Experimental Induction of Apoptosis by Salvia sahendica Extract Alone and in Combination with Doxorubicin in Human Prostate Cancer Cells, LNCaP

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ABSTRACT
Background: Previous chemical investigations on different species of Salvia have shown the presence of flavonoids, diterpenoids, triterpenoids, sesterterpenes and essential oils exhibits the antitumor and cytotoxicity effects on several cancer cell lines.

Methods: Prostate cancer cells, LNCaP were treated with Salvia.sahendica and doxorubicin (DOXO) alone and in combination (DOXO before S.sahendica, after S.sahendica and with S.sahendica). MTT assay to study the sequence and simultaneous combined effect of DOXO and S.sahendica, Ethidium Bromide / Acridine Orange (EB/AO) staining method to determine apoptosis or necrosis, and flow cytometry for the study of cell cycle were performed.

Results: Effective dose of DOXO and S.sahendica were determined 180±1.8 nm/ml, 130±2.37 µm/ml, respectively and their effective time were 24h. The most cytotoxic effect was for effective dose of DOXO following with effective dose of S.sahendica (3.17% viability). The greatest amount of apoptosis was for effective dose of DOXO following with effective dose of S.sahendica (27.34%). Synergistic interaction between S.sahendica extraction and DOXO in LNCaP cells was determined by CI (combination indices) value. Effective dose of DOXO following with effective dose of S.sahendica induced 44.9 % subG1 arrest that was higher than that with effective dose of DOXO (3.49%) or S.sahendica (35.33%) alone. Also cells treated with non-effective dose of DOXO following with effective dose of S.sahendica revealed 76.47% G2/M arrest that was higher than that with effective dose of DOXO (62.42%) or S.sahendica (11.09%) alone.

Conclusions: By using this combination the side effects of DOXO may be declined by reducing dosage from180 nm/ml to10 nm/ml.

Keywords: Combination Drug Therapy, Doxorubicin, Salvia, Prostate cancer cells

INTRODUCTION
Salvia species (sage) belong to the Lamiaceae family and include nearly 900 species spread throughout the world. 17 of which are endemic to Iran. These species are known for various biological activities in folk and modern medicine. S.sahendica is an aromatic, endemic perennial herb which grows wildly in Iran (Tabriz, East Azerbaijan province). Morphologically, the species is distinguished by simple, serrate and ovate leaves, with four to six white flowers in each verticillate inflorescence. This plant has many different biological activities; for example, it has been used as an antimicrobial agent in Azarbayejan province of Iran. S.sahendica has been used as both a medicinal and culinary plant for seasoning, flavoring, and preserving foods. Due to its ability to slow oxidization in foods, it has long been used as a food preservative.

Biological activities of S.sahendica such as antibacterial, antioxidant and anti-inflammatory have been reported as well. Considering the result of the HPLC analysis the plant extract has 264 ± 1 mg gallic acid equivalent as phenolics and 251 ± 17 mg catechin equivalent as flavonoids. These results confirmed the presence of luteolin (7.24 mg/g), catechine (3.52 mg/g), rosmarinic acid (67.12 mg/g) and rutin (1.85 mg/g) as the main compounds in the plant extract. Rosmarinic acid was the major compound of this plant extract. The anti-cancer effect of rosmarinic acid, extracted from the other plants, on cancer cells has been reported.

DOXO is widely used clinically for the treatment of various cancers such as prostate cancer. It is a complex drug in terms of its cytotoxic mechanisms of action. It appears to be cell cycle-specific in a variety of cell lines, inducing DNA damage predominantly in G2 phase.

In this study we used total extract of S.sahendica to observe it’s combined effect with doxorubicin in order to reduce effective dose (IC50) of DOXO, as so many side effects have been reported by using DOXO and considering S.sahendica is a natural drug with no or more less side effects than chemical drugs. We examined the invitro anti-proliferative and pro apoptotic effects of the methanol extract of S.sahendica and cytotoxic effect of DOXO on LNCaP cell line. We aimed to see if in combination manner effective dose of DOXO could be reduced.

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MATERIAL AND METHODS

Plant material

Aerial parts of *S. sahendica* were collected at Tabriz, Iran (June 2004; voucher herbarium specimen: MPH-848). The plant’s aerial parts were air-dried, protected from direct sunlight, and then powdered. The powder was kept in a closed container in a cold room. The powdered plant material (50 g) was extracted four times with methanol at room temperature overnight. The methanol extract was combined and concentrated under reduced pressure on a rotary evaporator, then filtered and lyophilized.

Cell line

The human estrogen-dependent prostate cancer cell line, LNCaP, obtained from Pasteur Institute, Tehran, Iran. Cells were cultured in RPMI 1640 media, supplemented with 10% fetal bovine serum (both from Gibco, Invitrogen, Paisley, U.K.) penicillin (100 U/ml) streptomycin (100 µg/ml; both from cinnagen, Tehran, Iran). Cells were harvested using trypsin (Invitrogen Life Technologies), DOXO and *S. sahendica* extraction.

*In vitro* cytotoxicity assays

The MTT cell viability assay detects the reduction of MTT [3-(4, 5-dimethylthiazol-2)-2, 5-diphenyl-tetrazolium bromide, Sigma] (a colorimetric technique) by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and hence for measuring the cytotoxicity cell and viability. The concentration of the crude extract that killed 50% of the cells (IC50) was calculated by excel software and the mean optical density (OD) ± SD for each group of the replicates calculated. Percent viability of cells exposed to treatments was calculated as follows: % Viability = (Test OD - blank/Non-treated OD - blank) x 100. Also LNCaP cells treated with different doses of DOXO (10, 50,10 and 200 nm/ml) and after 24 and 48h this listed process performed for them.

Determination of the type of combination

The isobologram and the Fa - CI plot are two sides of the same coin: the Fa - CI plot is effect-oriented, whereas the isobologram is dose-oriented. Evaluate the drug-drug interaction with fa - CI plot

Combination effects of crude extraction of *S. sahendica* and DOXO were investigated in prostate cancer cell, LNCap. Combination indices (CIs) were determined using the unified theory in various concentrations and mixing ratios (synergy: CI <0.9, additivity: 0.9 < CI < 1.1, and antagonism: CI > 1.1). By definition, synergism is more than an additive effect and antagonism is less than an additive effect. With CompuSyn software we obtained the Fa table for fa = 0.05 to 0.97; the dose required for each drug alone at a given effect (fa) and the -fold dose reduction if combined for a given effect (fa); DRI values for each of the actual combination data points; Fa-DRI plot for each drug and for each combination with computer simulation, as well as the actual combination data points located on the graph. DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect compared with the dose of each drug alone.

Evaluate the drug-drug interaction with isobologram analysis

The normalized isobologram can be constructed for the non-constant ratio combination design, but only the constant ratio combination design can yield the classic isobologram. Neither isobologram nor the Fx – CI plot graph is dependent on the mechanisms and sites of drug actions, since they are derived from mathematical induction and deduction. The diagonal line is the line of additivity. Experimental data points, represented by geometric shapes, located below, on, or above the line indicate synergy, additivity, or antagonism, respectively. In the denominator, (Dx) is for D1 “alone” that inhibits a system x%, and (Dx) for D2 “alone” that inhibits a system x%. The (Dx) and (Dx) values can be calculated from eq. 1.

![Figure 1: MTT assay for simultaneous combination of crude extract of *Salvia sahendica* and DOXO (n=3).](image-url)
\[ D = D_m \left[ \frac{f_a}{(1 - f_a)^{1/m}} \right] \]

(1)

\(D_m\) is the median-effect dose (e.g., IC50, ED50, or LD) that inhibits the system under study by 50%, and \(m\) is the coefficient signifying the shape of the dose-effect relationship, where \(m = 1, > 1, \) and < 1 indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curves, respectively.7

**Ethidium bromide / Acridine orange double staining**

Apoptotic cell death is known to be induced by many chemotherapeutic agents routinely used in cancer treatment regimens. Apoptosis is characterized by distinct morphological features including chromatin condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation.11 Apoptosis is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate number of cells in the body.12 The levels of apoptotic and necrotic cells were determined by EB/AO double staining. The cells were treated with different doses of salvia (5 and 130 as non-effective and effective doses, respectively) in one experiment and different doses of DOXO (10 and 180 as non-effective and effective doses, respectively) in another one. After 24h (as effective time), cells were

![Figure 2: MTT assays of LNCaP cells after the sequential combination treatment (first salvia, then DOXO): a) Non-effective dose of salvia following with non-effective dose of DOXO, b) Effective dose of salvia following with non-effective dose of DOXO, c) Effective dose of salvia following with effective dose of DOXO (n=3, *=p<0.05, **=p<0.01 and ***=p<0.001).](image-url)
staining with EB/AO, imaged using a fluorescent microscope (Carl Zeiss, Inc) and the number of apoptotic and necrotic cells were counted in 10 fields. Counts were expressed as the percentage of apoptotic and necrotic cells. The predominance of live, apoptotic, and advanced apoptotic and necrotic cells in the AO\textsuperscript{high}, AO\textsuperscript{low}-EB\textsuperscript{low}, and AO\textsuperscript{low}-EB\textsuperscript{high} populations, respectively, supports the interpretation that a downward shift in green fluorescence represents changes in nucleic acid accessibility by AO and/or a reduced overall amount of DNA in cells undergoing apoptosis compared with healthy, live cells. In addition, as cells enter into the later stages of apoptosis (or directly undergo necrosis), the membrane shows increased permeability to vital dyes, as demonstrated here by increased uptake and fluorescence with EB\textsuperscript{13}.

**Cell Cycle Analysis**

The cells were incubated with various concentrations of *S. sahendica* for 24 h. After treatment, cells were washed with phosphate-buffered saline (PBS) and fixed in ice-cold 70% ethanol, incubated with 5 μg/ml RNase I /1 μg/ml propidium iodide, and then cellular DNA content was determined by a flow cytometer. Apoptotic cells were
detected using PI staining of treated cells followed by flow cytometry to detect the so-called sub-G1 peak.

Treatment of cells

Following harvesting, cells were seeded (at 20,000/0.5 ml/well for LNCaP cells) in 96-well plates and incubated for 24hr prior to treatment. On the day of treatment, we added various doses of DOXO (10, 50, 100 and 200 nm/ml)
and in the other experiment the various doses of \textit{S. sahendica} (5, 25, 50, 100 and 150 µM/ml) added to LNCaP cells. For combination treatment, we designed both simultaneous and sequence treatments. For the simultaneous combination treatment, we treated according to this pattern: on the day of treatment we added effective or non-effective dose of DOXO for one experiment, with effective or non-effective dose of \textit{S. sahendica}.

Figure 7: Morphological observations of LNCaP cells after sequential combination treatment (first DOXO, then \textit{S. sahendica}). a) Control group b) Treatment with effective dose of DOXO shows granule and detached cells (arrows). c) Treatment with non-effective dose of DOXO following with effective dose of salvia shows detached cells and d) Treatment with non-effective dose of DOXO following with non-effective dose of salvia shows less detached cells (×100).

Figure 8: Morphological observations of LNCaP cells after sequential combination treatment (first \textit{Salvia sahendica}, then DOXO), a) Control group, b) Treatment with effective dose of DOXO shows granule and detached cells (arrows). c) Treatment with non-effective dose of DOXO following with effective dose of salvia, d) Treatment with non-effective dose of DOXO following with non-effective dose of salvia (×100).
simultaneously. For the sequential combination treatment, we did like this pattern: on the day of treatment we added non-effective dose of DOXO, after 24hr, as effective time, we washed the culture using 0.5 ml of sterile PBS and the culture medium containing non-effective dose of S.sahendica was replaced it. In the other group of experiment we added non-effective dose of DOXO and, after 24hr, as effective time, washed the culture and replaced the culture medium with effective dose of S.sahendica. The third group of experiment was treated with effective dose of DOXO, after 24hr of effective time, the culture was washed and replaced with culture medium containing effective dose of S.sahendica. We also examined the sequential combination treatment in the order in which we first added S.sahendica following with DOXO, after 24hr, as effective time.

Figure 9: Determination of apoptosis in LNCaP cells treated with Salvia.sahendica crude extract by ethidium bromide/acridine orange staining. a) Untreated LNCaP cells that were considered as control group (×10).

Figure 10: Determination of apoptosis in cells treated with salvia following with DOXO using ethidium bromide/acridine Orange staining assay in LNCaP cells. a) Control sample, are untreated LNCaP cells, b) Effective dose of salvia (130±2/37μm/ml) following with effective dose of DOXO (180±184 nm/ml), C) Effective dose of salvia (130±2 /37μm/ml) following with non-effective dose of DOXO (10±2/20 nm/ml), d) Non-effective dose of salvia (5±1/60μM) following with non-effective dose of DOXO (10±2/20 nm/ml) (×10).
STATISTICS
Results are expressed as mean±SD. In this study we used excel software to draw charts, flowJo to draw flow cytometry graphs, CompuSyn to define the type of combination. Statistical analyses were done with instate with significance at p<0.05.

RESULTS
Cytotoxicity assay
Calculating effective dose of *Salvia.sahendica* crude extraction and DOXO was performed using MTT assay. Through that we treated the LNCaP cells with different doses of and DOXO. Effective dose, which represents the concentration of a drug that is required for 50% inhibition of cells replication (IC50), of *S.sahendica* and DOXO was determined 130± 2.3 µm/ml, 180±1.8 nm/ml respectively, and effective time was 24h for both of them. MTT assay was performed to study the sequence [Figure 2 and 3] and simultaneous [Figure 1] combined effect of DOXO and *Salvia.sahendica*.

In simultaneous combination treatment there was no significant reduction in the viability of the LNCAp cells after treatment comparing with control group and drugs alone. Therefore, simultaneous combination treatment of DOXO and *Salvia.sahendica* extract was not effective in LNCaP cells. In sequential combination treatment (first salvia, then DOXO) non-effective dose of salvia following with non-effective dose of DOXO, effective dose of salvia following with non-effective dose of DOXO, and effective dose of salvia following with effective dose of DOXO showed meaningful reduction comparing with control group and drugs alone.

In sequential combination (first DOXO, then salvia) non-effective dose of DOXO following with non-effective dose of salvia, showed no reduction in viability of the cells, non-effective dose of DOXO following with effective dose of salvia, and effective dose of DOXO following with effective dose of salvia showed meaningful reduction comparing with control group or treatment with salvia or DOXO alone.

Determining the type of combination Using fa-CI graph
As it is important to know the type of interactions between two drugs if it is antagonistic, additive or synergistic, we used Fa-CI graph, generated by CompuSyn software. According to CI values, very strong synergism was seen when LNCaP cells were treated with DOXO at IC50 value (180 nM) following with salvia at IC50 value (130µM). Strong synergism was seen when cells treated with salvia (in effective or non-effective dose) following with DOXO (in effective or non-effective dose). Synergistic effect was seen when we added non-effective dose of DOXO (10nm) following with effective dose of salvia (130µM) and relatively additive effect was seen when we added non-effective dose of DOXO following with non-effective dose of salvia [Figure 4]. The other parameter obtained from CompuSyn was DRI value [Table1].

Isobologram analysis
Normalized isobologram for combination at different combination ratios, DOXO following with *S.sahendica* and vice versa generated by CompuSyn. The results show that the IC equivalent concentrations for various *Salvia.sahendica* /DOXO combinations were located below the line of additivity, indicating synergistic interaction at all combinations [Figure 5].

Morphologic effects of *S.sahendica* and DOXO on LNCaP cells

Figure 11: Determination of apoptosis in cells treated with the DOXO following with salvia by ethidium bromide / acridine Orange staining assay in LNCaP cells. a) The control group, is untreated LNCaP cells, b) Effective dose of DOXO (180±184 nm/ml) following with effective dose of salvia (130±2 /37µm/ml), c) non-effective dose of DOXO(10±2/20 nm/ml) following with non-effective dose of salvia(5±1/60µm/ml), d) effective dose of DOXO (10±2/20 nm/ml) following with non-effective dose of salvia (130± 2 /37µm/ml) (~10).
Through microscopic investigations we observed morphological changes in LNCaP cells treated with crude extraction of *S. sahendica* and DOXO. LNCaP cells were imaged using an optical microscope (CETI, made in Belgium). After treatment with effective dose of DOXO (180nm/ml), and salvia (130µm/ml) the number of granule cells that have been detached (marked with arrows) [Figure.6] increased in comparing with the control group. We studied the effects of sequential treatment on the morphology of LNCaP cells. We first added DOXO (its
The most increase was related to adding effective dose of DOXO following by DOXO compared with untreated cells (23.98%).

The effective dose of DOXO showed arrest in G2/M phase following by DOXO (62.42%) in comparing with untreated LNCaP cells (27.34%), and also with noneffective dose of DOXO following by DOXO was increased when dose of S.sahendica effective dose (44.9%). The most G2/M arrest was detected in the cells treated with noneffective dose of DOXO following by effective dose of crude extract (76.47%) that was more than cells treated with effective dose of DOX alone (62.47%). Based on this study treated LNCaP cells with DOXO and S.sahendica in the sequential manner can induce G2/M and subG1 arrest more than treatment with DOXO or S.sahendica alone [Table 3].

**DISCUSSION**

Chemotherapy plays a critical role in virtually every phase of cancer treatment. Clinical protocols for cancer chemotherapy rarely use a single drug but usually combine two or more drugs with different mechanisms of action. The purpose of using drugs in combinations is to achieve therapeutic effects greater than those provided by a single drug alone. An optimal protocol of combination chemotherapy may increase the therapeutic efficacy, decrease toxicity toward the host or non-target tissues, and minimize or delay the development of drug resistance14. However, when anticancer agents with similar or different modes of actions are combined, the outcome can be synergistic, additive or antagonistic. Synergism implies that two drugs may produce greater therapeutic efficacy than the expected additive effect, whereas antagonism implies that the actual therapeutic activity produced by two drugs may be smaller than the expected additive effect7. The major advantages of combination therapy are achieving improved therapeutic effects and avoiding unnecessary toxicity through drug synergism15. Doxorubicin is used in prostate cancer therapy that causes bone marrow suppression, gastrointestinal distress, and severe alopecia. Its most distinctive adverse effect is cardiotoxicity, which includes initial electrocardiographic abnormalities (with the possibility of arrhythmias) and slowly developing, dose-dependent cardiomyopathy and congestive heart failure16.

Compounds that have been used to reduce the effective dose or increase the efficiency of DOXO were reported in articles. In one study in vivo analyzing of combination of ketoconazole with DOXO resulted in 50% reduction in the

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**Table 2: Measuring the apoptosis and necrosis by AO/EB double staining.**

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<thead>
<tr>
<th>Samples</th>
<th>Apoptosis (%)</th>
<th>Necrosis (%)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.64</td>
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<tr>
<td>Effective dose of doxo</td>
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<td>11</td>
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<td>2.3</td>
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<td>1.2</td>
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Table 3: cell cycle analysis

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<td>Effective dose of doxo</td>
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<td>Effective dose of salvia</td>
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<td>11.5</td>
</tr>
<tr>
<td>salvia+effective dose of doxo</td>
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<td>26.4</td>
</tr>
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<td>pre-non effective dose of salvia+non effective dose of doxo</td>
<td>15.3</td>
<td>12.7</td>
</tr>
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</table>

Effective or non-effective doses and then S.sahendica (its effective dose). By adding DOXO first and then S.sahendica the number of floating round compressed cells were increased [Figure 7]. We first added S.sahendica and then DOXO (effective dose of both drugs, and alsoeffective dose of S.sahendica with non-effective dose of DOXO). The results showed morphological changes as floating round compressed cells (arrows in Figure 8).

*Ethidium Bromide/Acridine Orange double staining*

Apoptotic morphological changes such as DNA fragmentation and apoptotic bodies were measured by ethidium bromide/acridine orange double staining. We observed LNCaP cells treated with S.sahendica or DOXO in alone [Figure 9] or combination manner [Figure 10 and 11] with florescent microscope and counted apoptosis and necrosis cells [Table 2]. The data revealed the effective dose of S.sahendica was inducing 18.8% apoptosis, which was increased when cells treated with the effective dose of DOXO following by the effective dose of S.sahendica (27.34%), and also with noneffective dose of DOXO following by effective dose of S.sahendica (20.33%).

**Cell cycle analysis**

We obtained the percentage of treated cells during each phase of cell cycle by flow cytometry assay [Figure 12]. Table 3 showed comparing the quantity of cells in sub-G1 and G2/M phase in cells treated with DOXO and S.sahendica separately and the cells treated with combination of these two drugs. LNCaP cells treated with effective dose of DOXO showed arrest in G2/M phase (62.42%) in comparing with untreated LNCaP cells (23.98%). The cells treated with effective dose of S.sahendica led to arrest in subG1 phase (35.33%) in compare with untreated cells (1.64%). When crude extract following by DOXO were added, increasing in subG1 phase (as you can see in table) was achieved, although the most increase was related to adding effective dose of DOXO following by effective dose of crude extract (44.9%).
level of PSA (prostate specific antigen) in tested patients with androgen-independent prostate cancer. Another article reported combination of zoledronic acid with DOXO in a sequential combination treatment manner acted synergistic and induce apoptosis in PC3 (androgen-independent prostate cancer cell line) and LNCaP (androgen-dependent prostate cancer cell line) cells. It was also reported that treatment of LNCAP and PC3 cells with ciprofloxacin following with DOXO will led to 4-15 fold reduction in effective dose of DOXO in these cells. All this articles are reporting the combination of DOXO with the chemical agents that in turn can have side effects. Another study was about combination of DOXO and sulforaphane (one of the effective components of broccoli extract) that revealed this combination can target 60% of stem cells of prostate cancer. Salvia species are known for various biological activities in folk and modern medicine. There is not any reported literature about the effect of Salvia.sahendica on cancer cells. However, different reports have verified the cytotoxic and antitumor properties of some species belonging to this genus. We studied the cytotoxic and pro-apoptotic effect of S.sahendica on human prostate cancer cell line, LNCaP, and whether the combination of S.sahendica crude extraction and DOXO induces apoptosis in LNCaP cells. Based on these observations, the extraction of S.sahendica decreased viability in a time and concentration-dependent manner and induced apoptosis in LNCaP cells. The sequence combination treatment, involving effective dose of DOXO following with effective dose of S.sahendica, resulted in synergistic effect in LNCaP cells, leading to the reduce the cells viability, increase the apoptosis and increase the sub-G1 arrest in LNCaP cell comparing with treatment with effective dose of DOXO or S.sahendica alone. As we aimed to reduce the DOXO dosage, the significant result of this experiment was the sequence combination treatment in which we first added non-effective dose of DOXO and then effective dose of S.sahendica resulted in reducing the viability, and increasing the G2/M arrest in LNCaP cell cycle. Therefore, using S.sahendica may lead to reduce the DOXO dosage from 180 nm/ ml to 10 nm/ ml and through that reduce its side effects.

Multiple mechanisms of action about DOXO are reported including interference with topoisomerase II, induction of single-strand DNA breakage, interference of DNA unwinding, induction of differentiation and generation of oxygen-free radicals. It could be possible that S.sahendica working with DOXO in at least one of this actions, so even we add non-effective dose of DOXO following with effective dose of S.sahendica we can see more G2/M arrest comparing with adding effective dose of DOXO, alone. Being at the same phase of the cell cycle will make the cancer cells more sensitive to the chemotherapy drugs, so treatment with DOXO may synchronize cells into the same phase of the cell cycle and adding S.sahendica after that could have better effect than using DOXO or S.sahendica alone. As in division phase, cancer cells are so fragile against drugs, another possibility could be DOXO makes cells undergo division; therefore, adding S.sahendica next could have a better effect on them.

According to the log-kill hypothesis it proposed that the magnitude of tumor cell kill by anticancer drugs is a logarithmic function and the most effective drug is which can kill greater number of the cancer cells. Based on this theory, the outcome of this experiment could be interesting as we detected pre-treated LNCaP cells with DOXO and then S.sahendica, make increase in sub-G1 phase comparing with treatment with DOXO or S.sahendica alone that can be as a sign for apoptosis. Different reports have verified the cytotoxic and antitumor properties of some species belonging to this genus. There was not any similar investigation on S.sahendica. This is the first report about the cytotoxicity and pro-apoptotic effects of S.sahendica on LNCaP cells, hence further studies will be necessary to supplement our findings by fully recognizing the mechanism of cytotoxicity and cytotoxicity-conducted isolation of constituents to determine the main constituents that are responsible for the anti-proliferative effects.

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