an intensively applied method in current therapy. Current therapeutics such as cisplatin, gold and ruthenium are quite effective in killing cancer cells due to their biochemical properties on inhibiting the growth of cancer cells (5). How-

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<th>Cyclin B1</th>
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<th>2. Replication</th>
<th>3. Replication</th>
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<tbody>
<tr>
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<td>1</td>
<td>0</td>
<td>0.66</td>
</tr>
<tr>
<td>Vanadyl sulphate treated cells</td>
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<td>0</td>
<td>1</td>
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<tr>
<td>Vanadyl sulphate treated cells</td>
<td>1</td>
<td>2</td>
<td>2</td>
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<td>Vanadyl sulphate treated cells</td>
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Table 1. Semiquantitative evaluation of labelings of cyclins (B1, D1) and E-cadherin in untreated and vanadyl sulphate treated MCF-7 cells. In this table, unstained cells were indicated by a 0, slightly stained by 1, moderately stained by 2 and intensively stained cells were indicated by the number 3.
ever, their strong side effects and development of resistance make their application and effectiveness on cancer therapy limited (19). Thus, novel agents for cancer treatment are really needed. Vanadium and its compounds have recently been reported as good candidates for anti-cancer potency (5). Based on this, in this study, we evaluated the anti-cancer potency of a vanadium salt, vanadyl sulphate, with a multi-mode assessment approach on its antiproliferative, cytotoxic and proapoptotic properties on human breast cancer MCF-7 cells. According to the MTT findings, the viability of MCF-7 cells decreased dependent on dose when applied for 24 hours with different vanadyl sulphate concentrations. As shown in Figure 1, the half maximal inhibition concentration (IC_{50}) of vanadyl sulphate on MCF-7 cells was determined to be 85 µM for this length of exposure. Similarly with our results, in L929 mouse fibrosarcoma cells and human hepatocarcinoma HepG2 cells vanadyl sulphate was shown to act as an antiproliferative agent according to dose and time (14). In addition, in another study, A549 human lung adenocarcinoma cells were inhibited with an IC_{50} value of 15 µM. In the same study, human prostate cancer cells DU145 were totally inhibited by the application of IC_{50} concentration of 15 µM (20,21). This can be attributed to the dependence of the antiproliferative activity of vanadyl sulphate on the cells type and application dose and time. This property of vanadium and vanadium compounds has been well described in a previous study (14).

Apoptosis triggering activity on malign cells is the most common property of vanadium compounds (14). For vanadium compounds, it is described that they are proapoptotic via DNA fragmentation (9,14). Based on the investigations into finding an apoptosis stimulating agent for cancer therapy (19), we evaluated the mode of cell death triggered by vanadyl sulphate application to MCF-7 cells. On the morphological analyses on confocal microscopy, on MCF-7 cells exposed to IC_{50} value of vanadyl sulphate for 24 hours clear apoptotic sparks such as chromatin condensation, fragmentation of cell and the nuclei, hole formation in the cytoskeleton and horse-shoe nucleus formation were detected (Figure 2). In line with our results, on A549 and DU145 cells exposed to vanadyl sulphate researchers have shown apoptotic cells with fragmented nuclei (21).

In one piece of research it was reported that increased intracellular basal calcium level lead to apoptosis (22). The death of T-cell hybridoma cells is found to be related to the increased intracellular calcium level (23). In accordance with these findings in our study, the basal intracellular calcium level of MCF-7 cells exposed to vanadyl sulphate for 24 hours was found to be augmented (Figure 3). The apoptosis triggering activity of vanadyl sulphate on MCF-7 is underlined in our study based on the morphological and ultrastructural changes (Figure 4) of the applied cells as well as annexin V-FITC and PI evaluations of cell death mode (Figure 5) in the same group of cells. In annexin-V analyses results, apoptosis was shown in 51.7 % of vanadyl sulphate applied cells. In addition the detected increase in basal calcium levels of the treated cells suggest apoptosis in accordance with the findings of other researchers on vanadate applied cells that reporting inhibition of Ca-ATPase activity, and in turn causing intracellular calcium accumulation and apoptosis (24). Results of a study on H35-19 rat hepatoma cells exposed to VOSO_4, NaVO_3, and NaVO_3 salts, showed undulations in the nuclear membrane, trabecular nucleus, fragmentations on the cisternae of Golgi apparatus as ultrastructural changes (25). Similarly, a loss of cristae and swelling of mitochondrion, shrunken cell structure, secondary lysosome formation, chromatin condensation and pyknotic nuclei were detected as ultrastructural changes after exposure to vanadyl sulphate for 24 hours.

Sodium orthovanadate was reported to stop cell cycles by causing increased cyclin B1 expression on HepG2, Sk-Hep-1 and Hep3B liver carcinoma cells relative to dose (14). Moreover, oxovanadium compounds were declared to inhibit cell cycles at a concentration of 100 µM over the course of a 24 hours application (26). In our study, in vanadyl sulphate treated MCF-7 cells, cyclin B1 expression was relatively decreased (Table 1) compared to the control cells. On the contrary, cyclin D1 expression on these cells was significantly increased (Table 1).

The expression of E-cadherin in a variety of aggressive human cancers has been reported to be reduced (27). In breast cancer cells the expression of this protein was declared to be partly or totally absent. The absence of E-cadherin expression makes breast cancer cells more invasive and metastatic (28). Stimulating expression of E-cadherin in breast cancer cells was determined to significantly inhibit the proliferation of cells in vitro and in vivo (20). Herein, it was detected increased E-cadherin expression in MCF-7 cells caused by vanadyl sulphate application. Our result is supported by a study that declared vanadium (IV) compounds to inhibit the invasion and adhesion of osteosarcoma cells relative to dose (29).

In conclusion, vanadyl sulphate was found to be cytotoxic and antiproliferative on MCF-7 cells when applied for a short time. It triggered apoptosis by changing the morphology and ultrastructure of the exposed cells. Thus, vanadyl sulphate might be suggested for further investigations for its usage in cancer therapy due to its anti-cancer properties considered above.

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