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Assessment of cranberry bush on MCF-7 human breast cancer cells

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This research aimed to study the therapeutic potential of *Viburnum opulus* L. (VO) powder extracts on human breast cancer and normal breast epithelial cell lines. The half-maximum inhibitory concentration IC_{50} value was 51.32 µg/mL for 24 h using an MTT assay. The total apoptotic induction was found to be 40.50% for MCF-7 cells exposed to 100 µg/mL VO powder extract for 48 h. The genotoxic effect in MCF-7 cells was statistically significantly increased in a dose-dependent manner after exposure to VO powder extracts. P1 class glutathione-S-transferase (GSTP1) expression level was noticed protective feature in drug resistance metabolism. GSTP1 activities were found statistically significant differences between all of the groups (**P <0.01). GSTP1 overexpressed in MCF-7 control cells and expression levels decreased in the MCF-7 groups that were treated with VO powder extract at different concentrations of 25, 50 and 100 µg/mL for 24, 48 and 72 h. These results suggest that VO powder extracts suppress the proliferation and GSTP1 expression of MCF-7 cells and this suppression is attributed to both induction of apoptosis and DNA damage. This study provided the information that VO powder extract may be suitable for drug therapy efficacy at the molecular level.

Keywords: Alternative medicine, Apoptosis, Genotoxicity effects, Metabolic protein level

Breast cancer is a type of cancer that is very common among women with different symptoms[1] which has a highly heterogeneous disease involving genetic and environmental factors[2] but its onset mechanisms are still unclear[3]. It has been reported that there are various mechanisms in the initiation and progression of breast cancer at the cellular level by researchers[4]. The standard treatment for breast cancer is chemotherapy but a combination therapy of chemotherapy and anticancer drugs count on for alternative treatment[5]. In the treatment of breast cancer, specific enzymes that reduce the cytotoxic activity of drugs regardless of intracellular drug concentration can induce resistance to cancer[6]. Among them, enzymes, glutathione S-transferase P1 (GSTP1) handles targeted drug resistance in a wide variety of chemotherapeutic agents. GSTP1 is an important isozyme belonging to the glutathione *S*-transferase (GST) family. This enzyme catalyzes the conjugation of the reduced form of glutathione to xenobiotic substrates for detoxification[7]. They have reported that tumour cell lines over-expressing GSTP1 have shown resistance to too many of the chemotherapeutic drugs and also achieved that GSTP1 inactivates chemotherapeutic drugs by conjugating them to glutathione (GSH)[8]. Although many anti-cancer compounds are not good substrates of GSTP1, because of the high levels of GSTP1 are still unclear. Recent research has suggested that GSTP1 has different functions on cancer cells apart from detoxifying chemicals or drugs[9].

Anticancer therapies, including pathway inhibitor therapy and cytotoxic chemotherapy, can induce apoptosis in many cancer cells. Apoptosis is programmed cell death and is triggered by the activation of a genetically controlled self-destruct mechanism. Some cancer cells do not undergo apoptosis and may survive longer than the cells from which they originated[10]. When cancer patients are treated, resistance to treatment develops in cancerous tissues thanks to the mechanisms of escape from "apoptosis to resistance"[11]. Resistance to chemotherapy in cancer cells has been attributed to multiple mechanisms that often act together, including escape resistance from apoptosis[12].

While breast cancer death rates decrease in developed countries, breast cancer density increases due to changing life conditions[13]. At this point,

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, Ethacrynic acid; GST, glutathione S- transferase; GSTA1, glutathione S-transferase A1; GSTP1, glutathione S-transferase P1; MCF-10A, Michigan Cancer Foundation-10A-non-tumorigenic human breast epithelial cell line; MCF-7, Michigan Cancer Foundation-7-human breast adenocarcinoma cell line; VO, *Viburnum opulus*

researchers have focused on early detection, advanced and new therapeutics and more effective treatment methods for breast cancer[14]. It also has been reported that antioxidants provide a reduction in different diseases such as liver diseases, inflammation, cancer and ageing since the 20th century. Antioxidants are effective as a daily supplement in the prophylactic treatment of different diseases, in reducing the side effects of chemo and radiotherapy and in reducing oxidative stress in cancer[15]. However, there is controversy about the effectiveness of these compounds in the treatment of breast cancer due to having both antioxidant and pro-oxidant properties depending on the tumour type, time and environment at the same molecular concentration.

Viburnum opulus (VO) is a plant belonging to the Adoxaceae family, this plant with red coloured, chickpea-sized fruit, which is distributed in a wide range from South America to South East Asia and has over 230 species, most of which are endemic[16]. It is called as guelder rose, European guelder, European cranberrybush, water elder, rose elder, rose ebru, cherry-wood, crampbark, snowball tree, and as gilaburu in Turkey. Many studies have investigated the chemical contents of VO[17] considering the phenolic compounds in the VO fruit, it is rich in gallic acid, catechin, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, vanillic acid and quercetin, and besides these, there are organic acids malic, succinic, tartaric, fumaric and acetic acids in the VO's composition fruit. In research examining the juice of VO fruit, some state that VO fruit juice has a stimulating effect on the immune system with some sugars, such as glucose, fructose, sucrose and vitamin C[18]. The researchers have investigated the biodiversity and biochemical properties of VO which also characterized the total phenolic compounds and ascorbic acid amounts. The presence of these active compounds in large amounts in the fruit of the VO species suggested that could use this species as powerful antioxidants[19] also is used for medicinal because the antioxidants it contains prevent some stomach and renal diseases of the VO fruit juice. It has been used traditionally to treat colds, ulcers, nervous system disorders, diabetes and hypertension[20].

However, anti-cancer drugs can directly kill not only cancer cells but also healthy cells. However, the side effects of chemotherapy vary depending on the dose of the drugs used, and these side effects may cause tumour regeneration or display resistance to the anticancer drugs. Especially, the high death rate in treated patients with breast cancer considers the effectiveness of anti-cancer drugs.This study, it was aimed to investigate VO powder extract to MCF-7 breast cancer cell line and also MCF-10A non-tumoral breast cell line after the application and the effect of VO powder extract on both drug resistance metabolism and anti-tumoral properties.GSTP1 expression level which has a protective feature in drug resistance metabolism was studied by immunocytochemistry method and GSTP1 and total GSTA1 metabolic protein level spectrophotometer method. The anti-tumoral properties of VO powder extract were determined by applying cytotoxicity, genotoxicity and apoptosis.

Materials and Methods

Plant material and preparation of extract

VO was identified by Prof. Dr Ahmet Aksoy at the Department of Botanic, Akdeniz University. The bunches with fruits of VO were collected from the region of Kayseri in Turkey around October 2020, after being combined and packed in plastic bags furthermore removed the stalks and crushed the fruits with a glass stick. The supernatant of clear juice was poured into glass bottles after centrifuging the mash at 6000-gauge for 20 min. finally, were lyophilized with a Labconco freeze dryer (model 117-A65312906; FreeZone 2.5) for 24 h, and then stored in plastic vials at −80°C until analysis after centrifuged extracts. On the analysis day, VO powder extract was sterilized with 0.45 and 0.20 μm filter in which sterile phosphate buffer saline (PBS) and 1:1 diluted with injection water (for pH value control) for 24, 48 and 72 h. The cells were exposed to different concentrations (25, 50, and 100 μg/mL) of VO powder extract which sterilized with 0.45 and 0,20 μM filters.

Cell lines

The human breast cancer cell line, MCF-7, and the non-tumorigenic human breast epithelial cell line, MCF-10A, were used in this study. MCF-7 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) high medium containing 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL sodium pyruvate, 0,01 mg/mL human insulin and 1% penicillin/streptomycin and maintained at 37 °C in 5% CO_2 incubator. MCF-10A, epithelial normal cells were cultured in DMEM low medium containing 5% fetal bovine serum, 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor (EGF), 0,01 mg/mL human insulin and 500 ng/mL hydrocortisone maintained at 37°C in 5% CO_2 incubator changing every 48 h with corresponding assay medium.

Assessment of the cytotoxic effect of VO powder extract by MTT assays

The cell lines were dispensed 96 well plates at a density of 1×10^4/well. After 24 h incubation, the cells were exposed to different concentrations of extract (25, 50, 100 µg/mL) for 24, 48 and 72h, the end times cells per well were incubated with 10 µL of a 5 mg/mL MTT stock in phosphate-buffered saline (PBS) solution for 4 h. MTT formazan crystals were then resolubilized by adding 100 µL 100% dimethylsulfoxide (DMSO) to each well. The plates were shacked for 5 min. Absorbance at 560 nm was determined using 750 nm as the reference wavelength by using an ELISA reader (Bio-Tek Instruments, Inc., USA). All the experiments were done in triplicates in at least three cultures from cell lines and represented the data as per cent viability compared to control. The half-maximal inhibitory concentration (IC_{50}) was calculated with GraphPad Prism 6 software.

Induction of apoptosis to MCF-7 by VO powder extract using annexin V-FITC/PI staining

The percentage of cells showing changes in cell death was determined using the 'Annexin V & Dead Cell Assay kit (MCH100105)' according to the manufacturer's instructions (Merck Millipore, Darmstadt, Germany), at the same time quantitative analysis of living, early and late apoptosis and dying cells investigated with this assay. Annexin V is a calcium-dependent, phospholipid-binding protein and has a high affinity for phosphatidylserine (PS), a membrane component localized on the inner surface of the cell membrane. This method is based on the detection of PS on the outer membrane with labelled Annexin V-FITC/Propidium Iodide (PI) using the dead cell marker 7-Aminoactinomycin D (7-AAD). The cell lines were dispensed 6 well plates at a density of 1×10^6/well after 24 h incubation and also were exposed to different concentrations of extract (25, 50, 100 µg/mL) for 24, 48 and 72 h. Non-apoptotic cells:Annexin V (-) and 7-AAD (-) • early apoptotic cells: Annexin V (+) and 7-AAD (-) • late stage apoptotic and dead cells: Annexin V (+) and 7-AAD (+) • nuclear debris: Annexin V (-) and 7-AAD (+) type of four populations of cells can be distinguished in this assay were counted by the Muse Cell Analyzer (Merck Millipore).

Assessment of the DNA damages of MCF-7 and MCF-10A cells treated with VO powder extract

The cells were dispensed 6 well plates at a density of 1×10^5/well after 24 h incubation and exposed to different concentrations of extract (25,50,100 µg/mL) for 24, 48 and 72h. The alkaline Comet assay was performed as previously described which is associated with DNA damage compared with Comet tail length however indicates more DNA damage depending on longer tail length[21]. All the experiments were done in triplicates in at least three cultures from the cell line.

Assessment of the glutathione *S*-transferase activity of MCF-7 and MCF-10A cells treated with VO powder extract

The glutathione-S-transferase activity was measured by the optimized method of Habig[22]. At this method, GSTA1 for total GST activity, GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (ε = 9.6 $mM^{-1}cm^{-1}$), Etacrynic acid (EA) (ε = 5.0 $mM^{-1}cm^{-1}$) were used as substrates for total GSTP1 activity determinations at 340 nm, 270 nm, respectively. Activity determinations were performed with comprised reduced glutathione (GSH) concentrations of 1 mM CDNB with 1 mM GSH; in a 1000 µL assay reaction mixture. During the experiments, the continuous absorbance readings were obtained for 5 min intervals by spectrophotometer. All the experiments were done in triplicates in at least three cultures from the cell line.

Assessment of GSTP1 expression in MCF-7 and MCF-10A cells treated with VO powder extract by immunocytochemistry confocal microscopy assay

The cells were seeded on coverslips in six plates at a density of 3×10^5 cells/well in DMEM-High and incubated in a humidified CO_2 incubator (5% CO_2, 37°C) and washed three times using 500 µL 1X PBS, were fixed with 400 µL of 4% paraformaldehyde (pH 7.4) in PBS for 10 minutes at 37°C and washed three times 500 µL 1X PBS. Fixed cells were permeabilized with 0.1% Triton X-100 in 1X PBS for 15 min at room temperature. Then the cells were washed three times again with 500 µL 1X PBS. To block cells, the cells can be incubated overnight in 2% BSA. Cells were then incubated overnight at 4°C with primary antibody diluted in 500 µL of 0.1% BSA for MCF-10A and MCF-7, MCF-10A and MCF7 were treated with VO extract separately. The primary antibody was used at a dilution of 1:100 in 500 µL 0, 1% BSA/PBS and then incubated for 3 h in the dark at room temperature. The cells were washed three times with 500 µL of 1X PBS and added the secondary antibody. This antibody was diluted and incubated for 2 h at room temperature in the dark. Finally ICC characterization with FITC for GSTP1 in MCF-7

cells, cells were mounted with an anti-fade solution and viewed on a Nikon-Eclipse Ti-S microscope applied to a Nis-elements 20x objective.

Statistical analysis

GraphPad Prism 6 software was used for statistical analysis. Tukey's multiple comparisons test and the One-way Annova test were used for significance analysis. A p-value of <.05 was accepted as statistically significant.

Results

Assessment of the cytotoxicity effects

To detect cytotoxicity percentage, MCF-7 and MCF-10A cells were treated with different concentrations of VO powder extract for 24 48 72 h. MCF-7 and MCF-10A controls were without the extract treatment. The results of the cytotoxicity percentage were presented in (Fig. 1). The result shows that MCF-7 cells treated with different concentrations of the VO powder extracts and exposed to varying incubation times, such as 24, 48, and 72h, do not proliferate uniformly. However, MCF-7 and MCF-10A control groups had a 100% rate of cytotoxicity percentage. In this study, dose and time-dependent revealed that the group with the lowest concentration of 25 µg/mL from VO powder extract produced the lowest cytotoxicity percentage of MCF-7 cells of 111,3±0,8, 85,5±0,7 and 72,9±0,6 at 24, 48 and 72 h incubation periods, respectively. At the 50 µg/mL MCF-7/VO groups had cytotoxicity percentages of 98,4±0,5 73,8±0,9 and 68,5±1,2 at 24, 48 and 72 h incubation periods, respectively. Similarly, the group with the highest concentration of 100 µg/mL from VO powder extraction also had the highest cytotoxicity percentage 53,9±0,5 33,15±2,0 and 31,15±1,2 at 24, 48 and 72 h incubation periods, respectively (Table 1).The results show that VO powder extract applied different time and concentration-dependent activity on the proliferation of MCF-7 cells. When the half inhibitory concentration (IC_{50}) results were examined in the MCF-7/VO groups, the IC_{50} value was 51.32 µg/mL for 24 h (Fig. 2). In the MCF-10A/VO groups, proliferation was observed to increase in the groups applied at different concentrations at 24 and 48 h. In the highest concentration of 100 µg/mL from the VO powder extract group, it was observed that the cells

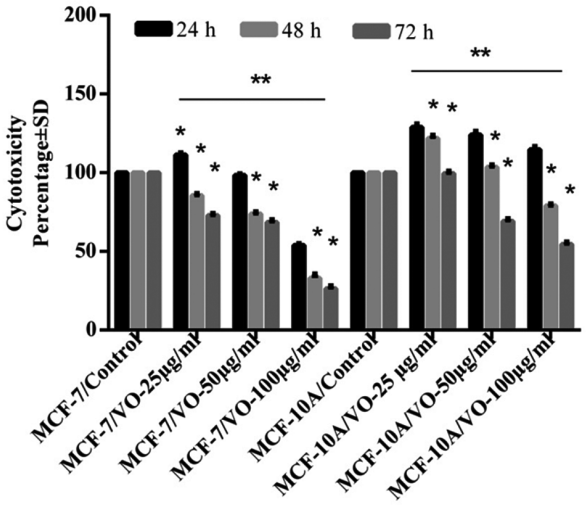

Fig. 1 — The MTT for cytotoxicity (%) assay results and effects of indicated different concentrations of VO powder extract for 24, 48 and 72 h, respectively, on the viability of the cells, were presented. The results of 1-way ANOVA analysis to compare fold differences of mean normalized % viability values among different concentrations of VO powder extract together and relative in MCF-7 and MCF-10A cells. Bars represent fold differences of mean normalized % viability values ± SD (n=3). **P* <0.05 compared to corresponding control compared to 25, 50 and 100 µg/mL concentrations of VO powder extract. ***P* <0.01 significant differences when compared with time, 24, 48 and 72 h

Table 1 — Cytotoxicity percentage of MCF-7 and MCF-10A cells cultured with and without VO powder extract. The statistical significance was indicated with letters. Activity levels were given with ± SD values. The cut of value for significance was taken as $P< 0.05$

MCF-10A Concentration (µg/mL)	24 h	48 h	72 h
		Percentage	
Control	100	100	100
25	128,9±1,^{7a,c}	121,9±1,4^{a,b}	99,5±1,1^{a,b,c}
50	124,1±1,6^{a,c}	103,5±0,9^{a,b}	69,9±1,1^{a,b,c}
100	111,6±1,6^{a,c}	79,0±0,7^{a,b}	54,5±1,0^{a,b,c}
MCF-7 Concentration (µg/mL)	24 h	48 h	72 h
		Percentage	
Control	100	100	100
25	111,3±0,8^{a,c}	85,5±0,7^{a,b}	72,9±0,6^{a,b,c}
50	98±0,5^{a,c}	73,8±0,9^{a,b}	68,5±1,2^{a,b,c,}
100	53,9±0,5^{a,c}	33,1±2,0^{a,b}	31,1±1,2^{a,b,c}

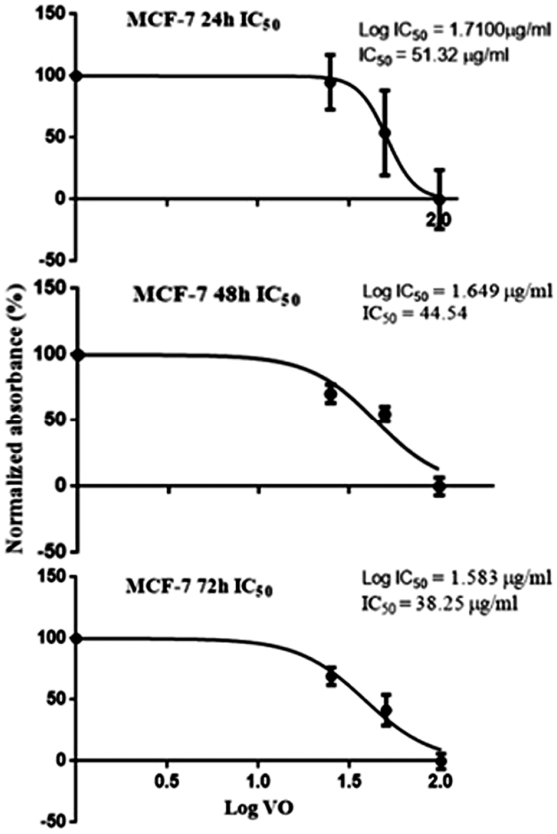

Fig. 2 — By using prism GraphPad 6, the curves of log IC_{50} of VO powder extract in the MCF-7 cells were illustrated for different time-dose dependent. Error bars represent the standard deviation from the mean

died due to prolonged waiting.The total cytotoxicity percentage of all of the groups that were dependent on time dose was found statistically significant difference between 24-48 h, 24 h-72 h and 48 h-72 h (*P <0.05) (Table 1).

Induction of apoptosis to MCF-7 cells treated with VO powder extract

The PI test was used to detect the percentage of late and early apoptotic cells. According to flow cytometry results, fluorescence signals from annexin V dye caused different binding of all conjugates to phosphatidylserine depending on different doses and time and different results emerged. The apoptotic cells were assessed by annexin V-FITC/PI staining. Figure 3 represents the percentages of annexin V positive cells in control and MCF-7/VO groups for 48 h. There were found statistically significant between the control and experiments groups that MCF-7/VO (****P <0.0001) (Fig. 4). The results of every group for different time-concentration dependent did not apoptosis evenly.

Assessment of the DNA damages of MCF-7 and MCF-10A cells treated with VO powder extract

Comet assay was used to detect the genotoxic effect by calculating the percentage of the tail length in the DNA of both cell groups during the test a comet appearance was observed in the DNA of the MCF-7/VO groups in which different concentrations of VO powder extract were applied for 48 h while no comet vision was observed in the control groups (MCF-7, MCF-10A and MCF-10A/VO) (Fig. 5) It was found significant differences of tail length as a percentage when compared with the control group at different concentrations (25, 50, 100 µg/mL) during 48 h (*P <0.05) nevertheless notable significant difference wasn't obtained in the other dose-time dependent groups. However, it was observed that VO powder extract caused damage to the DNA of cancer cells 24, 48 and 72 h after the treatment, compared to MCF-7 and MCF-10A/VO cells. According to the comet test results at the same time, the damage appeared in the DNA of MCF-10A cells, albeit very little, in the groups in which high doses (100 µg/mL) were applied for 72 h. As a result, it was revealed that the doses of VO powder extract in a short time damaged the DNA of cancerous cells, but in high doses for a long time, remarked side effects on healthy cells.

Assessment of GSTP1 and GSTA1 levels on MCF-7 and MCF-10A cells treated with VO powder extract

In this study, the goal was to determine GST isoenzymes levels in breast cell lines treated with VO powder extract. Enzymatic activities for the control groups of both MCF-10A and MCF-10A which treatment with VO powder extract (MCF-10A/VO) for 25 50 100 µg/mL for each separately 24 48 and 72 h, total GST mean (GSTA1) activities by using CDNB as substrate, were not found statistically significant difference at the between both of control groups (Table 2). On the other hand, statistically significant differences were found in the comparison of all the groups with one wayAnnova test (**P <0.01). GSTA1 activities for the MCF-7 and MCF-7/VO groups statistically significant differences found in the comparison with both MCF-10A and MCF-10A/VO from the control groups and also among themselves (Fig. 6A). GSTP1 activities by using EA as substrate were found statistically significant differences between all of the groups with oneway Annova test (**P <0.01) (Table 3). The statistically significant differences were found in the comparison of time and concentration before and after VO both in the 24, 48, and 72 h groups (Fig. 6B).

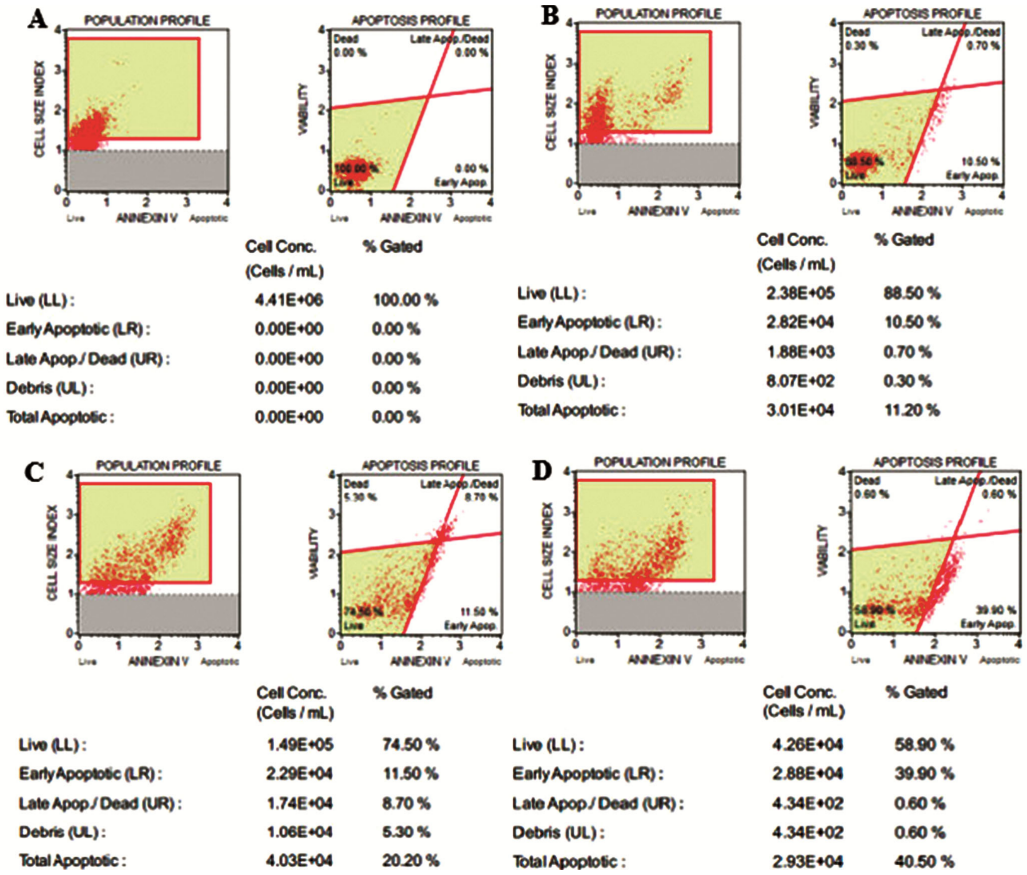

Fig. 3 — The percentage of Annexin V positive cells in control and VO powder exposed groups. Live cell population and early apoptotic, late apoptotic for MCF-7 cell treated with different concentrations (25, 50and 100 µg/µL) VO powder extract for 48 h. (A) control cells; (B) 25 µg/mL VO powder extract exposed MCF-7 cells; (C) 50 µg/mL VO powder extract exposed MCF-7 cells; and (D) 100 µg/mL VO powder extract exposed MCF-7 cells

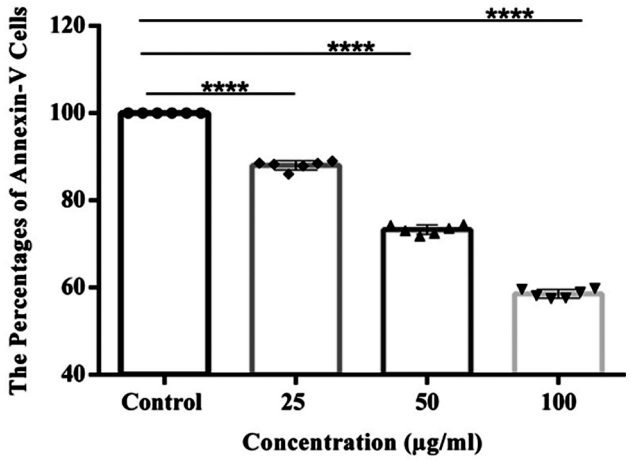

Fig. 4 — The percentage of Annexin V positive cells in control and VO powder extract exposed MCF-7 groups. MCF-7 cells were stained Annexin V-FITC/PI and analyzed by Muse Cell Analyzer. The data was showed that as the mean±SD from six independent experiments. The percentage of Annexin-V positive cells was statistically different between MCF-7 and MCF-7/VO groups (****P <0.0001). Error bars represent the standard deviation from the mean

Assessment of the expression of the GSTP1 protein in MCF-7 and MCF10-A cells treated with VO powder extract

To demonstrate the contribution of GSTP1 to cell protection and drug resistance by the treatment with VO powder extract in all of the groups, immunocytochemistry was performed using the GSTP1 antibody. GSTP1 overexpressed in MCF-7 control cells and expression levels decreased in the MCF-7 groups that were treated with VO powder extract at different concentrations of 25, 50 and 100 µg/mL for 24, 48, and 72 h. However, noticeable changes are not observed in GSTP1 expression levels in the MCF-10A cells and MCF-10A/VO powder extract cell groups at all doses and times.The cell protective feature of GSTP1 became active within 24 h of the application of VO powder extract and continued until the end of 72 h in the MCF-7 and MCF-7/VO powder extract groups. The lowest expression level of GSTP1 was seen at the MCF-7/100 µg/mL VO powder extract for 24 h when compared with the control group MCF-7. Images taken by the confocal microscope showed a remarkable

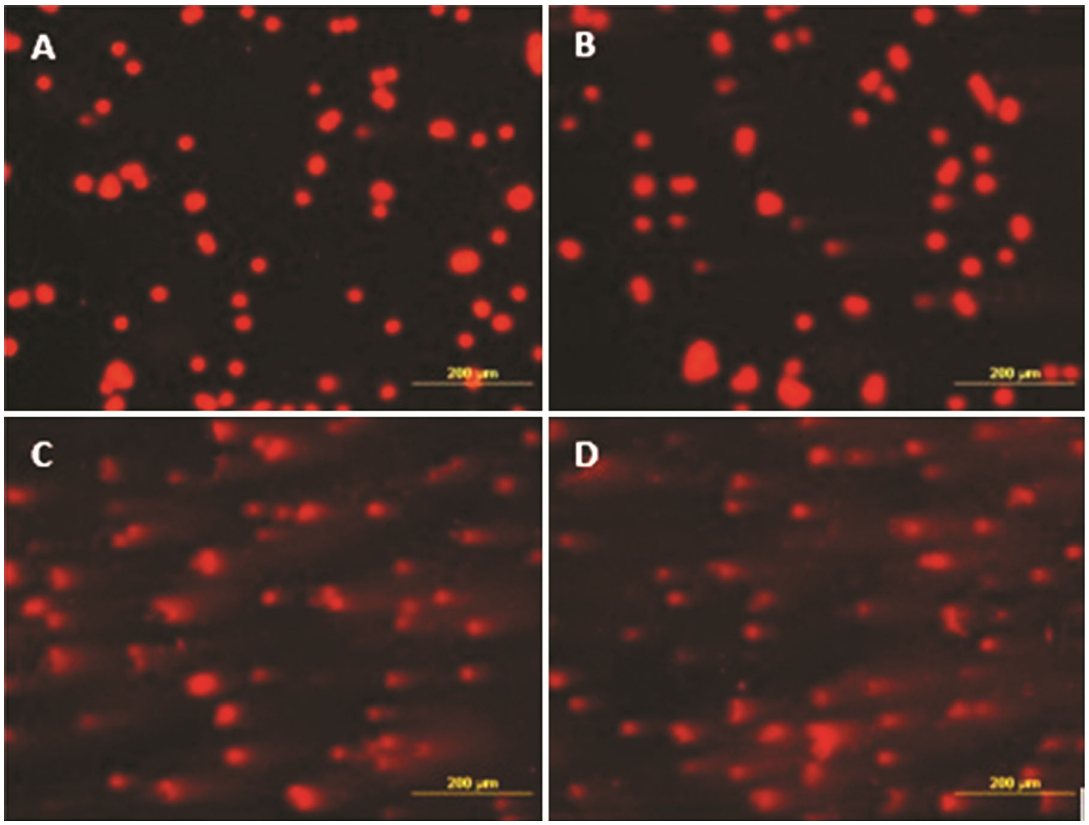

Fig. 5 — Evaluation of genotoxic effects by comet assay at different concentrations of VO powder extract damaged the DNA of MCF-7 cells. The percentage of tail length increased significantly statistically as dose-dependent for 48 h. (A) Control MCF-7 cells; (B) Treatment with 25 µg/mL VO powder extract for 48 h; (C) Treatment with 50 µg/mL VO powder extract for 48 h; and (D) Treatment with 100 µg/mL VO powder extract for 48 h. DNA comets were representative as seen under the fluorescent microscope (x200 magnification)

Table 2 — GSTA1 activity levels of MCF-7 and MCF-10A cells cultured with and without VO powder extracts. CDNB was used as a substrate. The statistical significance was indicated with letters. Activity levels were given with ± SD values. The cut of value for significance was taken as $P< 0.05$

MCF-10A GSTA1 Concentration (µg/mL)	24 h	48 h	72 h
		$nmole^{-1}min^{-1}mg$	
Control	2,7±0,2	$3,2±0,1^{d}$	$3,5±0,09^{e}$
25	$2,9±0,06^{b}$	$3,1±0,05^{f}$	2,8±0,2
50	$2,6±0,1^{b}$	$3,5±0,09^{f}$	2,2±0,06
100	$1,9±0,03^{b}$	$2,2±0,02^{f}$	2,7±0,1
MCF-7 GSTA1 Concentration (µg/mL)	24 h	48 h	72 h
		$nmole^{-1}min^{-1}mg$	
Control	$8,1±0,2^{a,b,c,f}$	2,3±0,1	1,3±0,03
25	$3,2±0,1^{a}$	$1,7±0,1^{b}$	$1,5±0,7^{c,d,e}$
50	$1,9±0,03^{a}$	$1,9±0,2^{b}$	$1,2±0,1^{c,d,e}$
100	$3,1±0,08^{a}$	$2,3±0,1^{b}$	$1,7±0,1^{c,d,e}$

reduction of the green FITC depending on dose time in MCF-7 cells (Fig. 7).

Discussion

Today, chemotherapeutic components damage healthy tissues and chemo-resistance of these components are still a major problem in cancer treatment. Chemotherapeutic drug resistance continues to hinder the effectiveness of cancer treatment.

Conjugation of glutathione with chemotherapy drugs is an important detoxification mechanism that reduces toxicity, limits the formation of DNA cross-

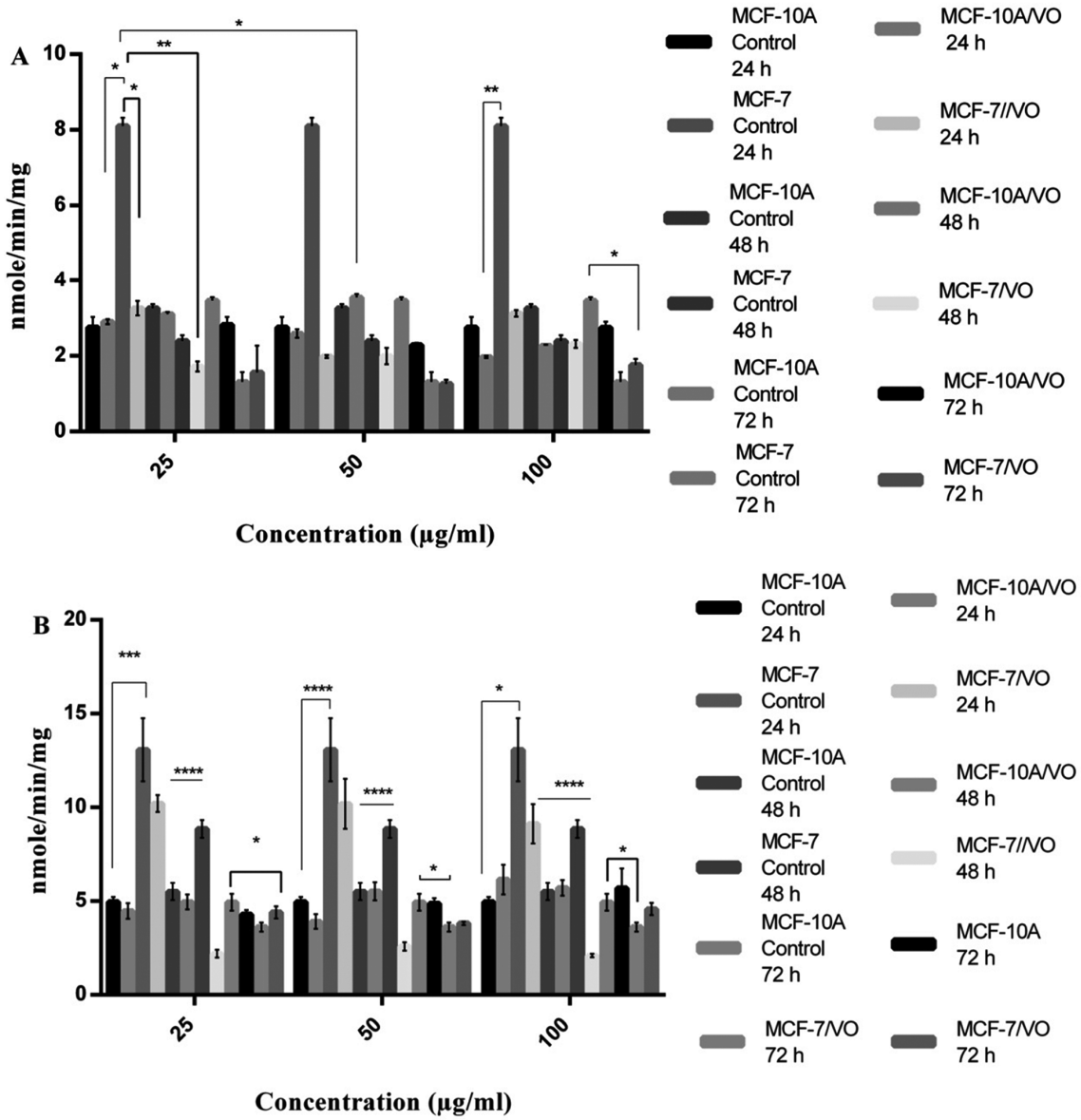

Fig. 6 — GST enzyme activities. (A) Comparison of GST A1 activities of MCF-10A cells and MCF-10A/VO powder extract, MCF-7 and MCF-7/VO powder extract cells by using CDNB as substrate; and (B) Comparison of GST P1 activities of MCF-10A cells and MCF-10A/VO powder extract, MCF-7 and MCF-7/VO powder extract cells by using EA as substrate (Bars represent fold differences of mean normalized nmole/min/mg±SD)

links and supports drug clearance[23]. Several studies have shown that GSTP1 is a crucial factor for tumorigenesis and tumour growth, based on the fact that negative effect on the viability or proliferation rate of cancer cells GSTP1[24]. GSTP1 expression levels were detected very high in many kinds of tumours and tumour cell lines including non-small cell lung, colon, pancreas ovarian and breast and in a wide range of drug-resistant cell lines and tumours for the reasons that are not always easily understandable. (When compared to normal tissues or wild-type cell lines, respectively)[25]. On the other hand, some researchers reported that GSTP1 expression level is lower in some cancers, including prostate cancer, than in the surrounding normal cells and this loss is due to abnormal hypermethylation of CpG islands in the promoter region of the GSTP1 gene. Therefore, loss of GSTP1 expression by hypermethylation of CpG islands in the GSTP1 promoter region, it has been reported that GSTP1 can serve as a molecular marker for the detection and diagnosis of prostate and some cancer types[26]. In addition, the level of GSTP1 protein

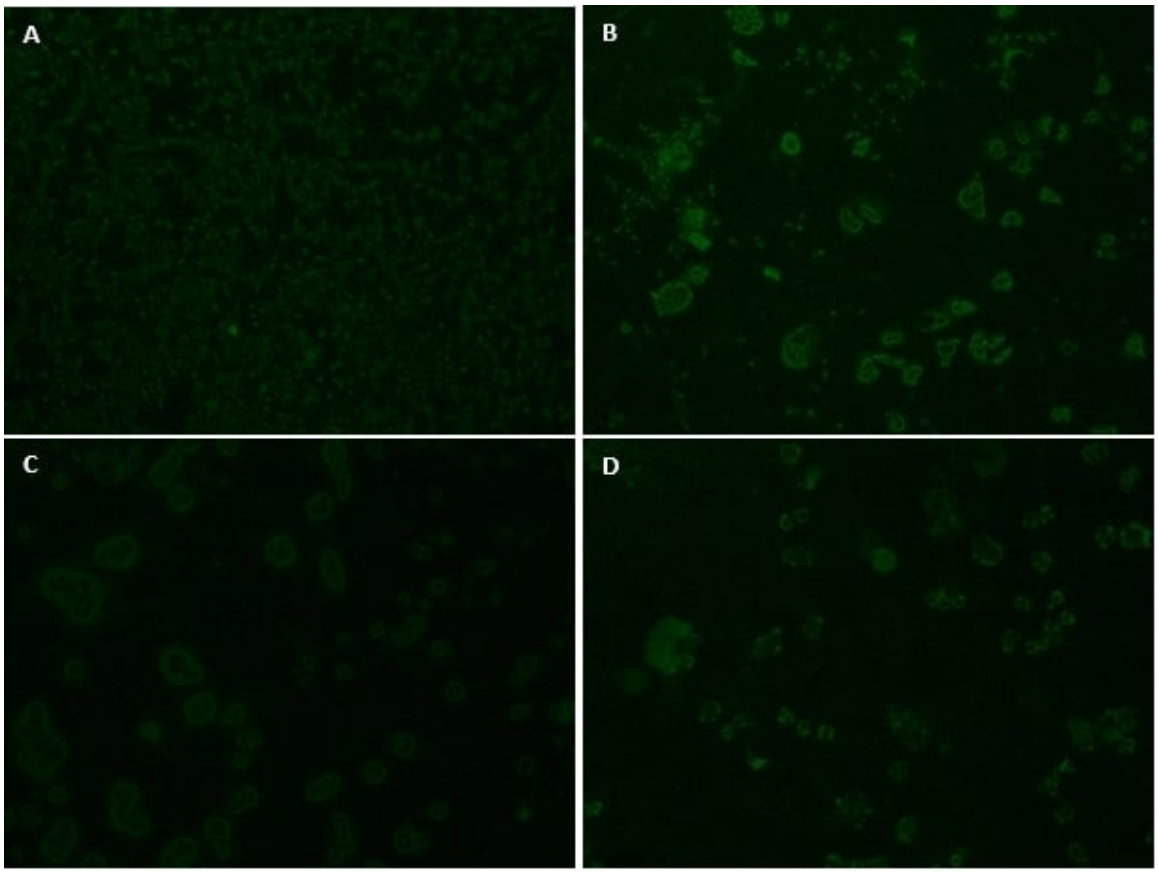

Fig.7 — Expression levels of the GSTP1 protein of GSTP1 antibody using the immunocytochemistry (ICC) method. The effect of VO powder extract on GSTP1 protein expression by ICC in MCF-7 cells. (A) GSTP1 expression levels in MCF-7 cells without VO powder extract; (B) MCF-7/VO powder extract with 25 µg/mL for 24 h; (C) MCF-7/VO powder extract with 50 µg/mL for 24 h; and (D) MCF-7/VO powder extract with 100 µg/mL for 24 h.The images were taken under the confocal microscope. (FITC, green signal) (x200 magnification)

Table 3 — GSTP1 activity levels of MCF-7 and MCF-10A cells cultured with and without VO powder extracts. EA was used as substrate. The statistical significance was indicated with letters. Activity levels were given with ± SD values. The cut of value for significance was taken as P <0.05

MCF-10A GSTP1 Concentration (µg/mL)	24 h	48 h	72 h
		$nmole^{-1}min^{-1}mg$	
Control	$4,96±0,8^{e}$	$5,5±0,4^{f}$	$3,6±0,2^{e}$
25	$4,48±0,1^{a}$	$4,96±0,4^{h}$	$4,4±0,3^{d,i,k}$
50	$3,91±0,3^{a}$	$5,5±0,4^{h}$	$3,8±0,09^{d,i,k}$
100	$6,15±0,7^{a}$	$5,7±0,4^{h}$	$4,5±0,3^{d,i,k}$
MCF-7 GSTP1 Concentration (µg/mL)	24 h	48 h	72 h
		$nmole^{-1}min^{-1}mg$	
Control	$13,0±1,5^{a,b,c,d}$	$5,5±0,4^{h,i,j}$	$4,9±0,4^{g}$
25	$10,2±0,4^{e,f,g}$	$4,9±0,4^{b,e,h,f,k}$	$4,2±0,2^{c,j}$
50	$10,1±1,3^{e,f,g}$	$5,5±0,4^{b,e,h,f,k}$	$4,8±0,2^{c,j}$
100	$9,08±1,0^{e,f,g}$	$5,7±0,4^{b,e,h,f,k}$	$5,6±1,0^{c,j}$

restored by epigenetic drugs has been suggested to be a marker of the efficacy of drug therapy in some tumour types[27]. For instance, it hasbeen stated that the increased GST expression in the drug-resistant cell line containing chlorambucil may be due to the GST-catalyzed formation of the thioether conjugate[28.] However, 50-fold higher GST expression was observed in the Adriamycin-resistant MCF-7 cell line than in the wild type MCF-7 cell line and this result cannot be easily explained by the GSTP catalytic

properties as Adriamycin GSH conjugates under physiological conditions[29]. In other words, some tumours and drug-resistant tumour cells can become protein-dependent due to the proliferative nature of tumour cells, the kinase pathways are not regulated and therefore tumour cells may try to compensate by increasing GSTP1 expression to offset the increased dysregulated kinase activity[30]. Moreover, GSTP1 has many functions in cancer and human pathologies, even drug addiction. It also has been shown to possess chaperone functions and regulated nitric oxide pathways apart from its glutathionylation and detoxification functions and control over kinase signalling[31]. Punganuru's group demonstrated that an enhancement in cell growth inhibitory effects of EA levels in human cancer cell lines which lung cancer cell line (H129), brain cancer cell lines (GBM10 and SF188), breast cancer cell lines (SKBR-3, MDA-MB-231, MDA-MB-468, MCF-7), colon cancer cell lines (HT29 and HCT116)[32]. Another research group examined protein levels of GST by using a CDNB substrate on breast cancer cell lines[33]. In vitro study has reported that the Piwil2 transfection, GST activity increased using CDNB as a substrate and decreased after using EA as a substrate. It has been suggested that the presence of numerical values, metabolic protein levels for drug discovery and the Piwil2 gene affect GST expression in the human breast cell line, MCF-7[34]. It was found significant similarity with our study by comparing decreased GSTP1 activities. In vivo study reported that GSTP1 expression levels were less in negative breast tumours than in positive breast tumours and were remarkably closer to docetaxel or paclitaxel reduction ratio[35]. According to the study, it can be thought that the chemoresistance is related to its enzyme activity as GSTP1a phase II detoxification enzyme which is involved in the conjugation of metabolites of some chemotherapic drugs with glutathione, raising the possibility that the increased metabolism of these agents induced by GST upregulation may reduce anti-tumour activity[36]. However, the mechanism of protection for normal cells of GSTP1 against chemotherapeutics remains unclear.

Due to the negative properties of these chemotherapeutic drugs, plants and their components have started to be included in the research in the treatment of diseases and continue to be studied. Previous studies have reported that VO is an antioxidant and all its extracts are potent radical scavengers due to high phenolic concentrations[37]. The study reported that VO extracts exhibited possible antitumor activity against Ehrlich ascites carcinoma (EAC) cells by modulating lipid peroxidation and enhancing endogenous antioxidant defence systems[38]. In the current study, VO powder extract was applied at different doses on MCF-7 and MCF-10A cell lines but GSTP1 enzyme activity was founded to be higher in MCF-7 control cells according to spectrometer readings despite that was decreased in the groups in different periods. GSTP1 and GSTA1 enzyme activities have not been seen any differences in MCF-10A groups which normal breast cells with and without VO powder extract. These results supported that VO powder extract does not cause dysregulation of kinase pathways in healthy cells and GSTP1 does not need a detoxification process therefore VO powder extract is not harmful to healthy cells. And also, this situation is thought to be due to EA which has good pharmacological properties and was used as a substrate for GSTP1 enzyme activity and is an inhibitor for other GSTs, its inhibitory effect on GSTP1 makes it active against human tumour cells. In the MCF-7 groups, however, GSTA1 enzyme activity was decreased in the groups in which only 24 h of VO powder extract was compared to the control group. At the same time, the expression level in MCF-7 and MCF-10A cells could not be observed by the immunofluorescence method using the GSTA1 antibody. The GSTA1 enzyme preserved the cell against oxidative stress by metabolizing bilirubin and also displayed the side effects of certain anti-cancer drugs in the liver. It also maintained the cells from reactive oxygen species and peroxidation products by exhibiting glutathione peroxidase activity[39]. Based on these proven features of GSTA1; although the GSTA1 enzyme activity results suggested that the VO powder extract is not harmful in both cell lines, the lack of GSTA1 expression results supports that GSTA1 cannot be a stand-alone marker in breast cancer. Some researchers even have emphasized that GSTA1 may be a marker in liver diseases[39]. Assessment of the expression of GSTP1 in this study indicated that VO powder extract substantially reduced the expression of GSTP1 in cancerous cells of the breast at 25 μg/mL in the 24 h and 50 μg/mL in the 24-48 h and 100 μg/mL all of the period. Overall, these findings suggest that VO powder extract induces and regulates apoptosis in cancer cells through the mitochondrial pathway. For the apoptosis detection, the PI test was used to detect the percentage of live cell population and early apoptotic, and late apoptotic for MCF-7 and

MCF-10A cell was treated with different concentrations and these apoptotic cells which was assessed by annexin V-FITC/PI staining. While late-stage apoptotic and dead cells (Annexin V (+) and 7-AAD (+)) were almost been absent in all the groups, the cells which were treated with VO powder extract become the highest total apoptosis of 40.50%. At the same time, unequal effects on MCF-7 cell survival at different concentrations of VO powder extract were displayed according to the findings. Because this study was dose and time-dependent, the control sample containing only MCF-7 cells had a proliferation rate of approximately 100% in all groups. In another study, VO The control sample contained only MCF-7 cells with a proliferation rate of approximately 100% in all groups because of the dose and time-dependent study. In another study, VO methanol extract was tested on a colorectal cancer cell line, which is utterly displayed serious health problems in the world, and its anti-cancer effect was studied. According to the results, it has been reported that the VO methanol extract reduces the cell viability and the cell number on cancer cell lines and also shown that apoptosis and necrosis increase and viability decreases between 5 and 2000 µg/mL doses of colorectal cancer cell in the range of 14.88–52.06% of concentration. In this study, in which the genotoxic effect was also examined, it was reported that high dose concentration caused DNA damage due to the proxy effect[40]. We observed that VO powder extract damaged the DNA of MCF-10A cells in which a high concentration was applied for 72 h. In general, antioxidants inhibit oxidative damage due to their capability of inhibiting intracellular ROS. It reported that high doses of antioxidants show pro-oxidant activity with the presence of transition metals (iron-II and iron-III)[41]. The results of this study showed that the effects of VO powder extracts were dose-time-dependent and this compound could reduce cell survival at doses above 100 µg/mL for 24 h and 25 µg/mL for 48 h. Besides, the lowest cell survival caused by VO powder extracts was related to 25 µg/mL and 100 µg/mL doses for 72 h. Koparal investigated the effect of VO juice on human cancer cell lines, A549, Caco-2, HeLa and normal cell lines, MDCK and HUVEC cells, and reported that VO fruit juice resulted in a dose-dependent decrease in cell viability between 10-100 µg/mL concentrations. And also with this study, it was known that the cell viability of VO fruit juice on healthy control cells decreased within 72 h[42]. Such therapeutic approaches to drug development involve the use of small molecules, arrays of different concentrations[43].

According to the results of the study, VO powder extract played an important role in MCF-7 cells by decreasing cell viability, and increasing DNA damage and apoptosis, while it did not cause side effects in MCF-10A cells. It was supported that this situation was caused by the chemical components of VO fruit extracts[44]. The study findings suggest that VO powder extract, as the inducer of apoptosis, affected breast cancer cells with and without estrogen and progesterone receptors at low doses. Furthermore, the presence of the VO powder extract decreased the enzyme activity of GSTs and GSTP1 expression levels in breast cancer cells with and without estrogen and progesterone receptors and also cancerous cells at low doses. This study investigated the effect of VO powder extract on both tumour cells and non-tumour cells at the metabolic protein level, using the in vitro method for the first time. In addition, this study also presents the investigation of the biological importance of intracellular GSTP1 activity, the detection of cancer cells and the evaluation of the effect of VO powder extract, and most importantly, for the first time, etacrynic acid and VO powder extract can be used together as a powerful chemical tool and can be more effective in cancer cells. These results provided references for future studies on the effects of extracellular GSTP1 on MCF-7 with and without VO powder extract and the regulatory effects of VO powder extract on metabolic protein GSTA1 and GSTP1 and also showed whether VO powder extract can have side effects on normal cells and cancerous cells at the metabolic level.

Conclusion

In this study, it was investigated the effect of VO powder extract on both tumour cells and non-tumour cells at the metabolic protein level, using the in vitro method for the first time. It must demonstrate and evaluate GSTP1 activity for cancer diagnosis and evaluation of the efficacy of drug therapy in cancer cells. These results provided the information that VO powder extract may be suitable for drug therapy efficacy at the molecular level. This study shows that DNA damage, intracellular cytotoxicity and apoptosis have increased due to the antioxidant properties of *V. opulus* fruit powder extract. And also, the current research is one of the cell culture components in the preparation of plant extracts in cell culture studies supports that the results can be interpreted more

precisely by eliminating different possibilities. This study is limited as it was done without fractioning the VO powder extract. Future studies are needed to investigate which compounds were effective and responsible for the antitumor activity of VO powder extract.

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Conflict of interest

The author declares no conflict of interest.

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