Dicle Tıp Dergisi/ Dicle Medical Journal (2016) 43 (4) :534-541 DOI10.5798/diclemedj.0921.2016.04.0725

## ORIGINAL ARTICLE

# Implications of enhanced effectiveness of vincristine sulfate/ε-viniferin combination compared to vincristine sulfate only on HepG2 cells

## HepG2 hücrelerinde Vinkristin sülfat/ε-viniferin kombinasyonunun vinkristin sülfat ile karşılaştırıldığında artmış etkinliğinin vurgulanması

Filiz Özdemir[1], Zerrin İncesu[1], Mesut Şena[1], Nur İpek Öndera[1], Miris Dikmen[2]

[1] Anadolu University, Faculty of Pharmacy, Department of Biochemistry, 26470, Tepebası, Eskisehir, Turkey
[2] Anadolu University, Faculty of Pharmacy, Department of Pharmacology, 26470, Tepebası, Eskisehir, Turky
**Corresponding author** : Filiz Özdemir, Anadolu University, Faculty of Pharmacy, Department of Biochemistry, 26470, Tepebası, Eskisehir, Turkey *e-mail: fozdemir3@anadolu.edu.tr*

**Abstract**
**Objective:** This study was designed to investigate the effects of ε-viniferin (ε-VNF) on the mitochondrial pathway of apoptosis and on late apoptosis in HepG2 cell lines. To observe these effects, ε-VNF and vincristine sulfate (VNC), anti-cancer drugs used for treatment on HepG2 cells, were administered either alone or in combination at different time intervals.
**Methods:** Mitochondrial membrane potential changes in the cells (ΔΨm) were evaluated using cationic dye JC-1, while Bax, Bcl-2 expression levels with RT-PCR and caspase-3 activity were analyzed using a kit. For detection of apoptotic activity, an in situ TUNEL assay was performed.
**Results:** When 98.3μM ε-VNF, 52.5μM VNC and the 11.25+15.8μM VNC+ε-VNF combination were compared with the control group, ΔΨm changes at the 6th hour were found to be 19.5%, 5.5%, 24.6%, and 3.5% , respectively. These finding show that the combination group (24.6%) resulted in early apoptosis of the cell at the 6th hour. Bax mRNA expression increased at the 24th hour in the VNC+ε-VNF group compared to control group (160%), and caspase-3 activation increased in the 1.25+15.8 μM[VNC+ε-VNF] group compared to the control group at the 48th hour. The detection of DNA fragments in HepG2 cells within 24 hours suggests direct apoptosis.
**Conclusion:** These findings demonstrate that the doses administered to the VNC+ε-VNF combination group were lower than those administered to groups using one agent alone (e.g. VNC), the results of which reduce the possibility of administering toxic doses.
**Key words:** viniferin, vincristine, HepG2 cell, apoptosis

**Özet**
**Amaç:** Bu çalışma HepG2 hücre hattında geç apoptoz ve mitokondriyal yolakta ε-viniferin (ε-VNF)'in etkisini incelemek için dizayn edildi. Bu amaç için, HepG2 hücre dizisinde anti-kanser ilaç olarak kullanılan vinkristin sülfat (VNC) ve ε-VNF farklı zaman aralıklarında tek başına ve kombine olarak verildi.
**Yöntemler:** Hücrelerin mitokondriyal membrane potansiyeli (ΔΨm) katyonik boya ile, RT-PCR ile Bax, Bcl-2, kaspaz-3 aktivitesi de kit kullanılarak değerlendirildi. Apoptotik aktivite in situ TUNEL ile tespit edildi.
**Bulgular:** 98.3μM ε-VNF, 52.5μM VNC ve 11.25+15.8μM VNC+ε-VNF kombinasyonu kontrol grubu ile mukayese edildiği zaman, ΔΨm değişimleri 6. saatte sırasıyla %19,5, %5,5, %24,6 ve %3,5 olarak bulundu. Bu sonuçlar 6. Saatte kombinasyon grubunun (%24,6) erken apoptoza gittiğini göstermiştir. Bax mRNA ekspresyonu 24. saatte VNC+ε-VNF grubu kontrol grubu ile mukayese edildiğinde (%160), kaspaz-3 aktivasyonu VNC+ε-VNF (11.25+15.8 μM) grubu kontrol ile mukayese edildiğinde 48. saatte artış göstermiştir. 24 saat içerisinde, HepG2 hücrelerinde DNA kırıklarının tespit edilmiş olması doğrudan apoptozis ile ilişkili oldugunu göstermektedir.
**Sonuç:** Bu bulgular apoptozise yol açan VNC+ ε-VNF grubunun, tek başına kullanılan ajanın (VNC) oluşturacağı muhtemel toksik dozu azaltabileceğini göstermektedir.
**Anahtar kelimeler:** viniferin, vincristine, HepG2 hücre dizisi, apoptosis

**Received:13 July 2016;Revised:27 Sept 2016; Accepted:20 Oct 2016**

## INTRODUCTION

Hepatocellular carcinoma (HCC), the world's fifth most common cancer type, is responsible for the death of 600,000 people per year [1,2]. It is essential therefore that new therapeutic and/or protective agents against HCC be developed for patients suffering from this disease [3]. Chemotherapy is one of the most commonly used strategies in the treatment of HCC [4]. Combined treatment with multiple drugs is a standard regimen for cancer treatment, as this is a more effective strategy compared to single drug usage, insofar it decreases the resistance of drugs and their side effects [5,6]. In recent years, antitumor drugs derived from plants have begun to garner much attention due to their potent effects and low toxicities [1].

The phenol, ε-viniferin, is an antioxidant derived from resveratrol via the oxidative process [7]. The hepatoprotective and antioxidant properties of ε-viniferin and its ability to induce apoptosis in leukemia B cells have been well proven over time [8]. Resveratrol and its oligomers, including ε - viniferin, have been reported to have anti-proliferative and pro-apoptotic effects in cancer cells, such as HepG2 cells and human colon cancer cells [7,9,10].

Apoptosis, or cell death, is a fundamental process for both the development and the repair of cells and for tissue homeostasis. Apoptosis, which is known to be stimulated in many cells via either the mitochondrial (intrinsic) pathway or the death receptor (extrinsic) pathway, leads, via both pathways, to cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies. Apoptotic cell death progresses, to a great extent, as dependent on or independent from caspase. Many studies have demonstrated that, like the apoptosis-inducing factor (AIF), mitochondria release cell death effectors independent from caspase [11].

In the studies performed in our laboratory, the combined administration of ε-viniferin has been found to effectively stimulate HepG2 cell apoptosis and shows a synergistic effect [10].

In this study, we have aimed to investigate the effects of either vincristine or ε-viniferin or a combination of both on the mitochondrial pathway of apoptosis and on the late apoptotic phases in HepG2 cells. To this end, mitochondrial membrane potential (ΔΨm) changes, Bax and Bcl-2 mRNA expression rates, caspase-3 activation and DNA fragmentation have been examined.

## METHODS

### Drugs and Chemicals

Vincristine sulfate was procured from Sigma Aldrich Co, St Louis, USA, and ε-Viniferin was procured from Actichem (France).

### Cell Culture

Human liver hepatoma (HepG2) cells obtained from DSMZ (Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH/Germany) were cultivated in petri dishes as 1x106 ml/cells and suspended in a DMEM medium containing 10% FBS and penicillin/streptomycin, at 37°C, in an incubator containing 5% CO2. All experiments were performed in 96 plates. HepG2 cells (2x104 cell/mL) were seeded in a 25 cm2 flask and treated with a dose of IC50 (50% lethal) and IC50/2 (half of the 50% lethal). The cells were treated with either VNC (IC50—52.5 μM and IC50/2—26 μM) or ε-VNF (IC50— 98.3 μM and IC50/2—49 μM) alone or with a combination of ε-VNF and VNC (IC50—15.8 + 11.25 μM and IC50/2—7.9 +5.6 μM, respectively) for 24 hours. The IC50 and IC50/2 viability doses used in this study were determined using the values measured by Ozdemir et al. with the MTT method [10]. Briefly, cells (2 x 104 cells/well) were seeded in 96-well plates. After 2 hours of incubation, cells were treated with VNC (8.95–286.5 μM) and e-VNF (12.5–400 μM), either alone or in combination (4.48 + 6.25– 143.2 + 200 μM). After 24 or 48 hours of incubation time, 20 μL

of 5 mg/mL MTT was added to each well, followed by incubation for an additional 2 hours. The medium was removed, and 200 µL of DMSO was added to dissolve formazan crystals. The absorbance of the wells were measured at 540 nm using a microplate reader (Bio-Tek, ELX 808 IU- serial number 235302). The signal generated is directly proportional to the number of viable (metabolically active) cells in the wells. The values of the blank wells were subtracted from each well of treated and control cells.

**Mitochondrial Membrane Potential (ΔΨm) Assay**

The ΔΨm was determined using JC-1 probes (BD™ MitoScreen (JC-1) (RUO); Cat no: 551302). In brief, HepG2 cells were treated with ε-VNF, VNC and a combination of VNC + ε-VNF for 6, 12, 24 and 48 hours, incubated with 5µM JC-1 at 37°C for 15 minutes, and then washed and resuspended in cold PBS. Cells were analyzed using FACS Vantage flow cytometer according to the instructions given in BD™. Finally, a dot plot of red fluorescence (FL2, living cells with intact ΔΨm) versus green fluorescence (FL1, cells with lost ΔΨm) was recorded.

**Quantitative Real-time PCR**

Total RNA isolation and cDNA synthesis HepG2 cell samples with treated ε-viniferin and vincristine specific concentrations were collected after 24 and 48 hours. Total cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche). The cDNAs were synthesized from 1 µg RNA with Oligo-dT primer using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH). All cDNAs were then used for amplification of Bcl-2 and Bax genes on the LightCycler® 480

**Instrument**

The cDNAs were amplified using Bcl-2 (anti-apoptotic gene, Gene Bank accession no.BC 027258, 290 bp), Bax (pro-apoptotic gen, Gene Bank accession no. AY 217036, 437 bp) and GADPH (Internal standard, Gene Bank accession no. NM 002046, 622 bp) specific primer sequences, according to LightCycler DNA master SYBR Green I Kit (Roche Applied Science) protocol. PCR amplifications and analyses were performed using a LightCycler® 480 instrument (Roche Applied Science) with software version 1.5.0. All reactions were performed in duplicates using the LightCycler FastStart DNA Master SYBR Green I Kit in a final 20 µl volume, with 4 µM of MgCl2, 0.5 µM of each primer and 2 µL cDNA or 2 µL DNA standard dilution. The relative amount of mRNA compared to GAPDH (housekeeping gene) level was calculated using Crossing point (Cp) values and scaled relative to control samples set at a value of 1. Results for gene expression in experimental samples were plotted and compared with the control samples.

**Caspase-3**

HepG2 cells were treated with varying concentrations of VNC (52.5 or 26 µM) , ε-VNF (98.3 or 49 µM) and a combination of the two drugs (15.8 ε-VNF+11.25 µM VNC or 7.9 ε-VNF+5.6 µM VNC) for 24 and 48 hours. Caspase-3 activity was measured using the Phycoerythrin Active Caspase-3 Apoptosis Kit (BD Biosciences Pharmingen; Cat.No. 550914), according to the manufacturer's instructions. Samples were analyzed on a Becton Dickinson FACS Aria flow cytometer (BD FACS AriaTM I Cell Sorter Flow Cytometer, Serial No: P07900204) using FACSDiva Version 6.1.2. Software.

**TUNEL Assay**

Cells were grown on 24-well microplates and treated with VNC, ε-VNF and a combination of the said two based on the IC50 value. The fragmented DNA of apoptotic cells was quantified by Tdt-mediated dUTP nick end labelling (TUNEL), using the In Situ Cell Death Detection Kit, Flourescein (Roche, Germany;

Cat No. 11684795910). To briefly summarise performance of the assay, the cells on each well were fixed with 4% paraformaldehyde and incubated at between 15°C and 25°C and then washed with phosphate-buffered saline (PBS) for 30 minutes. Next, each well was incubated in a permeabilisation solution on ice for 2 minutes before being washed with PBS twice. A 50 µl TUNEL Reaction Mixture was then added to each well.

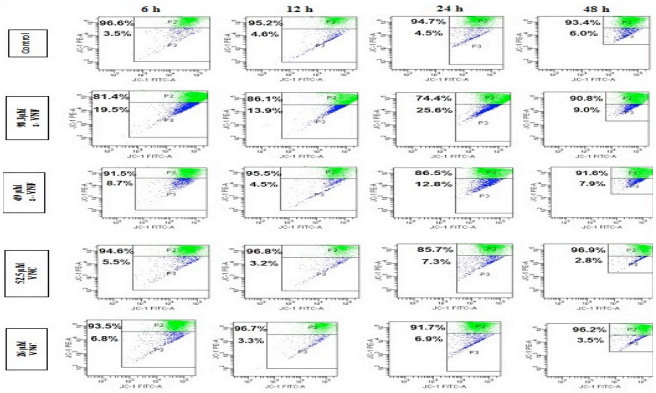


**Fig 1.** Mitochondrial membrane permeabilization (MMP) of 98.3 µM ε-viniferin, 49 µM ε-viniferin, 52.5 µM Vincristine and 26 µM vincristine in HepG2 cells. Mitochondrial membrane permeabilization (MMP) was determined by flow cytometry using JC-1. The percentages of cells with MMP (blue) were calculated.

The microplate was covered with parafilm, and the cells were incubated in a container for 60 minutes at 37°C to protect the cells from any light source. After incubation, each well was washed three times with PBS. After washing with PBS, the slides were dried, and fluorescent images were acquired with a fluorescence microscope (Leica DM-IL, 11101097).

## DISCUSSION

Loss of ΔΨm results in the release of pro-apoptotic factors to the cytoplasm, followed by the continuation of the apoptotic process on the basis of caspase activation [5]. There are numerous studies which have evaluated Mitochondrial Membrane Potential using flow cytometry [12,13]. Within the scope of this study, the integrity of the mitochondrial membrane was analyzed using JC-1, a cationic dye. The JC-1 dye turns green as indication of impaired ΔΨm in apoptotic cells. Changes in mitochondrial membrane potential are only observed when interruption, apoptosis or necrosis occurs in the cell cycle. A depolarized mitochondrial membrane generally indicates that the cell is in the process of early apoptosis [14]. In this study, the treatments performed with all of the agents administered to analyze early apoptosis during the process of cell death had incubation periods of 6, 12, 24 and 48 hours. Measurement of the mitochondrial membrane potential percentage changes of the cells undergoing agent-induced apoptosis showed that, when compared to control groups, a dosage of 98.3 µM ε-VNF administered at the 6th hour alone resulted in 19.5% depolarization, and that with a dosage of 52.5 µM VNC, 5.5% depolarization occurred. However, in the combined usage 11.25+15.8µM [VNC+ε-VNF], ΔΨm increased to 24.6%. At the 12th hour, though no significant change occurred in ΔΨm, depolarization percentages of 98.3 µM and 49.0 µM ε-VNF at the 24th hour were detected to be 25.6% and 12.8%, respectively, when used alone; but these effects started to decrease at the 48th hour. These findings are considered to be positive insofar as they show that the combined usage of VNC+ε-VNF at doses of 11.25+15.8µM at the 6th hour resulted in a decrease of the high dose of VNC when used alone, an increase of depolarization, a display of activity at the 6th hour, which is considered a short time, and also an early initiation of apoptosis (Figs 1 and 2, Table 1). In the study by Jiang et al. [15], mitochondrial membrane changes in HepG2 cells were examined after tectorigenin treatment, which involves anti-tumor and anti-angiogenic activities, and according to the results of the study, 20 mg/L of tectorigenin was found to induce apoptosis, a result indicated to be associated with the loss of mitochondrial membrane permeability. In another study, it was concluded that alantolactone induced apoptosis in HepG2 along the mitochondrial

pathway in the HepG2 cell line that was induced with 40 μM of alantolactone for 3, 6 and 12 hours, the results of which led to the opening of mitochondrial permeability transition pores upon the increase in the Bax/Bcl-2 ratio with alantolactone [2].

**Table 1:** Effects of collapse in ΔΨm on apoptosis of HepG2 cells. Data refer to the percentage of cells with intact ΔΨm (polarize) and fold ΔΨm (depolarize). HepG2 cells, were treated with /without drugs for 6, 12, 24 and 48 hours.

| | 6 hours | | 12 hours | | 24 hours | | 48 hours | |
|---|---|---|---|---|---|---|---|---|
| Treatment | Intact ΔΨm | Fold ΔΨm | Intact ΔΨm | Fold ΔΨm | Intact ΔΨm | Fold ΔΨm | Intact ΔΨm | Fold ΔΨm |
| Control | 96.6 | 3.5 | 95.2 | 4.6 | 94.7 | 4.5 | 93.4 | 6 |
| 98.3 μMε-VNF | 81.4 | 19.5 | 86.1 | 13.9 | 74.4 | 25.6 | 90.8 | 9 |
| 49.0 μMε-VNF | 91.5 | 8.7 | 95.5 | 4.5 | 86.5 | 12.8 | 91.6 | 7.9 |
| 52.5 μM VNC | 94.6 | 5.5 | 96.8 | 3.2 | 85.7 | 7.3 | 96.9 | 2.8 |
| 26 μM VNC | 93.5 | 6.8 | 96.7 | 3.3 | 91.7 | 6.9 | 96.2 | 3.5 |
| (11.25+15.8 μM) [VNC+ε-VNF] | 76.1 | **24.6** | 96.4 | 3.6 | 93.3 | 5.9 | 94.2 | 5.6 |
| (5.6+7.9 μM) [VNC+ε-VNF] | 90.6 | 9.5 | 95.5 | 4.5 | 94.9 | 4.4 | 91.4 | 8 |

Bcl-2 is an antiapoptotic protein and effector molecule that has functions on the mitochondrial pathway of apoptosis. Bcl-2 constitutes a heterodimer complex with Bax, which is a proapoptotic molecule. Bax mRNA expression is increased in apoptosis, and the Bax/Bcl-2 ratio is an important parameter for determining the life and death of cells [16]. The Bax and Bcl-2 mRNA expression levels of experiment groups at different times and concentrations and the target gene/reference gene (Bax /GAPDH or Bcl2 /GAPDH) expression ratios are shown as percentages in Table 2. Compared to the control group, the concentrations of the 26 or 52.5 μM VNC, 49 or 98.3 μM ε-VNF and 5.6+7.9μM VNC+ε-VNF groups at the 24th and 48th hours did not increase in Bax mRNA expressions. It was determined that the 11.25+15.8μM [VNC+ε-VNF] groups increased Bax mRNA expressions at the 24th hour in relation to the control group, and that the highest increase was found to be in the combination group administered 11.25+15.8μM [VNC+ε-VNF] (160%) (Table 2). At the 24th hour, compared to the control group, the highest increase of Bcl-2 mRNA expression was determined to be in the 49 μM ε-VNF and 26 μM VNC group (385% and 148%, respectively) HepG2 cells. However, Bcl-2 mRNA expression is downregulated in the event of apoptosis in the cell. In the concentrations of all groups at the 48th hour, no significant increase was seen in Bax mRNA expressions, even though Bcl-2 mRNA expression levels were significantly downregulated compared to the control group. At the 24th hour, HepG2 cells treated with 98.3 μM ε-VNF, or the combination of 11.25+15.8μM [VNC+ε-VNF], increased Bcl2 mRNA expressions in comparison to the control group (289% and 1373%, respectively), with the highest increase determined to be in the 11.25+15.8μM [VNC+ε-VNF] combination group. Lastly, it was observed that Bax mRNA expression significantly increased in the 11.25+15.8μM [VNC+ε-VNF] combination group at the 24th hour compared to control group. From these results, it can be suggested that for the groups whose Bax mRNA expression significantly increases, apoptosis may increase.

**Table 2:** Bax /GAPDH (target gene/reference gene) and Bcl 2 /GAPDH (target gene/reference gene) expressions % ratios in HepG2 cells at different times and concentrations.

| | Bax /GAPDH gene expressions % ratios | | Bcl2 /GAPDH gene expressions % ratios | |
|---|---|---|---|---|
| | 24 hour | 48 hour | 24 hour | 48 hour |
| Control | 100 | 100 | 100 | 100 |
| Vincristine **(52.5 μM)** | 7 | 38 | 43 | 22 |
| Vincristine **(26 μM )** | 85 | 8 | **148** | 1 |
| ε-viniferin **( 98.3 μM )** | 14 | 65 | **289** | 20 |
| ε-viniferin **(49 μM)** | 30 | 75 | **385** | 16 |
| [VNC+ ε-VNF] **(11.25+15.8μM)** | **160** | 6 | **1373** | 9 |
| [VNC+ ε-VNF] **(5.6+7.9μM)** | 50 | 27 | **81** | 1 |

Apoptosis is basically carried out with a class of cysteine proteases called caspases and is one of

the most effective approaches used in the removal of cancer cells.

In our study, all applications, except for ε-VNF incubation, produced results similar to those of caspase-3 activation, which control cells displayed after the 6th, 12th and 24th hour, following 6, 12, 24, 48 and 72 hours treatment of HepG2 cells with VNC, ε-VNF and a combination of the two. In the treatment of 24 hours, the 49 μM ε-VNF dose showed 7.1% caspase-3 activation. However, when HepG2 cells were left in incubation with 52.5 μM VNC, caspase-3 activation was found to be 11.2% (Table 3). This ratio did not show a significant change by reducing VNC concentration to 26 μM (10.1%). The combined application of the drugs increased caspase-3 activation in HepG2 cells in a manner independent of the dosage. While the combined administration of 5.6 +7.9μM (VNC+ε-VNF) resulted in 7.3% caspase-3 activation, this ratio rose up to 7.5% with an increase in 11.25+15.8μM [VNC+ε-VNF] concentration. When combined administration was compared to the control group at the 48th hour, though it increased over time, it was not found to be as effective as the usage of VNC alone in both doses. However, the fact that the combined group had the same activity at a lower dosage as the group in which the agents were administered alone may be considered to be an important indicator in respect to apoptosis.

There are a number of assays which are used to analyse DNA damage, with some tests measuring DNA damage directly, such as TdT-mediated-dUTP nick-end labelling (TUNEL) [17]. The formation of DNA fragmentation in HepG2 cells incubated with ε-VNF, VNC and combined drugs for 24 hours was determined with TUNEL (Fig. 3). In the untreated control group, no DNA fragmentation was observed (Fig. 3A); however, DNA fragmentation was seen after the treatment of HepG2 cells with 52.5 μM VNC (Fig. 3C), 98.3 μM ε-VNF (Fig. 3B) and combined 11.25+15.8μM [VNC+ε-VNF] (Fig 3F). Figure 3D, Figure 3E and Figure 3G show that in the cells treated with half doses, DNA fragments exhibit less apoptotic character. As a result, HepG2 cells were found to be directed to undergo apoptosis due to DNA fragments detected under the different doses of ε-VNF, VNC and combined agents (VNC+ ε-VNF) administered within 24 hours.

**Table 3:** Apoptotic Percentage of Caspase-3 activity of HepG2 cells after viniferin, vincristine and vincristine+viniferin [VNC+VNF] treatment in different concentrations.

| | 6 hours | | 12 hours | | 24 hours | | 48 hours | | 72 hours | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % Viability | % Apopt. | % Viability | % Apopt. | % Viability | % Apopt. | % Viability | % Apopt. | % Viability | % Apopt. |
| Control | 96.1 | 3.9 | 99.1 | 0.9 | 97.4 | 2.6 | 97.3 | 2.7 | 97.6 | 2.4 |
| ε-Viniferin ( 98.3μM ) | 95 | 5 | 97.3 | 2.2 | 95.5 | 4.5 | 97.8 | 2.2 | 98 | 2 |
| ε-Viniferin (49 μM) | 93.3 | 6.7 | 95.4 | 4.6 | 92.9 | 7.1 | 97.2 | 2.8 | 97.4 | 2.6 |
| Vincristine (52.5 μM) | 95.9 | 4.1 | 99.3 | 0.7 | 98 | 2 | 88.8 | 11.2 | 99 | 1 |
| Vincristine (26 μM ) | 96.5 | 3.5 | 98.8 | 1.2 | 98.8 | 1.2 | 89.9 | 10.1 | 98.9 | 1.1 |
| VNC + ε-VNF (11.25 +15.8μM) | 96.6 | 3.4 | 99.1 | 0.9 | 98.4 | 1.6 | 92.5 | 7.5 | 98.8 | 1.2 |
| VNC+ ε-VNF (5.6 +7.9μM) | 96.3 | 3.7 | 97.4 | 2.6 | 97.5 | 2.5 | 92.7 | 7.3 | 98.5 | 1.5 |

In many studies found in the literature, the anticancer effects of other natural compounds containing ε-viniferin and resveratrol were investigated alone [18] and also as combined with other chemotherapeutic agents [19] in lymphoid myeloid, leukemia, colon cancer and Merkel carcinoma on HepG2, HeLa, C6, MCF-7, HT-29 cell lines [20-27]. However, there were no studies found in which the combined usage of ε-VNF with VNC led to DNA fragmentation in HepG2 cells, which is an indicator specific to apoptosis. In a study carried out in China that was closely related to our study, DNA fragmentation was observed 24 hours after a 20 mg/ml application dose of extracts obtained from the China-grown roots of Paeoniae Radix (PRE), some of the compounds of which contain resveratrol, to HepG2 cells [28]. In another study performed in 2010, extracts obtained from the roots of the Oenothera species were used in combination with VNC on HeG2 cells, and a decrease in the amount of ATP used was

detected after an incubation of 24 hours, when DNA fragmentation of the cells occurred [29]. In the study by Machana et al. [30], the administration of 10 different derivatives with cytotoxic properties, obtained from 6 different plant species endemic to China and similar to vincristine, to HepG2 cells led to morphological nuclear changes specific to apoptosis and DNA fragmentation after an incubation period of 24 hours.

To conclude, the combined administration of VNC+ε-VNF was found to be more effective on the permeability of the mitochondrial membrane at the 6th hour compared to single drug administration. In addition, the stimulation of m-RNA expression level by Bax, one of the mitochondrial membrane proteins, in 24 hours, as a result of combined drug administration, suggests that this activity might be seen via the mitochondrial pathway. However, this finding has to be further supported with the examination of other mitochondrial proapoptotic protein expression levels. Single drug administration was not as effective as combined treatment in respect to caspase-3 activation and the formation of DNA fragmentation. This study has demonstrated that an effective dose of VNC can be reduced to lower concentrations with the combination of ε-VNF and stimulated mitochondrial dependent apoptotic pathway in HepG2 cells.

**Acknowledgements**

This work was funded by a grant from the Anadolu University (Project No. 090306). Authors wish to thank Anadolu University Plants Drugs and Scientific Research Center for technical support.

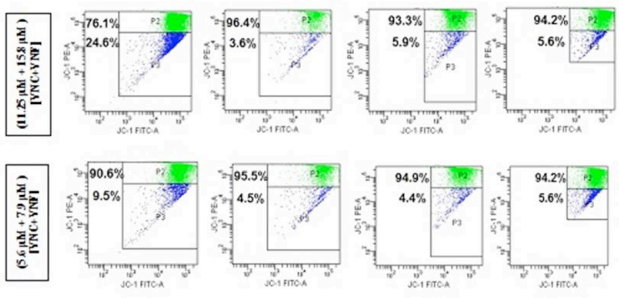


**Fig. 2.** Combined [VNC+ε-VNF] treatment induces mitochondrial membrane permeabilization in HepG2 cells. Cells were treated with [11.25 VNC+ 15.8 µM VNF] and [5.6 VNC+ 7.9 µM VNF] for 6, 12, 24, and 48 h. (A) Mitochondrial membrane permeabilization (MMP) was determined by flow cytometry using JC-1. The percentages of cells with MMP (blue) were calculated.

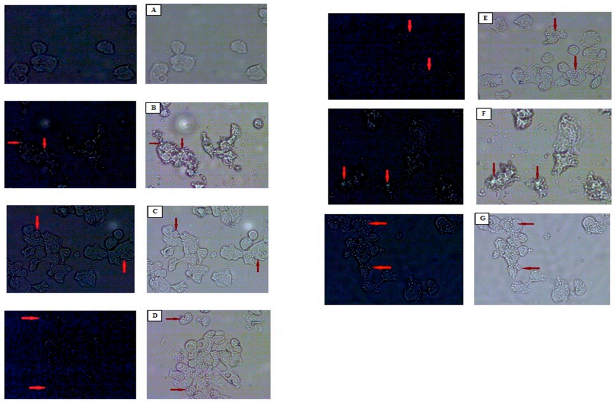

**Fig. 3.** Control group [A], HepG2 cells were treated with 98.3 µM ε-viniferin [B], 52.5 µM vincristine [C], 49 µM ε-viniferin [D], 26 µM vincristine [E], (11.25+15.8µM) [vincristine+ ε-viniferin] [F], (5.6 +7.9µM) [vincristine+ ε-viniferin] [G] for 24 hours. No treated HepG2 cells used as control group and thus gave TUNEL-negative results indicating no apoptotic signal. Arrows indicated cells with fragmented DNA because of which occured actively at the treatment and presence of apoptotic bodies at 24 hours. (Magnification X 40).

**Declaration of Conflicting Interests:** The authors declare that they have no conflict of interest.

**Financial Disclosure:** No financial support was received.

## REFERENCES

1. Ji Y, Ji C, Yue L, Xu H. Saponins isolated from Asparagus induce apoptosis in human hepatoma cell line HepG2 through a mitochondrial-mediated pathway. Curr Oncol. 2012;19 (Suppl 2):1.

2. Khan M, Li T, Ahmad Khan M.K, et al. Alantolactone Induces Apoptosis in HepG2 Cells through GSH Depletion, Inhibition of STAT3 Activation, and Mitochondrial Dysfunction, Hindawi Publishing Corporation. Biomed Res Int. 2013;2013:719858.

3. Ohgidani M, Komizu Y, Goto K, Ueoka R. Residual powders from Shochu distillation remnants induce apoptosis in human hepatoma cells via the caspase-independent pathway. J Biosci Bioeng. 2012;114:104.
4. Xu SP, Sun GP, Shen YX, et al. Synergistic effect of combining paeonol and cisplatin on apoptotic induction of human hepatoma cell lines. Acta Pharmacol Sin. 2007;28:869.
5. Cao MR, Li Q, Liu ZL, et al. Harmine induces apoptosis in HepG2 cells via mitochondrial signaling pathway. Hepatobiliary Pancreat Dis Int. 2011;10:599.
6. Fan LL, Sun GP, Wei W, et al. Melatonin and doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. World J Gastroenterol. 2010;28:16:1473.
7. Zghonda N, Yoshida S, Araki M, et al. Greater effectiveness of ε-viniferin in red wine than its monomer resveratrol for inhibiting vascular smooth muscle cell proliferation and migration. Biosci Biotechnol Biochem. 2011;75:1259.
8. Santamaria AR, Innocenti M, Mulinacci N, et al. Enhancement of viniferin production in Vitis vinifera L. cv. Alphonse Lavallée Cell suspensions by low-energy ultrasound alone and in combination with methyl jasmonate. J Agric Food Chem. 2012;60:11135.
9. Colin D, Lancon A, Delmas D, et al. Antiproliferative activities of resveratrol and related compounds in human hepatocyte derived HepG2 cells are associated with biochemical cell disturbance revealed by fluorescence analyses. Biochimie. 2008;90:1674.
10. Ozdemir F, Akalın G, Sen M, et al. Towards Novel Anti-tumor Strategies for Hepatic Cancer: ε-Viniferin in Combination with Vincristine Displays Pharmacodynamic Synergy at Lower Doses in HepG2 Cells. OMICS. 2014;18:324.
11. Yang L, Liu X, Lu Z, et al. Ursolic acid induces doxorubicin-resistant HepG2 cell death via the release of apoptosis-inducing factor. Cancer Lett. 2010;298:128.
12. Chen X, Cao Z, Zhang Y, et al. Fuzheng Qingjie Granules Inhibit Growth of Hepatoma Cells via Inducing Mitochondria-Mediated Apoptosis and Enhancing Immune Function. Integr Cancer Ther. 2016:1–10.
13. Todor I.N, Lukianova N.Yu, Shvets Yu.V, et al. Metabolic changes during development of Walker- 256 carcinosarcoma resistance to doxorubicin. Exp Oncol. 2015:37,1,19–22
14. Darzynkiewicz Z, Bedner E, Smolewski P. Flow cytometry in analysis of cell cycle and apoptosis. Semin Hematol. 2001;38:179.
15. Jiang CP, Ding H, Shi DH, et al. Pro-apoptotic effects of tectorigenin on human hepatocellular carcinoma HepG2 cells. World J Gastroenterol. 2012;21:18:1753.
16. Dikmen M, Ozturk N, Ozturk Y. The Antioxidant Potency of Punica granatum L. Fruit Peel Reduces Cell Proliferation and Induces Apoptosis on Breast Cancer. J Med Food. 2011;14:1.
17. Robinson L, Gallos ID, Conner SJ, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27:2908-17.
18. Xue Y-Q, Di J-M, Luo Y, et al. Resveratrol Oligomers for the Prevention and Treatment of Cancers. Oxid Med Cell Longev. 2014;(2014):765832.
19. Heiduschka G, Lill C, Seemann R, et al. The effect of resveratrol in combination with irradiation and chemotherapy: Study using Merkel cell carcinoma cell lines. Strahlenther Onkol. 2014;190:75.
20. Billard C, Izard J, Roman V, et al. Comparative Antiproliferative and Apoptotic Effects of Resveratrol, e-viniferin and Vine-shots Derived Polyphenols (Vineatrols) on Chronic B Lymphocytic Leukemia Cells and Normal Human Lymphocytes. Leuk Lymphoma. 2002;43:1991.
21. Ohguchi K, Akao Y, Matsumoto K, et al. Vaticanol C-induced cell death is associated with inhibition of pro-survival signalling in HL60 human leukemia cell line. Biosci Biotechnol Biochem. 2005;69:353.
22. Bhat KP, Pezzuto JM. Cancer chemopreventive activity of resveratrol. Ann N Y Acad Sci. 2002;957:210.
23. Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natura product derived from grapes. Science. 1997;275(5297):218.
24. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov. 2006;5:493.
25. Gonz´alez-Sarr´ıas A, Gromek S, Niesen D, et al. Resveratrol oligomers isolated from carex species inhibit growth of human colon tumorigenic cells mediated by cell cycle arrest. J Agric Food Chem. 2011;59:8632.
26. Barjot C, Tournaire M, Castagnino C, et al. Evaluation of antitumor effects of two vine stalk oligomers of resveratrol on a panel of lymphoid andmyeloid cell lines: comparison with resveratrol. Life Sci. 2007;81:1565.
27. Marel A.K, Lizard G, Izard J.C, et al. Inhibitory effects of trans-resveratrol analogsmolecules on the proliferation and the cell cycle progression of human colon tumoral cells. Mol Nutr Food Res. 2008;52:538.
28. Lee SM, Li ML, Tse YC, et al. Paeoniae Radix, a Chinese herbal extract, inhibit hepatoma cells growth by inducing apoptosis in a p53 independent pathway. Life Sci. 2002;27:71:2267.
29. Jaszewska E, Kosmider A, Kiss AK, Naruszewicz M. Oenothera paradoxa defatted seeds extract containing pentagalloylglucose and procyanidins potentiates the cytotoxicity of vincristine. J Physiol Pharmacol. 2010;61:637.
30. Machana S, Weerapreeyakul N, Barusrux S, et al. Cytotoxic and apoptotic effects of six herbal plants against the human hepatocarcinoma (HepG2) cell line. Chin Med. 2011;31:6:39.