

Carvacrol effect on topotecan cytotoxicity in various human cancer cells *in vitro*

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Abstract

Purpose: To investigate the modulatory effect of the natural phytochemical, carvacrol, on Topotecan (TOPO) cytotoxicity and cellular uptake in different cancer cell lines.

Methods: The cytotoxicity of the carvacrol/TOPO combination therapy was determined *in vitro* using crystal violet assay. Coomassie blue and DAPI fluorescent stains were used for cellular morphology and molecular cell death assessments, respectively. Additionally, TOPO cellular uptake after carvacrol/TOPO combination therapy was determined.

Results: Treatment of HeLa and HCT116 with carvacrol/TOPO resulted in 7.70- and 5.71-fold reduction in TOPO half maximal inhibitory concentration (IC₅₀), respectively, relative to TOPO single treatment. On the other hand, treatment of MCF-7, HepG2, SKOV3, and A549 cancer cells with carvacrol/TOPO resulted in increasing the IC₅₀ of TOPO by 1.49-, 1.33-, 1.50- and 1.26-fold, respectively, relative to TOPO single treatment.

Conclusion: Carvacrol had enhanced TOPO cytotoxicity and cellular uptake in HeLa and HCT116 cancer cells but might cause TOPO resistance in MCF-7, HepG2, SKOV3 and A549 cells.

Keywords

Apoptosis, Cellular morphology assessment, Cellular uptake, Combination therapy, Crystal violet assay

Introduction

In the twenty first century, cancer is still the leading cause of the death in all over the world (Bray et al. 2018). In order to fight cancer, there are three known therapeutic approaches; surgery, irradiation and chemotherapy. Among the previous therapeutic procedures, chemotherapy, used alone or in combination with other forms of therapy is the favorable approach for the treatment of cancer (Rang et al. 2012). Topotecan (TOPO), an inhibitor of the topoisomerase I, the enzyme responsible for DNA replication in cancer cells (O'Dwyer et al. 1994), is

the single-agent therapy of choice of many complicated types of cancer (Armstrong et al. 2005). However, like other chemotherapeutic agents, TOPO is associated with dose limiting toxicities such as neutropenia, thrombocytopenia, and anemia, and it has a low chance of being curative if used alone due to the development of topoisomerase I resistance (Bansal et al. 2017). Therefore, there is a need to combine TOPO with a safer agent that has an anticancer effect with a different target in order to enhance TOPO cytotoxicity and at the same time decrease its effective therapeutic dose and thereby its side effects (Wang et al. 2012).

Scientific studies nowadays are continuing to prove that many natural herbs and plant extracts; that have been used over generations as natural remedies, have phytochemicals (natural constituents), that exert chemoprevention and chemotherapeutic effects (Newman and Cragg 2016). The most important features of those natural ingredients are the safe nature of them relative to chemotherapy and their effectiveness against many diseases which allow them to offer an effective and safer alternative for cancer treatment (Lai and Roy 2004). Carvacrol (2-methyl-5-(1-methylethyl)-phenol), the liquid phytochemical isolated from the essential oil of thyme (*thymus vulgaris*) and other aromatic plants belonging to the family Lamiaceae, has been used for generations in folk medicine and the previous studies have proven that carvacrol has anti-cancer effects in addition to antioxidant, analgesic, anti-inflammatory, antimicrobial, antiapoptotic, antispasmodic, and antibacterial effects (Fachini-Queiroz et al. 2012; Khan et al. 2018). The strong cytotoxic and proapoptotic effect of carvacrol against various cancer cell lines had been reported in the literature (Sharifi-Rad et al. 2018). Moreover, the Food and Drug Administration (FDA) had assured the non-toxic nature of carvacrol through approving its use as food or chemical flavoring agent (Zotti et al. 2013).

Therefore, the aim of this study is to investigate the effect of carvacrol/TOPO combination treatment on the proliferation of different cancer cell lines relative to TOPO single treatment. Furthermore, assessment of cells morphological alterations and TOPO cellular uptake were performed to have an insight into the possible mechanism of action of this therapy combination.

Materials and methods

Drugs and chemicals

Topotecan (TOPO) hydrochloride and carvacrol were purchased from Sigma Aldrich Co. TOPO stock solutions were prepared by dissolving it in distilled water (D.W) and preserved at -20°C . Dullbecco's modified eagle's medium (DMEM), fetal bovine serum, trypsin/EDTA, penicillin G/streptomycin antibiotics, 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride solution, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and ethanol were obtained from Beijing Solarbio Science and Technology Co., (Shanghai, China). Crystal violet stain (CV) (from: s.d.fine-CHEM Ltd), Coomassie blue R-250 (CB R-250), sodium dodecyl sulfate (SDS), formaldehyde, acetic acid (AA), and methanol were gifted from King Fahd Medical Research Center (KFMRC).

Cancer cell lines and cell culture

MCF-7 breast, HeLa cervical, SKOV3 ovarian, HCT116 colon, HepG2 liver, and A549 non-small lung cancer cells were procured from the American Type Tissue Culture Collection (Manassas, VA, USA) and were gifted from the Regenerative Medicine unit at KFMRC.

All cell lines were grown as adherent monolayer cells in a (25 cm^2) culture flask and the growth medium (DMEM) was supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. The cancer cells were incubated in a 5% CO_2 / 95% humidified atmosphere at 37°C . The DMEM was removed from the cell culture flask and changed with new medium every 48 h. Cells were collected by trypsinization and passaged every 3–4 days after cells were fed to 90% confluence.

In vitro evaluation of cytotoxic activity

Cytotoxicity was determined using CV staining method, which is considered one of the simplest, quickest and most reliable methods to determine the viability of adherent cells especially for the assessment of the interactions between anticancer agents (Saotome et al. 1989; Sliwka et al. 2016).

Briefly, a $100\ \mu\text{l}$ of culture media containing 5×10^3 cells was added into each well of a flat-bottomed 96-well plate and incubated for 24 h at 37°C in a CO_2 incubator in order for cells attachment. After that, cells were treated with $100\ \mu\text{l}$ of the complete medium containing either six concentrations of TOPO-Sol in a range of (1.56–50 μM) or six concentrations of TOPO-Sol in a range (1.56–50 μM) in combination with a fixed concentration of carvacrol (166 μM), and incubated for 24 h at 37°C in a CO_2 incubator. Then, the culture media was discarded, followed by washing the wells carefully with a $100\ \mu\text{l}$ of PBS. Then $50\ \mu\text{l}$ of 0.1% CV stain was added and incubated in the dark hood for 10 minutes. After the incubation time, CV stain was removed and the wells were washed with tap water using the immersion technique and were left to dry. Finally, $100\ \mu\text{l}$ of 1% SDS was added to each well followed by manually plate agitation for 10 minutes. The absorbance (A) was read at 570 nm using a microplate reader (BioTek, Synergy HT microplate reader, USA). Wells containing negative and positive controls included culture media without cells (blank) and culture media containing cells without treatment (control) respectively. Half maximal inhibitory concentration (IC_{50}) values were determined experimentally for each treatment. Experiments for each sample were done in triplicate.

The percentages of growth inhibition were calculated by the following equation:

$$\text{Growth Inhibition (\%)} = \left(1 - \frac{(\text{A of treated cell} - \text{A of blank})}{(\text{A of positive control} - \text{A of blank})} \right) \times 100$$

Assessment of combination therapy synergism

The growth inhibition percentages resulted from the CV assay were inserted into CompuSyn software (Combosyn, Paramus, NJ, USA), in order to determine the combination index values of the carvacrol/TOPO combination therapy in different cell lines based on the combination index theorem of Chou-Talalay (Chou 2010). When $\text{CI} > 1$, the effect is antagonism and when $\text{CI} < 1$, the effect is synergism.

Assessment of TOPO cellular uptake

Topotecan cellular accumulation was assessed in cells by using spectrofluorometer according to the method of Lei et al. (2010). Cells were plated in 24-well plates at cell density of 25×10^3 cells/well in DMEM supplemented medium. Twenty-four hours later, cells were incubated for additional 24 h with different TOPO concentrations, selected previously from the CV staining assay, in the absence or presence of a fixed concentration of carvacrol (166 μM). After 24 h treatment, the cell medium was removed and the wells were washed with 300 μl of ice-cold PBS, then 1 ml of DMSO were added for 10 minutes in order to lyse the cells. Then, the supernatants were col-

lected and centrifuged at 14,000 rpm for 15 minutes to remove cell debris and to obtain cell lysate. The unique intense fluorescence of TOPO (Francis et al. 2015), in the supernatant was measured by a spectrofluorometer (F-2000 Fluorescence spectrophotometer, Hitachi, Japan) at excitation and emission wavelengths of $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 560$ nm, respectively to determine TOPO concentration. To adjust the background fluorescence from cellular components, different concentrations (0.0125, 0.25, 0.5, 1, 2, 4 μM) of TOPO were dissolved in DMSO and added to the untreated cells. Then the fluorescence intensities of supernatants were measured in order to prepare TOPO calibration curve in the presence of untreated cell lysates.

$$\text{TOPO cellular accumulation ratio} = \frac{\text{TOPO concentration in cells treated with carvacrol/TOPO combination therapy}}{\text{TOPO concentration in cells treated with only TOPO}}$$

Cell morphology characterization under light microscope

To evaluate the morphological changes of the treated MCF-7, HCT116, HeLa, HepG2, A549 and SKOV3 cell lines, cells were plated at a density of 5×10^3 cells per well into each well of the flat-bottomed 96-well plate and were incubated overnight in a CO_2 incubator at 37 °C. After cells attachment, 100 μl of drug concentrations selected earlier based on the IC_{50} values of TOPO solutions measured by the CV assay, were added after discarding the old medium and were incubated for 24 h at the same previous conditions in the absence or presence of (166 μM) carvacrol. Finally, cell morphology was evaluated by light microscope (TH4-200, Olympus optical Co-Ltd, Japan) after staining the cells with 0.02% CB R-250, according to the method of Alkhatib et al. (2017).

Morphological assessment of apoptotic cells using fluorescent microscope

The DNA fragmentation and nuclear abnormalities of the treated cells undergoing apoptosis were detected by using the DAPI stain. Cells were seeded at a density of 5×10^3 cells per 100 μl of DMEM into the wells of the 96 well-plates. Then cells were treated with the different TOPO concentrations (IC_{50}) selected according to the results measured by the CV assay, in the absence or presence of (166 μM) carvacrol. Following incubation for 24 h at 37 °C in a CO_2 incubator, cell morphology of the DAPI stained cells was assessed by a fluorescent microscope with blue filter at 437 μm (Leica CRT6000, Germany) according to the method of Alkhatib et al. (2018).

Statistical analysis

Statistical analysis was implemented using MegaStat Excel (version 10.3, Butler University, Indianapolis, IN). All data were expressed as mean \pm standard deviation (SD) for triplicate measurements. Independent *t*-test was used for the comparison between two independent groups and one-

way analysis of variance (ANOVA) followed by Tukey's test for post hoc analyses were used for multiple comparisons. Statistical differences were considered significant, highly significant and very highly significant when $0.01 \leq P < 0.05$, $0.001 \leq P < 0.01$ and $P < 0.001$, respectively.

Results

Effect of carvacrol on TOPO cytotoxicity in different cancer cell lines

The cytotoxicity of TOPO in the absence or the presence of (166 μM) carvacrol in HeLa, HCT116, MCF-7, HepG2, A549, and SKOV3 cancer cells were expressed as the percentages of growth-inhibiting rates (Fig. 1). The (166 μM) carvacrol concentration, which is smaller than the resulted carvacrol IC_{50} concentration in each cell line, was chosen and unified for all cancer cells. The IC_{50} s of the different treatment protocols, and their combination indexes were also evaluated as presented in Table 1. Six TOPO concentrations ranged from

Table 1. Effect of carvacrol/TOPO combination therapy on the growth of different cancer cell lines and their combination indexes (CI). Data were expressed as mean \pm SD, in triplicate.

Cancer Cell Line	TOPO IC_{50} (μM)	(TOPO + carvacrol (166 μM)) IC_{50} (μM)	(TOPO + carvacrol (166 μM)) CI-value
HeLa	26.57 \pm 0.50	3.45 \pm 0.31 ****	1.23E-19 Synergism
HCT116	15.88 \pm 0.08	2.78 \pm 0.25 ****	0.20 Synergism
MCF-7	12.65 \pm 0.05	18.91 \pm 0.37 ****	9.80 Antagonism
HepG2	5.5 \pm 0.5	7.35 \pm 0.31 *	36.80 Antagonism
SKOV3	32.50 \pm 2.50	48.83 \pm 1.61 ***	3.19 Antagonism
A549	10.63 \pm 0.32	13.39 \pm 0.53 **	14.89 Antagonism

Notes: the significant differences between TOPO IC_{50} s in the absence or presence of a fixed concentration of carvacrol (166 μM). IC_{50} in each cell line assessed by measuring the *P*-values using the independent *t*-test, were classified to * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

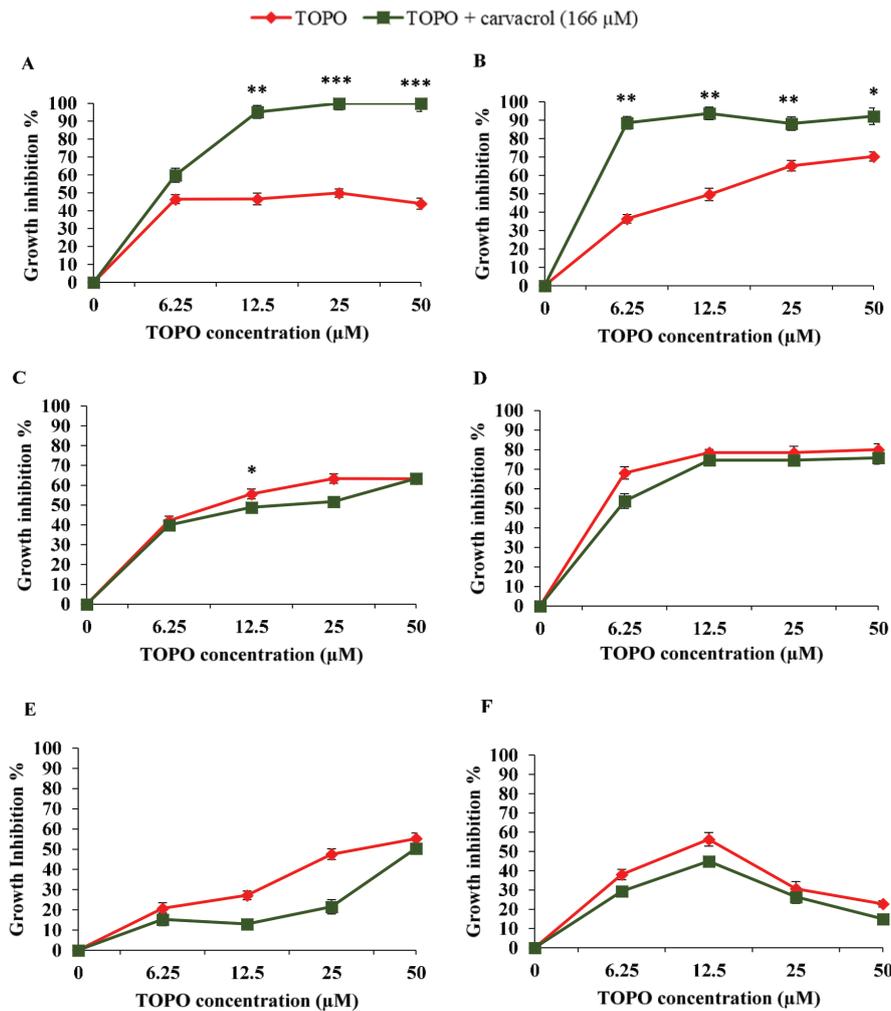


Figure 1. The curves of the growth inhibition percentages after 24 h treatment with different concentrations of TOPO in the absence or presence of a fixed concentration of carvacrol (166 μM) in (A) HeLa, (B) HCT116, (C) MCF-7, (D) HepG2, (E) SKOV3 and (F) A549 cell lines. Data were expressed as mean ± SD (error bars), $n = 3$. The significant differences between TOPO and (TOPO + carvacrol (166 μM)) combination at each time assessed by measuring the P -values using the independent t -test, were classified to * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

1.56 to 50 μM were added to cells, either alone or combined with (166 μM) carvacrol, then the cytotoxicity was estimated after 24 h by CV assay. The statistical comparisons between different treatment protocols at the same TOPO concentration were performed by the independent t -test.

Simultaneous addition of carvacrol to TOPO in cancer cells for 24 h was found to sensitize or inhibit TOPO growth inhibition percentage, depending on the type of the treated cancer cells. In HeLa and HCT116 cells, the growth inhibitory curves of TOPO combined with (166 μM) carvacrol were significantly increased relative to the curves of TOPO given alone at

TOPO concentrations of 25 and 50 μM ($P < 0.001$) in HeLa cells and at TOPO concentrations of 6.25, 12.5 and 25 μM ($P < 0.01$) in HCT116 cells. However, the growth inhibitory curves of the combination therapy were found to be decreased in comparison with TOPO alone in MCF-7, HepG2, A549, and SKOV3 cancer cells.

In terms of IC_{50} as illustrated in Table 1, HeLa and HCT116 cells treated with TOPO and (166 μM) carvacrol combination, were having a highly significant smaller IC_{50}

than TOPO solution at P -value < 0.0001 , which means that carvacrol addition to TOPO may enhanced its cytotoxic effect. The IC_{50} values were reduced by factors of 7.7 and 5.7 relative to TOPO single treatment in HeLa and HCT116 cells, respectively. Moreover, the combination indexes of cells treated with the combination treatment were less than one in both cell lines, indicating a synergistic effect between TOPO and carvacrol.

On the other hand, the addition of carvacrol to TOPO has significantly increased the IC_{50} values relative to TOPO single treatment in MCF-7 ($P < 0.0001$), HepG2 ($P < 0.05$), A549 ($P < 0.001$), and SKOV3 ($P < 0.01$). In other words, carvacrol addition to TOPO may reduced its cytotoxic effect as understood from their combination index values which are all larger than one, indicating antagonism.

Effect of carvacrol on TOPO cellular uptake in different cancer cell lines

The different cell lines were treated with two concentrations of TOPO (2 and 5 μM) in the absence or presence of

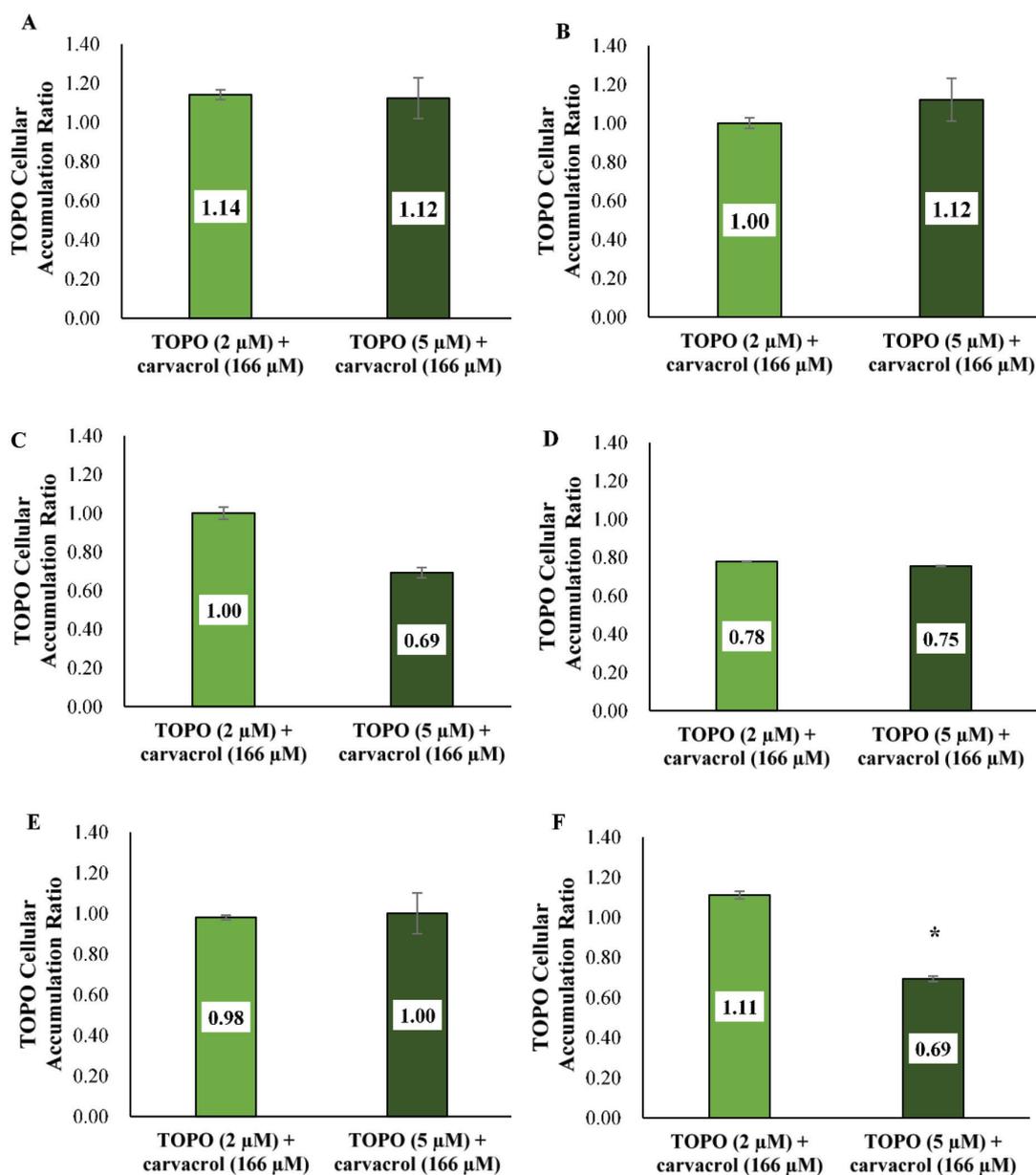


Figure 2. The effect of the 24 h treatment with (TOPO (2 or 5 μM) + carvacrol (166 μM)) combination on TOPO cellular uptake in (A) HeLa, (B) HCT116, (C) MCF-7, (D) HepG2, (E) SKOV3 and (F) A549 cell lines. Data were expressed as mean ± SD (error bars), $n = 3$. The significant differences between the different (TOPO + carvacrol (166 μM)) combinations at each time assessed by measuring the P -values using the independent t -test, were classified to * $P < 0.05$.

(166 μM) carvacrol. TOPO intracellular uptake concentrations in HeLa, HCT116, MCF-7, HepG2, SKOV3, and A549 cancer cells and their accumulation ratios after treatment with TOPO alone or in combination with carvacrol, were illustrated in Table 2 and Fig. 2, respectively. In HeLa and HCT116 cells, carvacrol co-administration with TOPO has increased its TOPO intracellular concentration especially when given with the higher (5 μM) TOPO concentration. The accumulation ratio of TOPO was 1.12 when both cell lines were treated with (5 μM) TOPO and (166 μM) carvacrol combination, which means that carvacrol may enhanced TOPO cellular uptake.

In contrast to the effect of carvacrol on TOPO cellular uptake in HeLa and HCT116 cells, carvacrol caused a slight decrease in TOPO intracellular concentration when

Table 2. Effect of carvacrol/TOPO combination therapy on TOPO cellular uptake in different cancer cell lines. Data were expressed as mean ± SD, in triplicate.

Cancer Cell line	TOPO intracellular uptake (μM)			
	TOPO (2 μM)	TOPO (2 μM) + carvacrol (166 μM)	TOPO (5 μM)	TOPO (5 μM) + carvacrol (166 μM)
HeLa	0.068 ± 0.000	0.077 ± 0.017	0.077 ± 0.017	0.087 ± 0.017
HCT116	0.066 ± 0.018	0.066 ± 0.018	0.086 ± 0.018	0.097 ± 0.018
MCF-7	0.059 ± 0.019	0.059 ± 0.019	0.070 ± 0.033	0.049 ± 0.019
HepG2	0.085 ± 0.016	0.066 ± 0.000	0.075 ± 0.016	0.057 ± 0.016
SKOV3	0.055 ± 0.017	0.054 ± 0.015	0.046 ± 0.017	0.046 ± 0.015
A549	0.096 ± 0.018	0.106 ± 0.018	0.138 ± 0.018	0.096 ± 0.018*

Notes: Significant differences between the intracellular uptake in cells treated with only TOPO and with (the same TOPO concentration + carvacrol (166 μM)) combination in each cell line assessed by measuring the P -values using the independent t -test, were significant at * $P < 0.05$.

carvacrol was added to 5 μM TOPO in MCF-7, HepG2 and A549 cancer cells (Table 2). The decrease in TOPO intracellular concentration when (166 μM) carvacrol was added to (5 μM) TOPO, was more obvious relative to its effect on the lower (2 μM) TOPO concentration. TOPO accumulation ratios were 0.69, 0.75 and 0.69 when MCF-7, HepG2 and A549 cancer cells treated with (5 μM) TOPO and (166 μM) carvacrol combination, respectively. Accordingly, carvacrol may reduce TOPO cellular uptake.

Cell morphology characterization under light microscope

Cell morphologies were assessed under light microscope for HeLa, HCT116, MCF-7, HepG2, A549, and SKOV3 cancer cells. As displayed in Figs 3 and 4, the images of control (untreated) HeLa, HCT116, MCF-7, HepG2, A549, and SKOV3 cancer cells, revealed the whole cells with no evidence of segmentation or fragmentation, but when cells subjected to TOPO alone or in combination with (166 μM) carvacrol, morphological changes were observed.

In all cell lines (Figs 3, 4), the images of cells treated with TOPO showed a clear signs of nuclei shape changing and apoptotic characteristics such as cell enlargement with cytoplasm shrinkage, membrane blebbing (outward pulse of cytoplasmic membrane), chromatin condensation (chromatin margination without nuclear condensation), intercellular space increase and cell structure loss. Furthermore, in HeLa and HCT116 cells (Fig. 3A, B), the cell number population was decreased in TOPO treated cells relative to control, and that decrease in addition to

the other apoptotic characteristics, were more dramatic in cells treated with TOPO and (166 μM) carvacrol combination therapy. In contrast, the addition of (166 μM) carvacrol to TOPO in MCF-7, HepG2, A549, and SKOV3 cancer cells (Fig. 4A–D), caused no change or a slight increase in cell population in comparison to cells treated with only TOPO.

Morphological assessment of apoptotic cells using fluorescent Microscope

Fluorescent nuclear staining with the cell permeable nucleic acid dye (DAPI) was used to assess alterations in nuclear morphology after treatment of HeLa, HCT116, MCF-7, HepG2, A549, and SKOV3 cancer cells with the IC_{50} concentrations of TOPO in absence or presence of (166 μM) carvacrol and incubated for 24 h (Figs 5, 6). Although the nuclei of the healthy control cells of all the different cell lines were large and displayed smooth, uniform and diffused staining under the fluorescent microscope, the nuclei of treated cells showed clear changes of nuclear morphology. All the different cell lines when treated with their IC_{50} TOPO concentrations, which were selected based on the results of the growth inhibition curves illustrated in Fig. 1, clearly represented an early induction of apoptosis.

In HeLa and HCT116 cells (Fig. 5A, B), obvious nucleus enlargement and decrease in cell population were seen after TOPO treatment, and these apoptotic characteristics were more dramatic when carvacrol was added to TOPO. Also, in MCF-7, HepG2, A549, and SKOV3 cancer

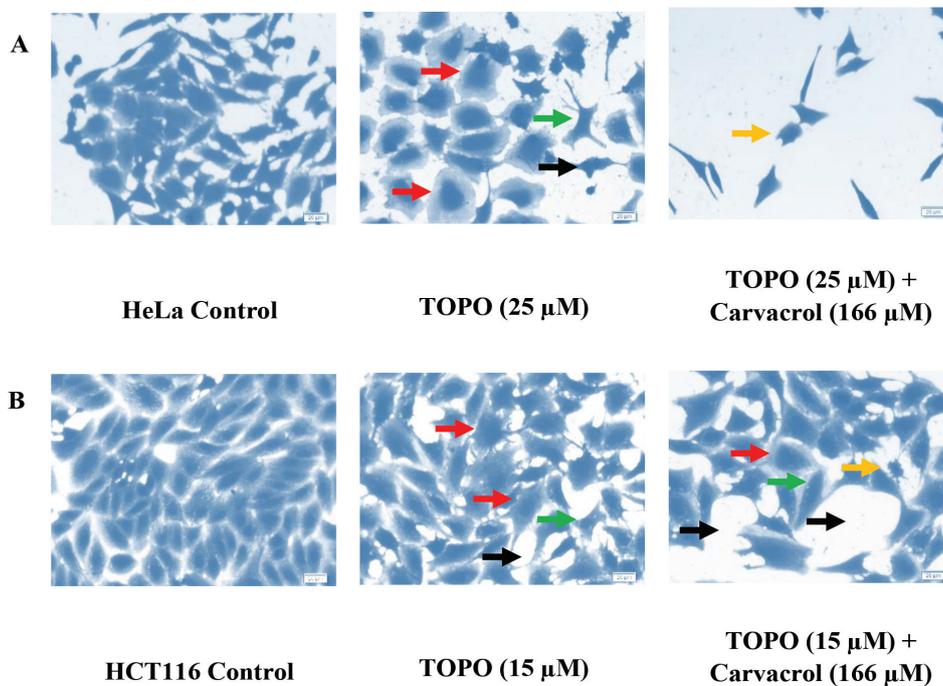


Figure 3. Light microscopy images (Scale bar: 20 μm) of (A) HeLa and (B) HCT116 cell lines treated for 24 h with TOPO IC_{50} in the absence or presence of carvacrol (166 μM). Images were magnified at 20 \times . The red, green, orange and black arrows represented cell enlargement with cytoplasm shrinkage, membrane blebbing, apoptotic bodies and intercellular space increase, respectively. Images were taken from at least three independent experiments with similar conditions.

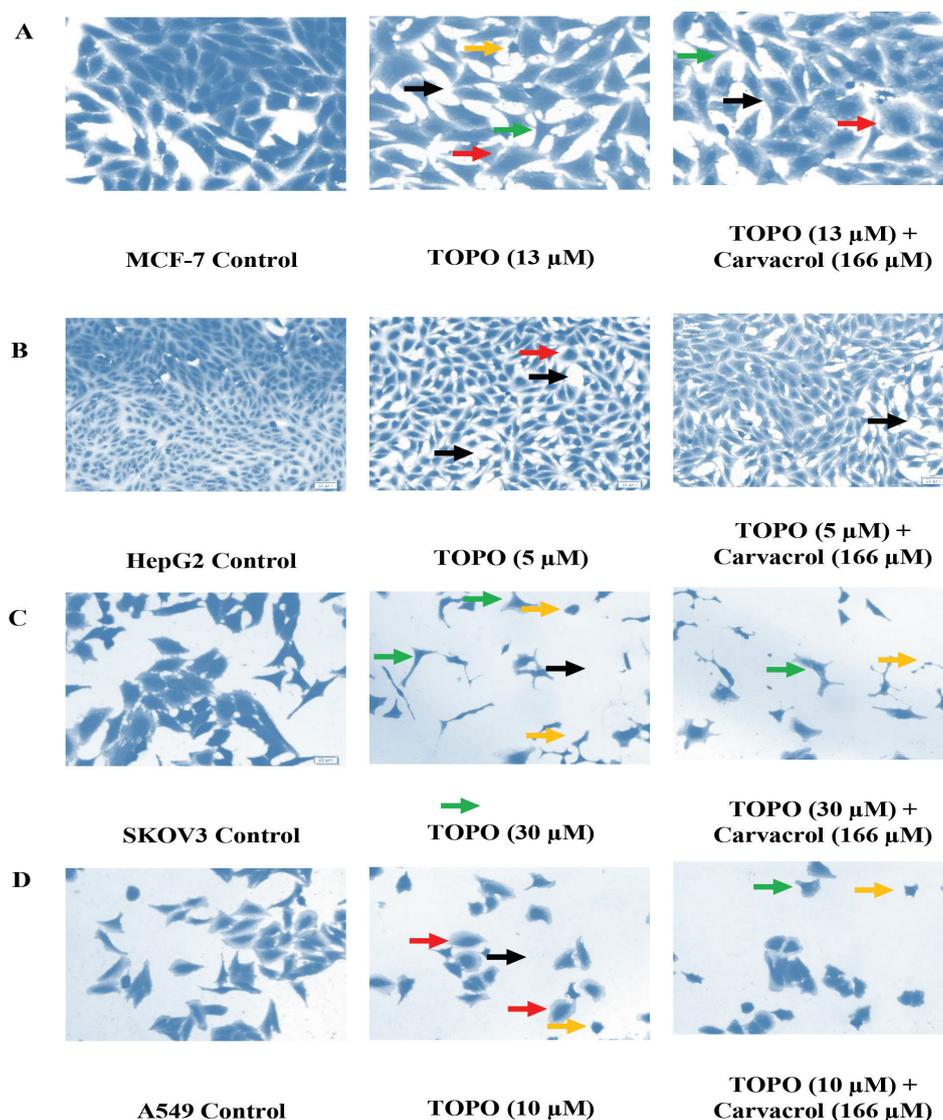


Figure 4. Light microscopy images (Scale bar: 20 μm) of (A) MCF-7, (B) HepG2, (C) SKOV3 and (D) A549 cell lines treated for 24 h with TOPO IC_{50} in the absence or presence of carvacrol (166 μM). Images were magnified at 20 \times . The red, green, orange and black arrows represented cell enlargement with cytoplasm shrinkage, membrane blebbing, apoptotic bodies and intercellular space increase, respectively. Images were taken from at least three independent experiments with similar conditions.

cells (Fig. 6A–D), nuclear abnormalities and cell population decrease were observed, but when cells were treated with the TOPO and (166 μM) carvacrol combination, the previous apoptotic features were diminished relative to TOPO lone treatment.

Discussion

Because treatment with TOPO alone is unlikely to be curative and is prone to resistance and severe toxicities, there is an interest in combining TOPO with natural and safer anticancer agents that has a different mechanism of cell death (Wang et al. 2012). Despite this interest, relatively few *in vitro* studies examining TOPO-phytochemical combinations have been performed. Therefore, in this study we combined carvacrol, which is a natural anticancer

agent, with TOPO treatment, then, we investigated the modulatory effect of it on TOPO cytotoxicity in various cancer cell lines. The possible modulatory mechanisms were assessed by examining TOPO cellular uptake and cellular morphological changes after treatment with different TOPO concentrations in the presence or absence of (166 μM) carvacrol.

Carvacrol, the monoterpenoid phenolic phytochemical, has demonstrated cytotoxic effects in several human cancer cells such as cervical cancer (Potocnjak et al. 2018), colon cancer (Fan et al. 2015), breast cancer (Mari et al. 2020), hepatoma (Yin et al. 2012; Elshafie et al. 2017), ovarian cancer (Elbe et al. 2020), and non-small cell lung cancer (Koparal and Zeytinoglu 2003; Jung et al. 2018). In contrast to TOPO mechanism of action, the mechanism of carvacrol antiproliferative effect was through reactive oxygen species induced apoptosis in most of the tested

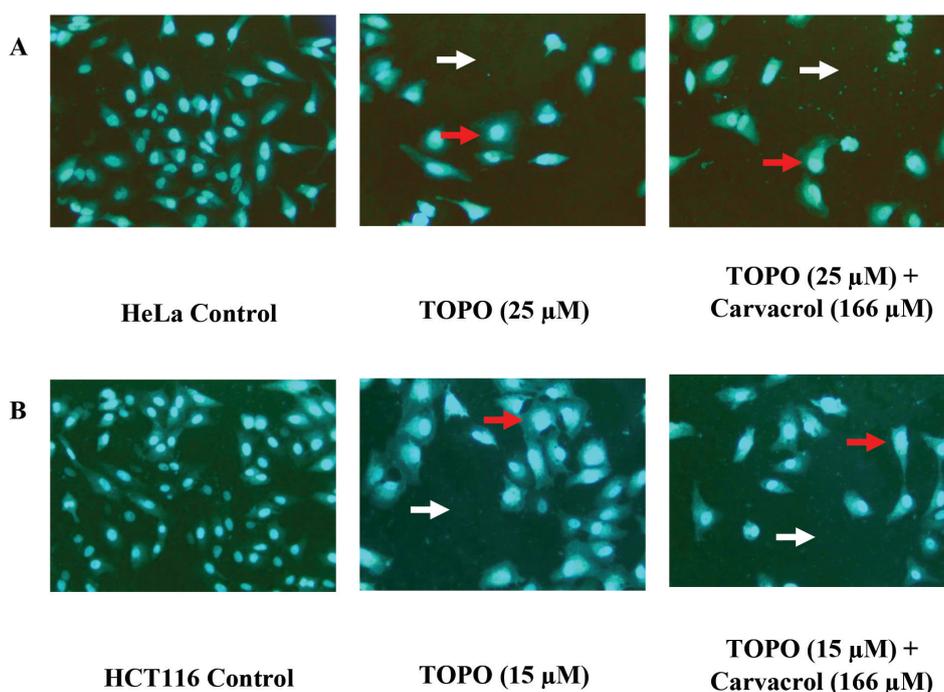


Figure 5. Fluorescent microscopy images (Scale bar: 20 μm) of A) HeLa and B) HCT116 cell lines treated for 24 h with TOPO IC_{50} in the absence or presence of carvacrol (166 μM). Images were magnified at 20 \times . The red, and white arrows represented nuclear enlargement or irregular shape, and intercellular space increase, respectively. Images were taken from at least three independent experiments with similar conditions.

cancer cell lines (Potocnjak et al. 2018). Therefore, in this study we studied the modulatory effect of carvacrol on TOPO cytotoxicity, cellular uptake and induced cellular morphology changes.

The results indicated that HeLa cervical cancer cells and HCT116 colon cancer cells showed an increase in their sensitivity when treated with carvacrol/TOPO combination therapy relative to the free-TOPO. Moreover, the addition of carvacrol to TOPO caused a highly significant 7.70- and 5.71-fold decrease in the IC_{50} concentrations (P -value < 0.0001) relative to TOPO single treatment in HeLa and HCT116 cells, respectively. The decrease in IC_{50} value, indicated that the combination treatment was more cytotoxic than TOPO alone because lower TOPO dose was needed to exert 50% cell death, than the dose needed to exert the same effect in both cell lines when treated with free-TOPO. To explore the mechanism behind the previous results, light and fluorescent microscopy images were taken after 24 h of the TOPO-carvacrol combination treatment. The images illustrated an increase in all the apoptotic features noticed in TOPO single treatment in both types of cancer cells especially the reduced cell population (Alkhatib et al. 2020). Our findings agreed with previous studies that showed carvacrol induced apoptosis through DNA fragmentation in HeLa cells (Mehdi et al. 2011), and through mitochondrial apoptotic pathway and the MAPK and PI3K/Akt signaling pathway in HCT116 cancer cells (Fan et al. 2015).

Furthermore, our data indicated an increase in TOPO intercellular levels after the addition of carvacrol, which was in accord with the decreased TOPO IC_{50} concen-

trations, and the increased apoptotic features exhibited earlier in treated HeLa and HCT116 cells. In fact, TOPO was proven to be a substrate of P-glycoprotein, the ATP dependent active transporter in cancer cells, and multi-drug resistance associated protein 1 (MRP1) drug transporters (Tian et al. 2006), while carvacrol was proven to be an inhibitor of P-glycoprotein mediated transport in LLC-GA5-CoL150 renal cancer cell line (Yoshida et al. 2006). Accordingly, carvacrol may inhibit TOPO efflux by inhibiting P-glycoprotein drug transporter and MRP1, resulting in an increase TOPO intercellular concentration compared to cells treated with single TOPO treatment.

The cytotoxic effect resulted from an interaction between two agents is considered synergistic, either when it is greater than the expected effect from one of these agents alone or when it is equal to the cytotoxic effect resulted from one of the two single agents but at better tolerated reduced drug concentration (Ramsay et al. 2005; Chou 2010). Therefore, a synergistic interaction between TOPO and carvacrol may be concluded, due to all the previous findings and due to the calculated CI values, which were less than one, in HeLa and HCT116 cancer cells. A previous study confirmed that some herbal phytochemicals were found to inhibit P-glycoprotein and at the same time cause direct cytotoxic effect, thereby, exerting synergistic interaction with camptothecins (the parent compound of TOPO) (Bansal et al. 2009).

On the other hand, in MCF-7, HepG2, A549, and SKOV3 cancer cells, our findings exhibited a decrease in TOPO induced cellular growth inhibition percentages after the addi-

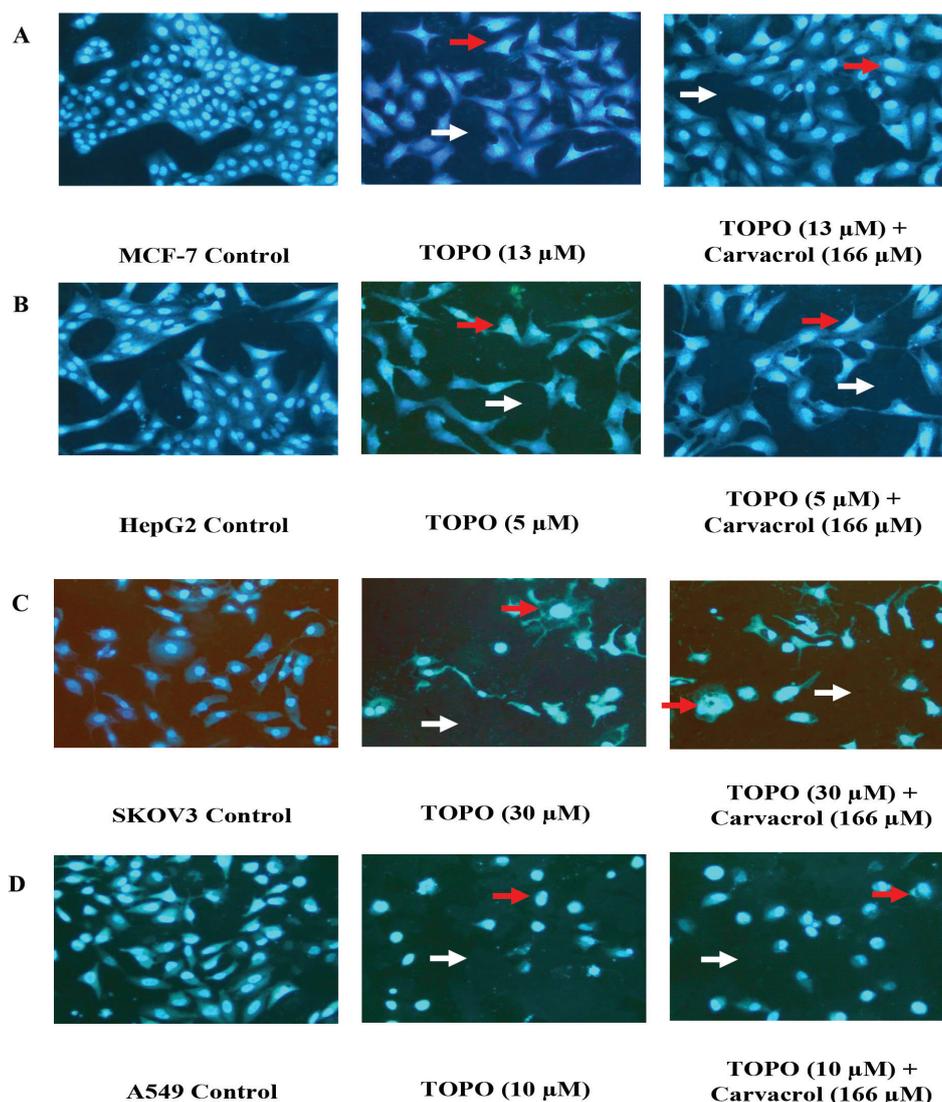


Figure 6. Fluorescent microscopy images (Scale bar: 20 μm) of (A) MCF-7, (B) HepG2, (C) SKOV3 and (D) A549 cell lines cell lines treated for 24 h with TOPO IC_{50} in the absence or presence of carvacrol (166 μM). Images were magnified at 20 \times . The red, and white arrows represented nuclear enlargement or irregular shape, and intercellular space increase, respectively. Images were taken from at least three independent experiments with similar conditions.

tion of carvacrol in comparison with TOPO alone. Also, the IC_{50} concentrations were significantly increased relative to the IC_{50} of TOPO single treatment in the same cell line. In addition, CB R-250 and DAPI stained images illustrated no difference or slight increase in cell viability relative to cells treated with TOPO alone. These results can be explained because carvacrol may exert different types of interaction with anticancer agents depending on the treated cancer cell line and the combined anticancer drugs. For example, carvacrol was found to be cytotoxic against HeLa cancer cells but when combined with cisplatin (the DNA damaging anticancer drug) it induced resistance through apoptosis and autophagy modulation (Potocnjak et al. 2018).

Moreover, the TOPO accumulation ratios were decreased when TOPO-carvacrol treatment protocol was used, which was more significant when higher TOPO concentrations were combined with carvacrol especially in MCF-7 and A549 cancer cells. The reduced TOPO in-

tercellular concentration was indicated by the decreased cytotoxic effect and the increased IC_{50} noticed after the addition of carvacrol to TOPO, suggesting a possible induction of TOPO efflux from the intercellular compartment through P-glycoprotein or other efflux pump, which is a quite common mechanism of resistance to chemotherapeutic agents (Ughachukwu and Unekwe 2012).

It can be concluded from the carvacrol/TOPO combination therapy protocol in MCF-7, HepG2, A549, and SKOV3 cancer cells that carvacrol exhibited an antagonistic effect when combined with TOPO, although it was proven to be cytotoxic when given alone in the previous literature (Koparal and Zeytinoglu 2003; Yin et al. 2012; Elshafie et al. 2017; Jung et al. 2018; Elbe et al. 2020; Mari et al. 2020).

These conflicting findings between the effect of carvacrol-TOPO combination on HeLa and HCT116 cells and its effect against MCF-7, HepG2, A549, and SKOV3 cancer cells can be explained because TOPO was found

to exert different kinds of interactions with other anticancer agents, depending on the type of cancer cell line being treated (Kaufmann et al. 1996). Additionally, some of the famous P-glycoprotein inhibitors like cyclosporine A and verapamil can induce P-glycoprotein expression in colon cancer cell line (Herzog et al. 1993). Sometimes the same phytochemical can cause an opposite effect on cytotoxicity and cellular uptake of the combined anticancer agent in the same cancer cell line depending on the treatment sequence, this happened when resveratrol (natural phytochemical) was combined with doxorubicin, the chemotherapeutic agent, either simultaneously or 24 h before doxorubicin in MCF-7 cancer cells (Osman et al. 2012). Therefore, carvacrol can cause a synergistic or antagonistic interaction effect with TOPO and it may inhibit or increase the expression of P-glycoprotein drug transporter, depending on the cancer cell type being examined.

Conclusion

The current study revealed that carvacrol can modulate the cytotoxic effect of TOPO either synergistically like in

HeLa and HCT116 cancer cells, or antagonistically like in MCF-7, HepG2, A549, and SKOV3 cancer cells. After the carvacrol-TOPO combination treatment, TOPO cellular uptake was either increased (HeLa and HCT116 cells) or decreased (MCF-7, HepG2, A549, and SKOV3 cells) depending in the cell type being treated. The mechanism of cell death in both types of interactions was through induction of apoptosis, but the intensity of apoptosis was in accord with the intercellular concentration of TOPO that was modulated by carvacrol. Further studies should be applied *in vitro* and *in vivo* to confirm the results of this study that showed a possible beneficial effect of carvacrol and TOPO combination in the treatment of cervical and colon cancer, but antagonistic effects were showed for the same combination in breast, liver, ovarian and lung cancer.

Also, this research calls for further studies to investigate the effect of TOPO, carvacrol and their combination on P-glycoprotein mediated resistance and hence by the TOPO intracellular accumulation and to explore the molecular mechanism of the apoptotic cell death after the carvacrol-TOPO combination therapy in various cancer cell lines.

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