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# Effects of Valproic Acid with Different Drugs in Ovarian Cancer Cell Lines: An *in vitro* Study

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**Abstract**: Ovarian cancer is one of the most lethal malignancies, ranked seventh among all cancers globally. In recent trends, there has been an increased use of natural compounds combined with conventional drugs in clinical trials to combat chemotherapeutic resistance. Valproic acid, a short-chain fatty acid, Resveratrol, Silymarin, and Cyclophosphamide are some of the compounds widely studied for various cancer treatments. Hence, an *in vitro* attempt at the combinatorial effect of drugs was investigated in human ovarian adenocarcinoma cells OVCAR-3 and SKOV-3. Cytotoxicity, apoptosis, and antioxidant scavenging activity were studied by MTT, AO/EB, and DPPH assay, respectively. In addition, analysis of quantitative residual protein of treated cell culture supernatants was evaluated in OVCAR-3 and SKOV-3. The combination of valproic acid with resveratrol and silymarin showed effective $IC_{50}$ and apoptosis when compared with cyclophosphamide. The antioxidant activity of valproic acid in combination with resveratrol and silymarin was 88% and 81%, respectively, at 50µg/ml. The study highlights the effective treatment strategy of combinatorial drugs for ovarian cancer. However, the limitations extend towards a molecular approach in the future for further validation with various other *in vitro* methods and preclinical studies.

**Keywords**: valproic acid; cytotoxicity; resveratrol; silymarin; ovarian cancer.



## 1. Introduction

Ovarian cancer is a complex neoplastic assembly considered the most lethal gynecological malignancy [1]. According to the survey per year, the rate of women getting new cases of ovarian cancer is 11.2, and the death rate is 6.7 per 100000 women [2]. Numerous chemotherapeutic studies have been done to treat advanced ovarian cancer. Treatment options for high-grade serous carcinoma include chemotherapeutic drugs with increased side effects. The development of chemo-resistance mechanisms has decreased the survival rate, and there is a need for more combinations of drugs with natural compounds to avoid resistance and increase survival with minimal side effects. Natural compounds have been on the market for several years and are replacing conventional drugs in clinical trials [3]. Over the years, combination therapy paved the way for diverse therapeutic strategies. The ancient medicine system has used

combinatorial drugs to treat various diseases, including cancer [4]. A study states that the synergism of Valproic acid (VPA) with Resveratrol (RES) has reduced brain injury during Ischemic stroke with lesser side effects [5]. RES has also been known as a neuroprotective agent for combating oxidative stress autism upon treatment with VPA in swiss albino mice [6] and plays a major role in preventing inflammatory factors such as IL-6, IL-1, TNF-α, etc. [7]. Silymarin (SIL), with VPA-induced liver damage, was attenuated by SIL in rats, proving its hepatoprotective [8-9]. In a similar study, monomethyl fumarate and SIL combination have also shown hepatoprotective results with VPA-induced toxicity [10]. Cyclophosphamide (CPA) is used as a drug for recurrent ovarian cancer. Recent studies state that metronomic CPA has proven to be effective in 48% of clinical patients with advanced ovarian cancer [11, 12]. However, the drug becomes resistant and has severe side effects with a high relapse rate. VPA is a well-established drug for epilepsy and bipolar disorders [13]. Several combinations of VPA with drugs like paclitaxel [14], carboplatin, and azacitidine [15], have proven to be effective in treating ovarian cancer, which is already in phase 1 and phase 2 trials. VPA has proven to be a promising drug for treating breast cancer, glioma, and acute myeloid leukemia individually and in combination [16, 17]. Thus in the present study, VPA is compared individually and in combination with RES, SIL, and CPA. The investigations included cytotoxicity, apoptosis, measuring the cells' residual protein content, and the drugs' antioxidant activity *in vitro*. An overview of the study is shown in figure 1.

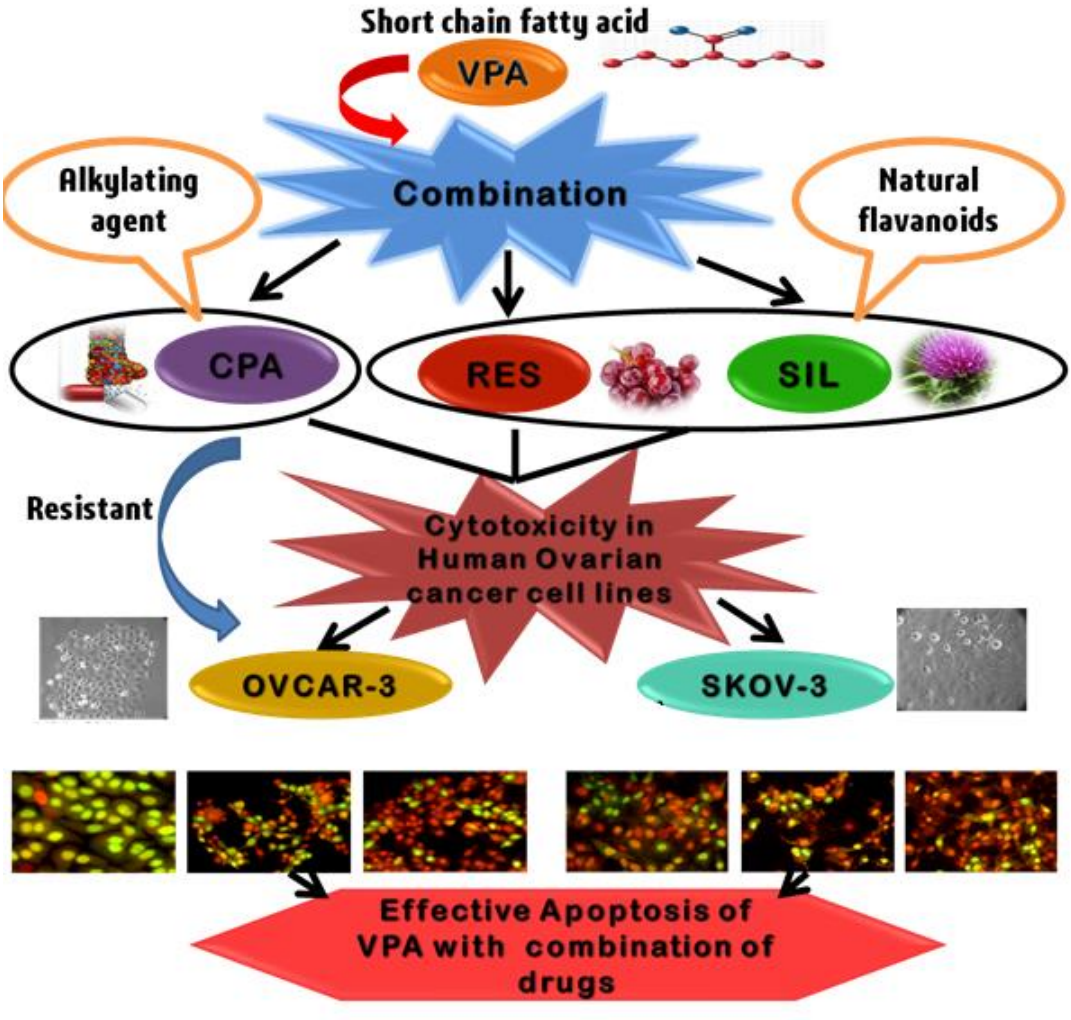


**Figure 1.** Overview of the study.

## 2. Materials and Methods

*2.1. Cell Culture.*

Human ovarian adenocarcinoma cell lines OVCAR-3 and SKOV-3 were included in the study. OVCAR-3 was kindly gifted by Dr. Ramray Bhat from the Indian Institute of Science (IISc), Bangalore, India. SKOV-3 was procured from National Center for Cell Science (NCCS), Pune, India. OVCAR-3 cell line was maintained in RPMI-1640 medium with Sodium bicarbonate, 1mM Sodium pyruvate, 0.01mg/mL Insulin, 2.5g/L Glucose and 10% Fetal Bovine Serum (FBS) and 1% Pen/Strep solution (Gibco). SKOV-3 cell line was maintained in McCoy's 5A medium (Himedia) with Sodium bicarbonate and 10% FBS. The cell lines were incubated in a $CO_2$ incubator (Thermo Heraeus, Germany) at 37˚C, 95% air and 5% $CO_2$ for propagation.

*2.2. Cell Viability Assay.*

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess the cytotoxic effect of drugs in OVCAR-3 and SKOV-3 cells. The cells at a density of $5\times10^3$cells/well were seeded in 96 well plates in triplicates and incubated for 24h for adherence. The synchronized cells were treated in serum-free media for 24h and 48h. At the end of treatment, the cells were washed with PBS. 20µL of MTT (5 mg/mL) (Sigma) was added to each well, and cells were incubated for 4h. An organic solvent dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance was measured at 570nm and 630nm using a spectrophotometer (MultiskanGO Thermo Scientific, USA). Cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = (\text{Absorbance}_{\text{test-blank}} / \text{Absorbance}_{\text{control-blank}}) \times 100 \text{ [18]}$$

*2.3. Apoptosis assay.*

Live/Dead cell assay was done by dual Acridine Orange/Ethidium Bromide (AO/EB) fluorescent staining method. The cell density of $5\times 10^4$ cells/well was seeded in a 6-well plate and treated with respective $IC_{50}$ concentrations as given in Table 1. The cells were assayed for 24h and 48h. After the incubation period, the cells were deprived of spent medium, and washed with PBS. 50µL of AO/EB dye mix (100µg/mL AO and 100µg/mL EB; Sigma) was added. The stained cells were visualized under a Trinocular inverted phase-contrast fluorescence microscope (Carl Zeiss Axiovert 40 CFL, Germany) using a UV filter at 20X magnification [19].

*2.4. Quantification of Residual Protein contents in Cell culture Supernatants by Bradford's assay.*

The cell culture supernatants of treated cells at 48h were isolated and stored at -20°C until further use. The supernatants were centrifuged at 10,000 g for 35min at 4°C. The obtained pellet was washed with PBS at 10,000g for 20 min at 4°C. The final pellet was resuspended in RIPA lysis buffer and stored at -20°C [20]. To quantify the protein content in the residual supernatant, the Bradford assay was performed with standard Bovine serum albumin (BSA) ranging from 2.5 - 20µg/mL. Bradford reagent was prepared by 50 mg of Coomassie Brillant blue (G-250) in 50 mL of methanol, 100 mL of 85% (w/v) phosphoric acid, and finally made

up to 1000 mL with water. 50 µL of the sample with 150 µL of Bradford reagent was added, mixed, and incubated for 15 minutes in the dark. After incubation, the reading was taken at 595 nm using a spectrophotometer (Multiskan GO Thermo Scientific, USA) [21].

*2.5. Antioxidant assay.*

The antioxidant activity was determined by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay by estimating the free radical-scavenging activity [22]. 100 µL of drugs at different concentrations was added to a volume of 100 µL from ethanolic solution (0.1mM). This reaction mixture was incubated for 30 min at room temperature in the dark, and absorbance was measured at 517 nm using a spectrophotometer (Multiskan GO Thermo Scientific, USA). Ethanol was taken as blank and ascorbic acid as a positive control. The rate of percentage of inhibition of DPPH radical by the drug was calculated using the formula:

$$\text{DPPH scavenging effect (\%)} = 100 - [(\text{Absorbance}_{control} - \text{Absorbance}_{test})/\text{Absorbance}_{control}] \times 100$$

The concentration of drugs required to scavenge 50% of DPPH free radicals was represented as an $IC_{50}$ value [23].

## 3. Results and Discussion

*3.1. Effect of VPA with CPA, RES, and SIL in OVCAR-3 and SKOV-3.*

The cytotoxicity of VPA, CPA, RES, and SIL individually and in the combination of VPA with CPA, RES, and SIL was investigated for 24 h and 48 h in OVCAR-3 and SKOV-3 cell lines. It was evident that an increase in concentration decreased the viability of the cells with increased cytotoxicity. The dose and duration-dependent cytotoxicity suggested that OVCAR-3 and SKOV-3 are responsive to the flavonoids and drugs. It also clearly determined that flavonoids are more effective than the drug. Thus, the inhibitory concentration was further taken for apoptosis study to assess the further characteristic features and functional activity of cancer cell lines. The VPA, which was conventionally used for ovarian cancer treatments, included side effects, notably liver damage. The study aimed to determine the cytotoxicity of ovarian cancer cells *in vitro*. VPA was compared with CPA, RES & SIL individually and in combination at 24 and 48 h. The comparative study with ovarian cancer-resistant drug CPA attempted to investigate flavonoids' resistance and influence. The results determined that the response of the drugs in OVCAR-3 was resistant only to CPA and not to other flavonoids. Flavonoids were shown to be effective in treating ovarian cancer by targeting various mechanisms such as autophagy, ROS damage, apoptosis, multiple drug resistance, etc., with fewer side effects from the literature [24, 25]. Flavonoids showed a dose and duration-dependent cytotoxicity in OVCAR-3 and SKOV-3 cells when treated individually or in combination.

The increasing side effects and development of resistance provoked investigation of the combined effect of the drugs for ovarian cancer. However, CPA resistance activity was reported in OVCAR-3 cells. Hence to study of CPA in non-resistant cell line SKOV-3 was attempted. CPA is widely used in recurrent ovarian cancer. This study used equal concentrations of VPA, CPA, RES, and SIL. VPA was very well known for inhibiting Histone deacetylases (HDACs) [26], and various studies report the combination of VPA with platinum drugs to modulate cell

cycle and epigenetics [27]. RES has been known to regulate SIRT-1 gene, which was known for longevity [28, 29]. RES targets the apoptosis pathway via inhibition of STAT3, which was involved in the autophagy mechanism [30, 31]. The combination of VPA and RES has already been studied in brain injury [5] and has proven effective in reducing the side effects of VPA. VPA effects have been known to induce liver damage; thus, SIL, a well-known flavonoid, is used in treating drug-induced liver injury [32, 33] and was also chosen for the study. Thus, this study studied a similar combination of VPA with RES, SIL, and CPA in ovarian cancer cells.

3.1.1. Cell cytotoxicity by MTT assay in OVCAR-3.

From figure 2, upon 24 h and 48 h, the $IC_{50}$ of VPA alone was found to be 183.3 µg/mL and 105.5µg/mL at 24 and 48 h, respectively. The VPA combination with RES showed effective $IC_{50}$ at 70.23 µg/mL and 69.62 µg/ml at 24 and 48 h, respectively. The VPA combination with SIL showed effective $IC_{50}$ at 77.17 and 71.62 µg/mL at 24 and 48 h, respectively. The combination of VPA with RES and SIL showed significance against VPA with $p<0.05$. Due to the resistance of CPA in OVCAR-3 cells, the combination of VPA+CPA has shown no effective cytotoxicity. Thus, VPA treatment in combination with RES and SIL was proven to be more effective than VPA alone.

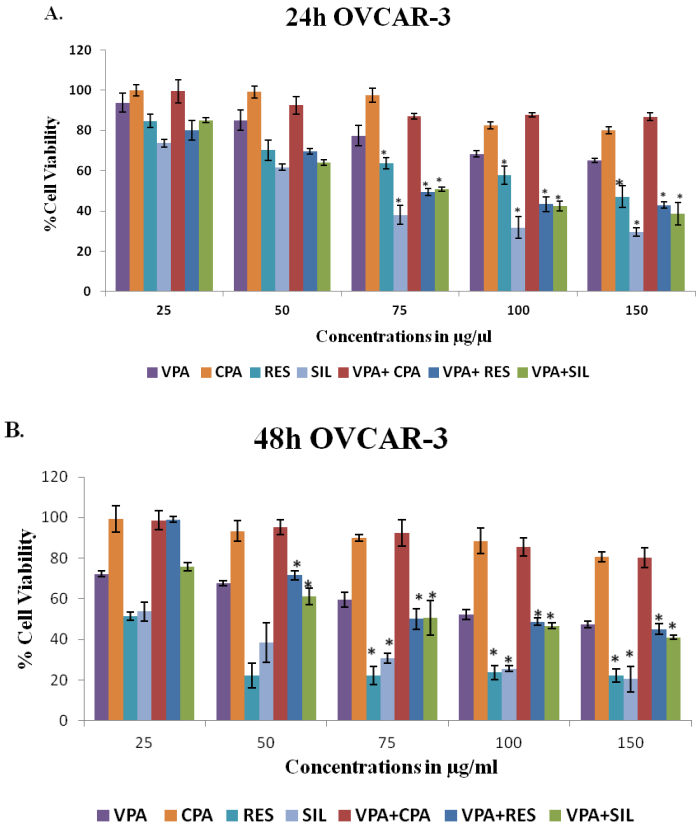


**Figure 2**. Effect of cytotoxicity in OVCAR-3 cells treated with different concentrations of VPA, CPA, RES & SIL and their combinations at (**A**) 24 h; (**B**) 48 h was determined by MTT assay. Data are presented as the mean ± standard deviation (n = 6). * represents significance $p<0.05$ (VPA: Valproic acid; CPA: Cyclophosphamide; RES: Resveratrol; SIL: Silymarin).

**Table 1.** Represents the half inhibitory concentrations of the groups studied in OVCAR-3 cells at 24 and 48h.

| **OVCAR-3** | **$IC_{50}$ (µg/mL)** | |
|---|---|---|
| | **24h** | **48h** |
| VPA | - | 105.5 ± 0.23 |
| CPA | - | - |

| OVCAR-3 | $IC_{50}$ (µg/mL) | |
|---|---|---|
| | **24h** | **48h** |
| RES | 122.6 ± 0.75 | 36.74 ± 0.66 |
| SIL | 46.63 ± 0.47 | 28.07 ±0.39 |
| VPA+CPA | - | - |
| VPA+RES | 70.23 ± 0.36 | 69.62 ± 0.51 |
| VPA+SIL | 77.17 ± 0.22 | 71.62 ± 0.14 |
| | | |

[1] The individual data points were expressed as mean ± standard deviation (mean ± SD).
- Denotes $IC_{50}$ beyond the desired range of concentration chosen.

3.1.2. Cell apoptosis by live/dead staining by Acridine orange/Ethidium Bromide (AO/EB)-48h in OVCAR-3.

A live/dead staining assay was performed by Acridine orange/Ethidium bromide at concentrations of 75 µg/mL of VPA, CPA, RES, and SIL to assess the cytotoxicity further and in combination, showed effective killing and apoptosis as shown in Figure 3. The RES and SIL individual and in combination with VPA, has shown signs of blebbing and morphology deformation of cells. Thus, our cytotoxic results correlate with the apoptosis study performed.

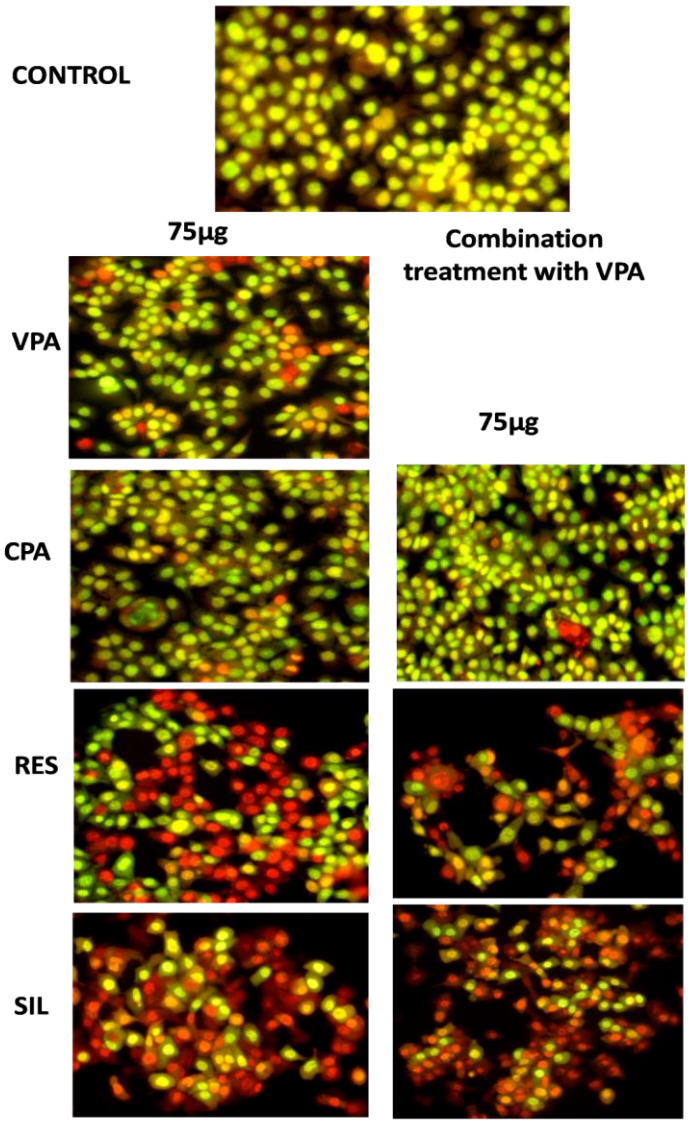


**Figure 3**. AO/EB staining in OVCAR-3 cells treated with VPA, CPA, RES, and SIL at 75µg and a combination of drug treatment with VPA at 48h (VPA: Valproic acid; CPA: Cyclophosphamide; RES: Resveratrol; SIL: Silymarin) Magnification: 20X.

3.1.3. Cell cytotoxicity by MTT assay in SKOV-3.

The cytotoxicity of VPA was determined along with CPA, RES, and SIL for 24 and 48h in SKOV-3 cells. Upon treatment of 24 and 48 h, the $IC_{50}$ of VPA alone was found to be 284.5 µg/mL and 220.8 µg/mL at 24 and 48 h, respectively. The VPA combination with RES showed effective $IC_{50}$ at 71.74 µg/mL and 48.38 µg/mL at 24 and 48 h, respectively. The VPA combination with SIL showed effective $IC_{50}$ at 97.17 and 66.86 µg/mL at 24 h and 48 h, respectively. The combination of VPA with RES and SIL showed significance against VPA with $p<0.05$. CPA showed cytotoxic activity in SKOV-3 at $IC_{50}$ of 194.7 µg/mL and in combination with VPA at 161.3 µg/mL at 24 h. The response of cytotoxicity, when treated with CPA at 24 h, was appreciative with the half inhibitory concentration, whereas the $IC_{50}$ increased with an increase in duration at 48h.

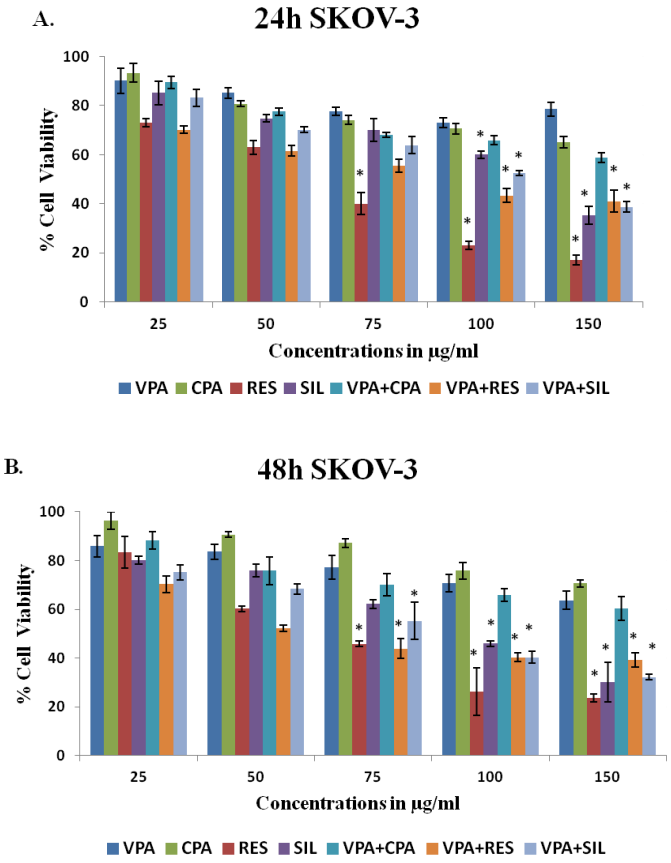


**Figure 4**. Effect of cytotoxicity in SKOV-3 cells treated with different concentrations of VPA, CPA, RES & SIL and their combinations at (**A**) 24 h; (**B**) 48 h was determined by MTT assay. Data represent the mean ± Standard deviation (SD) from triplicate experiments. * represents significance $p<0.05$ (VPA: Valproic acid; CPA: Cyclophosphamide; RES: Resveratrol; SIL: Silymarin).

**Table 2**. Represents the half inhibitory concentrations of the groups studied in SKOV-3 cells at 24h and 48h.

| **SKOV-3** | **$IC_{50}$ (µg/mL)** | |
|---|---|---|
| | **24h** | **48h** |
| VPA | - | - |
| CPA | - | - |
| RES | 55.07 ± 0.54 | 43.78 ± 0.49 |
| SIL | 93.67 ± 0.45 | 76.52 ± 0.35 |
| VPA+CPA | - | - |
| VPA+RES | 71.74 ± 0.33 | 48.38 ± 0.17 |
| VPA+SIL | 97.17 ± 0.25 | 66.86 ± 0.29 |

[1] The individual data points were expressed in the form of mean ± standard deviation (mean ± SD).
- Denotes $IC_{50}$ beyond the desired range of concentration chosen.

3.1.4. Cell apoptosis by live/dead staining by Acridine orange/Ethidium Bromide (AO/EB) - 48h in SKOV-3.

Similarly, in SKOV-3, a live/dead staining assay was performed by Acridine orange/Ethidium bromide, at a 75 µg/mL concentration of VPA, CPA, RES, and SIL and in combination with effective killing and apoptosis, as shown in Figure 5.

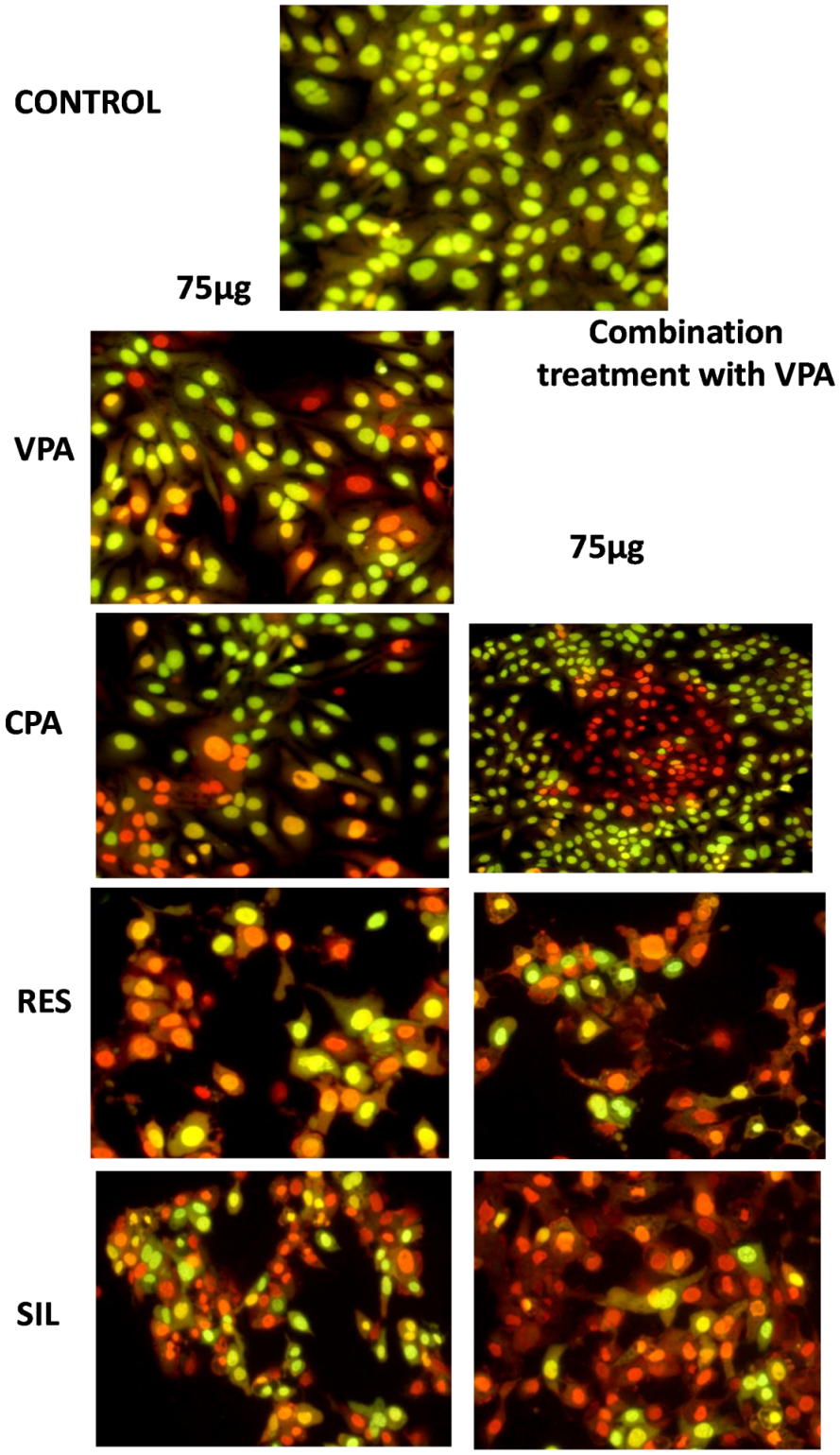


**Figure 5**. AO/EB staining in SKOV-3 cells treated with VPA, CPA, RES, and SIL at 75µg and a combination of drug treatment with VPA at 48h (VPA: Valproic acid; CPA: Cyclophosphamide; RES: Resveratrol; SIL: Silymarin) Magnification: 20X.

*3.2. Quantification of Residual protein content from treated cell culture supernatants of OVCAR-3 and SKOV-3.*

A Bradford assay was performed to quantify the residual protein content (RPC) from treated cell culture supernatants. Cell viability is one of the important methods to determine the vitality of cell cultures. Cell lysis is one of the significant characteristic features to determine the intracellular contents of the cells released by the cultured cells. During the process, mammalian cell cultures undergo loss of membrane integrity, and host cell proteins are released into the cultures supernatant.

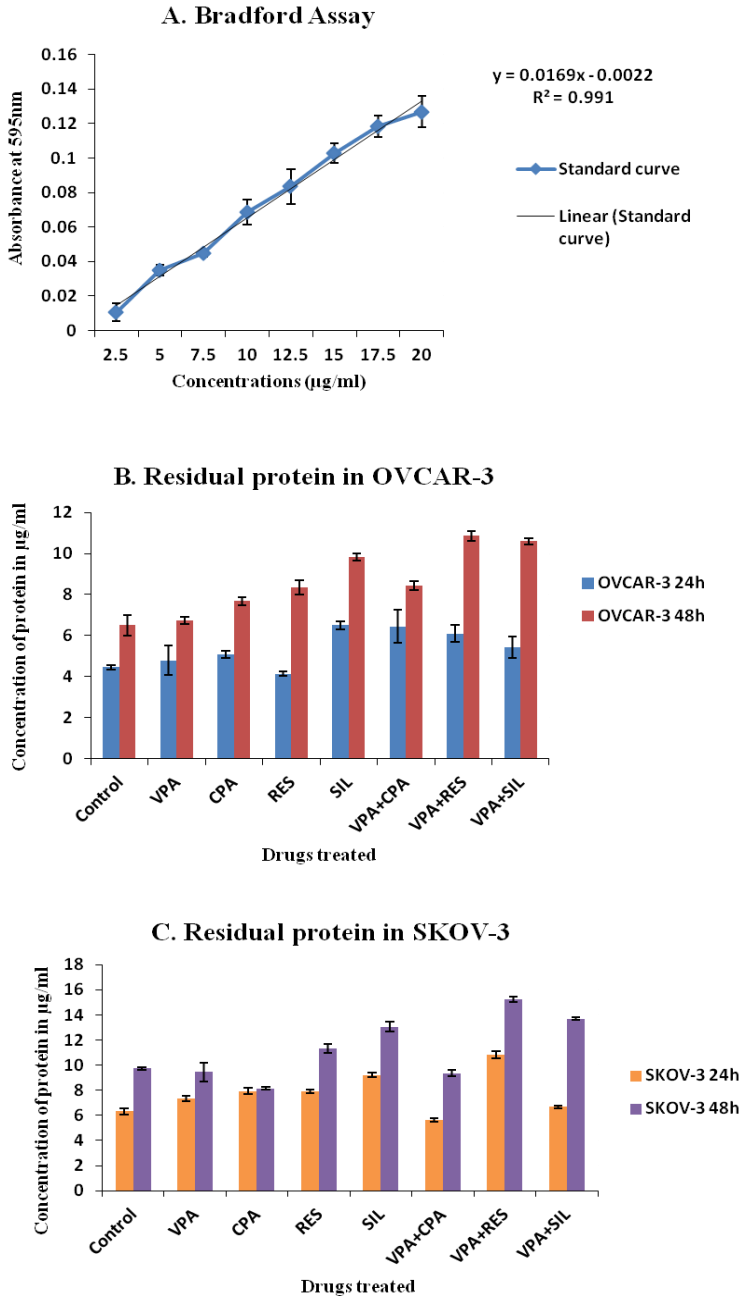


**Figure 6**. (**A**) Represents the standard curve of bovine serum albumin (BSA). The line represents the linearity of the graph. (**B**) Residual protein content in cell culture supernatants of OVCAR-3 for 24 h and 48 h. (**C**) SKOV-3 for 24 h and 48 h. Data represent mean ± SD (n=6).

The host cell proteins play a critical role and attribute vital information to cell lysis research [34, 35]. Thus, in the present study, the cells treated with VPA, RES, SIL, CPA, and their combinations were analyzed in OVCAR-3 and SKOV-3 to quantify the residual protein

content. The treated cells supernatant were collected time-dependently 24h and 48h after treatment and processed. Bradford assay was performed to measure the amount of protein released during the lysis process. Figure 6A represents the standard curve of bovine serum albumin (BSA), ranging from 2.5-20 µg/mL, BSA is the standard protein used for protein estimation. The unknown protein concentrations from treated samples were quantified based on the standard curve. Figure 6B. represents the residual protein content in OVCAR-3 for 24 h and 48 h. The control cells have 4.44 ± 0.1 µg/ml and 6.5 ± 0.5 µg/mL protein content in 24 h and 48 h of OVCAR-3, respectively. The RPC release shows the difference in both 24 h, and 48 h treated cells. The RPC content is higher in VPA+RES and VPA+SIL, which correlated with the cell viability assay, as VPA combination with RES and SIL showed effective cytotoxicity. However, the release in VPA, CPA, and RES shows less RPC when compared to the control. Thus, the release profile of the drug varies time-dependently, which further affects the RPC of the cells. Figure 6C. represents the residual protein content in SKOV-3 for 24 h and 48 h. The control cells have 6.31 ± 0.2 µg/mL and 9.72 ± 0.1 µg/mL protein content in 24h and 48h in SKOV-3 respectively. The release of RPC is higher in SKOV-3 control cells when compared to OVCAR-3. The combination of VPA+RES and VPA+SIL has shown higher RPC, similar to OVCAR-3. However, in SKOV-3, the cells treated with CPA showed no difference between 24h and 48h. Thus, the cell viability study correlates with the RPC released into the cells, which shows the prominent activity of the cell lysis mechanism happening during 24 and 48 h duration. The release of the drug and its mechanical action varies among different cell cultures, and the intracellular release profile affects the RPC of the cell.

*3.3. Antioxidant activity by DPPH assay.*

The antioxidant activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was performed at different concentrations with standard Ascorbic acid.

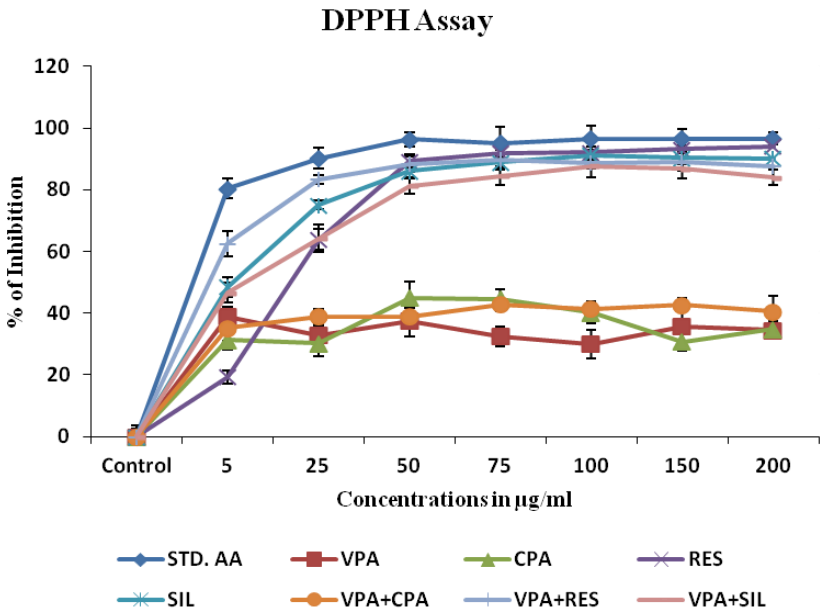


**Figure 7**. Determination of radical scavenging activity by DPPH inhibition of Standard ascorbic acid, VPA, CPA, RES, and SIL with combinations at different concentrations. Data represent mean ± SD (n=6).

Figure 7 denotes the antioxidant activity of VPA, CPA, RES, and SIL along with VPA+CPA, VPA+RES, and VPA+SIL combinations. VPA is a short fatty acid chain, and CPA is an alkylating agent. Thus, only 35-45% of inhibition activity was seen. However, RES and

SIL and their combinations with VPA have shown better scavenging activity of 88% and 81%, respectively, with increasing concentrations equivalent to standard ascorbic acid. The experiment was performed in triplicates (n=6).

**Table 3**. Percentage (%) of inhibition at 50 µg/mL.

| Drugs | % inhibition at 50 µg/mL |
|---|---|
| STD. AA | 96% |
| VPA | 37% |
| CPA | 45% |
| RES | 89% |
| SIL | 86% |
| VPA+CPA | 39% |
| VPA+RES | 88% |
| VPA+SIL | 81% |

## 4. Conclusions

Thus we conclude from the study that the *in vitro* activity of the combination of VPA with the flavonoids, RES, and SIL was proven to be cytotoxic in ovarian cancer cell lines OVCAR-3 and SKOV-3 when compared with the conventional drug cyclophosphamide. The apoptotic activity of cells showed cell death and blebbing and correlated with cytotoxic effects studied. The residual protein content from the treated supernatant was found to increase time-dependent. The combination of VPA with RES and SIL has shown efficient antioxidant activity compared to VPA and CPA. Further, molecular studies *in vitro* and preclinical *in vivo* experiments need to be performed to elucidate the mechanism of apoptosis induced by the drugs in combination.

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## Conflicts of Interest

The authors declare no conflict of interest.

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