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Anticancer Activity of *Papaver somniferum* L.

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Abstract: This work describes the pharmacological activity of extracts of *Papaver somniferum* L., a poppy species. *P. somniferum* L. products are still considered as a unique source of drug for many diseases. The present study was designed to determine antiproliferative and cytotoxic effects of *P. somniferum* L. extracts on HeLa (Human Cervix Carcinoma), HT29 (Human Colorectal Adenocarcinoma), C6 (Rat Brain Tumor Cells), and Vero (African Green Monkey Kidney) cell lines. Alkaloid-rich extracts of *P. somniferum* L. exhibited antiproliferative effects on various cancer cell lines, especially at high concentrations. We assessed the ability of extracts of *P. somniferum* L. to give harm to the membrane of the cells. Results indicated that *P. somniferum* L. extracts destroy cellular membrane in tumor cell lines at high concentrations. Remarkably, the LDH test results disclosed that cytotoxicity of *P. somniferum* L. on cells was low at middle concentrations. This may indicate its cytostatic potential. The results of this study support the efficacy of *P. somniferum* L. extracts as an anticancer agent.

Keywords: *Papaver somniferum* L., HT29, HeLa, Anticancer activity, Cytotoxic activity.

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INTRODUCTION

Cervical and colon cancers are the most common form of human cancer in the world. There were over two million new cases of cervical and colon cancer diagnosed in 2012 [1]. Therefore, cancer is increasingly becoming a global burden on individuals and social health systems. However, complex pharmacological studies encompassing several disciplines showed that the natural products lead to a significantly decreased death rate, including cancer. This was mainly due to the protective or healing effects of natural products such as fruits, vegetables and spices [2, 3]. Almost every time when the diverse biological activities of natural products are studied intensively; the significant bioactive molecules of herbs and spices display antioxidant, antiinflammatory, immunomodulatory, anticarcinogenic, antithrombotic, and anticoagulant effects [4-6]. Especially the antitumor activity of natural products has recently attracted considerable attention by synthetic chemists. There have also been a variety of studies addressing chemotherapeutic usage for the adjuvant therapy. Natural products were shown to boost the antiproliferative effect of some clinic chemotherapeutics in certain cancer cells [7, 8]. *P. somniferum* L. (the opium poppy) belongs to the family *Papaveraceae* and is widely used for medicinal purposes because it contains various alkaloids such as morphine, noscapine, narcotine, codeine, papaverine, and others [9-11]. Current studies show that alkaloids obtained from *Papaver* species could not only be used as analgesic or sedative but also in other areas such as cancer treatment [12]. *P. somniferum* L. alkaloids that show promise in cancer treatment include noscapine, which interacts with α -tubulin and has anticancer and antiangiogenetic properties [13, 14]; codeinone, an oxidative product of codeine which has apoptotic effects through fragmentation of DNA [15]; morphine, which shows anticancer activities by inhibiting NF- κ B; and (-)-3-acetyl-6 β -(acetylthio)-N-(cyclopropyl-methyl)-normorphine (KT-90), a derivative of morphine [16]. To the best of our knowledge, pharmacological effects of *P. somniferum* L. extracts on HT29, HeLa, C6 cancer cell lines and Vero normal cell lines have not been investigated in detail. In the present work, extracts of *P. somniferum* L. from Turkey (Ofis 8 variety produced by Afyonkarahisar Agricultural Enterprise) was investigated for its antiproliferative and cytotoxic activities against HT29, HeLa, C6 and Vero cells. We conducted in-depth analysis of the inhibitory effect on proliferation, cytotoxicity and morphology.

MATERIALS and METHODS

Preparation of extracts

A Turkish poppy (*Papaver somniferum* L.) cultivar, namely Ofis 98, was used in this study. The stem, leaf, capsule and root parts of the plant material were dried at shade and then extracted (each of 10 g) with hexane, ethyl acetate, and methanol (each of 150 mL) for 1 day. The solvents were dried to yield the hexane, ethyl acetate, and methanolic extracts (Table 1).

Table 1. *Papaver somniferum* L. extracts

<i>Solvent system/P. somniferum</i> L. parts	Root	Stem	Leaf	Capsule
Hexane	HPSR	HPSS	HPSL	HPSC
Methanol	MPSR	MPSS	MPSL	MPSC
Ethyl acetate	EPSR	EPSS	EPSL	EPSC

Preparation of cell culture

The anticancer potential of *Papaver somniferum* L. extracts was investigated on cancerous HT29 (ATCC® HTB-38™), HeLa (ATCC® CCL-2™), and C6 cells (ATCC® CCL-107™) and nontumorigenic Vero cells (ATCC® CCL-81™). The cell lines were cultured in a cell medium (Dulbecco's modified eagle's) enriched with 10% (v/v) fetal bovine serum and 2% (v/v) Penicillin-Streptomycin (10,000 U/mL). First, old medium was removed out of the flask while cells had reached approximately 80% confluence. Next, cells were taken from the flasks surface using 4-5 mL of trypsin-EDTA solution and neutralized by the addition of at least 15 mL supplemented DMEM and then subjected to centrifugation. Following, the cellular pellet was suspended with 4 mL of DMEM working solution and was counted to obtain a final concentration of 5×10^4 cells/mL, and inoculated into wells (100 μ L cells/well).

Cell proliferation assay (CPA)

A cell suspension containing approximately 5×10^3 cells in 100 μ L was seeded into the wells of 96-well culture plates. The cells were treated with *Papaver somniferum* L. extracts and control drug, 5-fluorouracil (5FU), dissolved in sterile DMSO (max 0.5% of DMSO) at final concentrations of 25, 50, 100, 150, 200, 250, 375, and 500 μ g/mL at 37 °C with 5% CO₂ overnight. The final volume of the wells was set to 200 μ L by medium. Cell proliferation assay was evaluated by ELISA BrdU methods as described previously [17].

Calculation of IC₅₀ and % inhibition

IC₅₀ value is a concentration that inhibits half of the cells in vitro. The half maximal inhibitory concentration (IC₅₀) of the *Papaver somniferum* L. extracts and control compounds was calculated using XLfit5® or Microsoft Excel® spreadsheet and represent in μ M at 95% confidence intervals. The proliferation assay results were expressed as the percent inhibition according to the following formula: % Inhibition = [1 - (Absorbance of Treatments / Absorbance of DMSO) x 100].

Cytotoxic activity assay

The cytotoxicity of the *Papaver somniferum* L. extracts and 5 fluorouracil on HeLa, C6, HT29, and Vero cells was determined through a Lactate Dehydrogenase Assay Kit according to the manufacturer's instructions (Roche, LDH Cytotoxicity Detection Kit). Approximately 5×10^3 cells in 100 μ L were placed into 96-well plates as triplicates and treated with IC₅₀ (μ g/mL) concentrations of *Papaver somniferum* L. extracts at 37°C with 5% CO₂ for 24 h. LDH activity was obtained by determining absorbance at 492 - 630 nm using a microplate reader. The

cytotoxicity assay's results were noted as the percent cytotoxicity according to the following formula: % Cytotoxicity = [(Experimental Value - Low Control / High Control - Low Control) x 100].

Cell imaging

Cells were plated in 96-well plates at a density of 5.000 cells per well and allowed 24 h. IC50 values of the *Papaver somniferum* L. extracts were administered and morphology alters of the cells were screened by phase contrast microscopy for 24 h every 6 h. Images of control and *Papaver somniferum* L. extracts treated cells were photographed at the end of the process using a digital camera attached to an inverted microscope.

RESULTS AND DISCUSSION

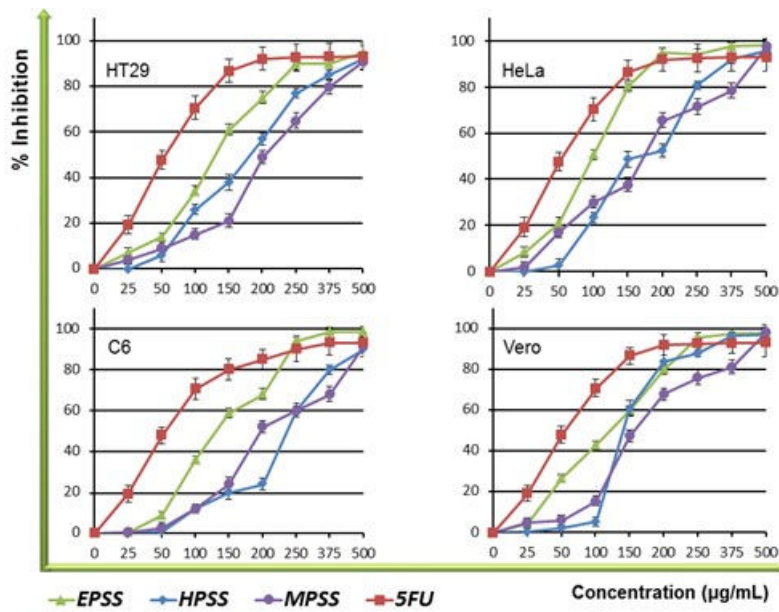
Antiproliferative effect of the *Papaver somniferum* L. extracts

The inhibitory effects of stem, capsule, root, and leaf extracts of the opium poppy on cancer cell lines were determined according to anticancer activity tests performed by using ELISA BrdU cell proliferation kit.

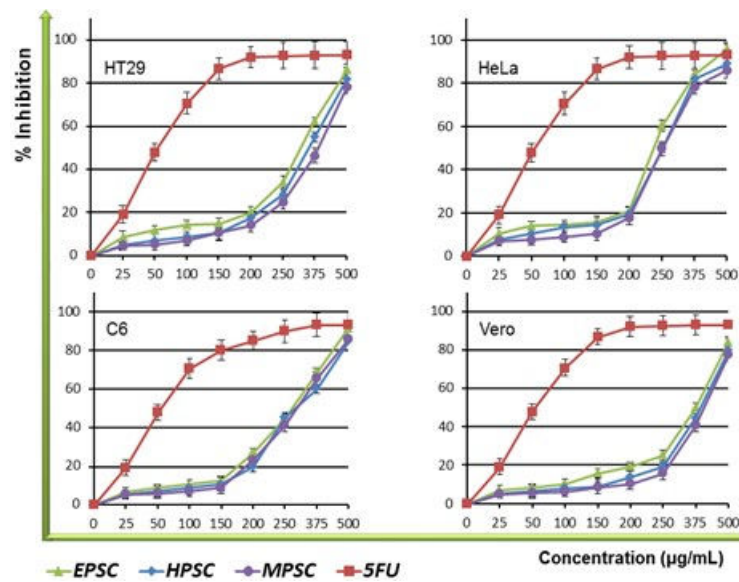
Table 2. IC50 ($\mu\text{g}/\text{mL}$) values of ethyl acetate (EPSR, EPSS, EPSL, and EPSC), hexane (HPSR, HPSS, HPSL, and HPSC), and methanol (MPSR, MPSS, MPSL, and MPSC) root, stem, leave and capsule extracts, respectively, of *Papaver somniferum* and positive control compound, 5FU on HeLa, HT29, C6, and Vero cell lines.

IC50 ($\mu\text{g}/\text{mL}$)	HT29	HeLa	C6	Vero
EPSR	225.62	120.52	170.60	113.98
HPSR	186.17	226.01	218.57	201.39
MPSR	128.90	118.76	203.49	170.30
5FU	91.23	82.16	94.21	88.74
EPSS	174.72	130.70	176.71	153.92
HPSS	219.97	209.60	271.50	190.82
MPSS	247.37	210.25	261.31	210.62
5FU	88.24	83.80	95.09	84.57
EPSL	195.17	264.40	146.69	134.34
HPSL	189.58	131.61	378.70	289.96
MPSL	212.95	174.62	155.38	143.25
5FU	84.26	86.54	91.44	85.71
EPSC	325.04	271.83	299.93	350.20
HPSC	348.79	290.18	323.67	376.11
MPSC	371.85	301.06	318.25	390.64
5FU	87.06	82.37	83.79	85.43

5-fluorouracil (5-FU) was used as a positive control for anticancer activity. Stem, root, and leaf extracts displayed significantly more anticancer activity than capsule extracts (Figure 1-2). IC50 values were determined by XLfit5 software using ELISA data (Table 2).

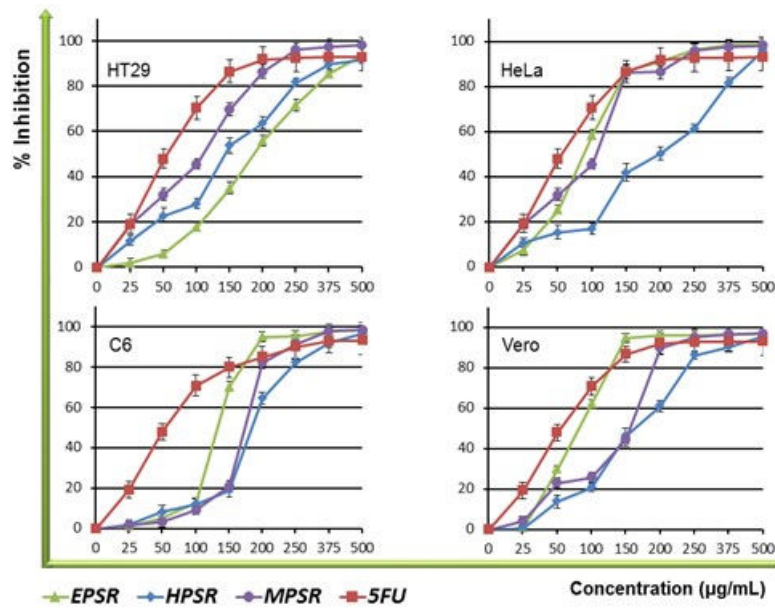


(A)

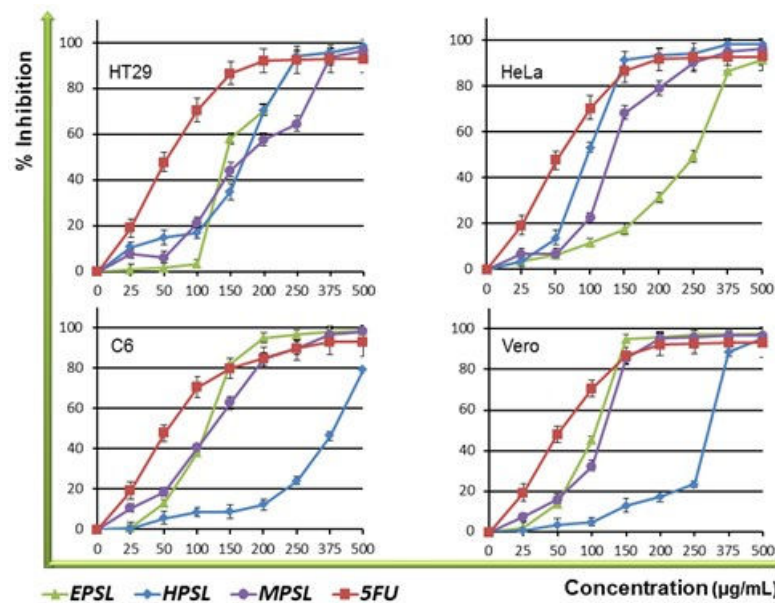


(B)

Figure 1. Antiproliferative effects of ethyl acetate (EPSS, EPSC), hexane (HPSS, HPSC), and methanolic (MPSS, MPSC) opium stem extracts (A) and opium capsule extracts (B), respectively, and positive control 5FU on HeLa, HT29, C6 and Vero cell lines. Cell proliferation measurement was carried out with BrdU cell ELISA kit. Inhibition percentage was reported as \pm SEM value of three independent measurements ($P < 0.05$). Each experiment was triplicated for each cell line and $r^2=0.85$ to 0.97 .



(A)



(B)

Figure 2. Antiproliferative effects of ethyl acetate (EPSR, EPSL), hexane (HPSR, HPSL), and methanolic (MPSR, MPSL) opium root extracts (A) and opium leaf extracts (B), respectively, and positive control 5FU on HeLa, HT29, C6 and Vero cell lines. Cell proliferation measurement was carried out with BrdU cell ELISA kit. Inhibition percentage was reported as \pm SEM value of three independent measurements ($P < 0.05$). Each experiment was triplicated for each cell line and $r^2=0.85$ to 0.97 .

Data showed that cell lines were resistant to extracts up to at least 5FU. In addition, there was statistically significant difference ($P < 0.05$) among cancer cell lines compared to Vero cell

lines. According to IC₅₀ values in Table 1, opium stem ethyl acetate extract (EPSS) displayed very strong anticancer activity on all cell lines. Opium stem hexane extract (HPSS) was effective only on Vero (190.82 µg/mL) cell line. Opium capsule extracts (EPSC, HPSC, MPSC) showed the least anticancer activity but low cytotoxicity. It is interesting to note that opium capsule displayed the lowest anticancer activity against the cells. The pharmacological activity of opium poppy is due to the major bioactive compounds such as morphine, codeine, tebaine, noscapine, papaverine and the capsule had the highest content of these bioactive compounds. However, one of them, noscapine, was responsible for their anticancer activity [18, 19]. Opium root ethyl acetate extract (EPSR) had anticancer activity on HeLa (120.52 µg/mL), C6 (170.60 µg/mL) and Vero (113.98 µg/mL) cell lines. Opium root hexane extract (HPSR) was effective only on HT29 (186.17 µg/mL) cell line, whereas the opium root methanol extract (MPSR) had quite a strong effect on both HT29 (128.90 µg/mL) and HeLa (118.76 µg/mL) cell lines. Opium leaf ethyl acetate extract (EPSL) showed highest anticancer activity on HT29 (195.17 µg/mL), C6 (146.69 µg/mL) and Vero (134.34 µg/mL) cell lines, while opium leaf methanolic extract (MPSL) had high anticancer activity on HeLa (174.62 µg/mL), C6 (155.38 µg/mL) and Vero (143.25 µg/mL) cell lines. Opium leaf hexane extract (HPSL) was effective only on HT29 (189.58 µg/mL) and HeLa (131.61 µg/mL) cell lines.

Cytotoxic activity of the *Papaver somniferum* L. extracts

Cytotoxic activities of various concentrations of opium plant extracts (25, 50, 100, 150, 200, 250, 375, and 500 µg/mL) on HeLa, HT29, C6 and Vero cell lines were measured using the LDH cytotoxicity kit. It was determined that there was cell membrane damage on high concentrations. Cytotoxicity results showed that only membranes of cells treated with high concentrations sustained significant damage and the same effect was not observed in lower concentrations (Figure 3-6). Figure 3 shows that opium stem hexane and methanol extracts (HPSS, MPSS) were not cytotoxic against HeLa, HT29 and Vero cells, but may induce necrosis against C6 cells. Probably, opium stem ethyl acetate extract (EPSS) was necrotic against all cells at 150 µg/mL or above concentrations. However, there is a significant application opportunity below that dose (Figure 3). Opium capsule ethyl acetate extract (EPSC) showed therapeutic promise at 200 µg/mL and below doses. Opium capsule hexane extract (HPSC) was safe for HT29 cell line at 250 µg/mL, but was quite toxic for C6 cells. The opium capsule methanolic extract (MPSC) at 250 µg/mL was likely not cytotoxic for HeLa, HT29 and Vero cell lines but was cytostatic (Figure 4). Opium root ethyl acetate extract (EPSR) had a cytotoxic effect on HeLa, HT29 and Vero cells at 150 µg/mL and above doses, but was cytotoxic at 50 µg/mL for C6 cell line. Opium root hexane and methanolic extract (HPSR, MPSR) were cytostatic at 100 µg/mL and below. It is possible that, they were necrotic for C6 cells at 150 µg/mL while necrotic for HeLa, HT29 and Vero cells at 200 µg/mL (Figure 5).

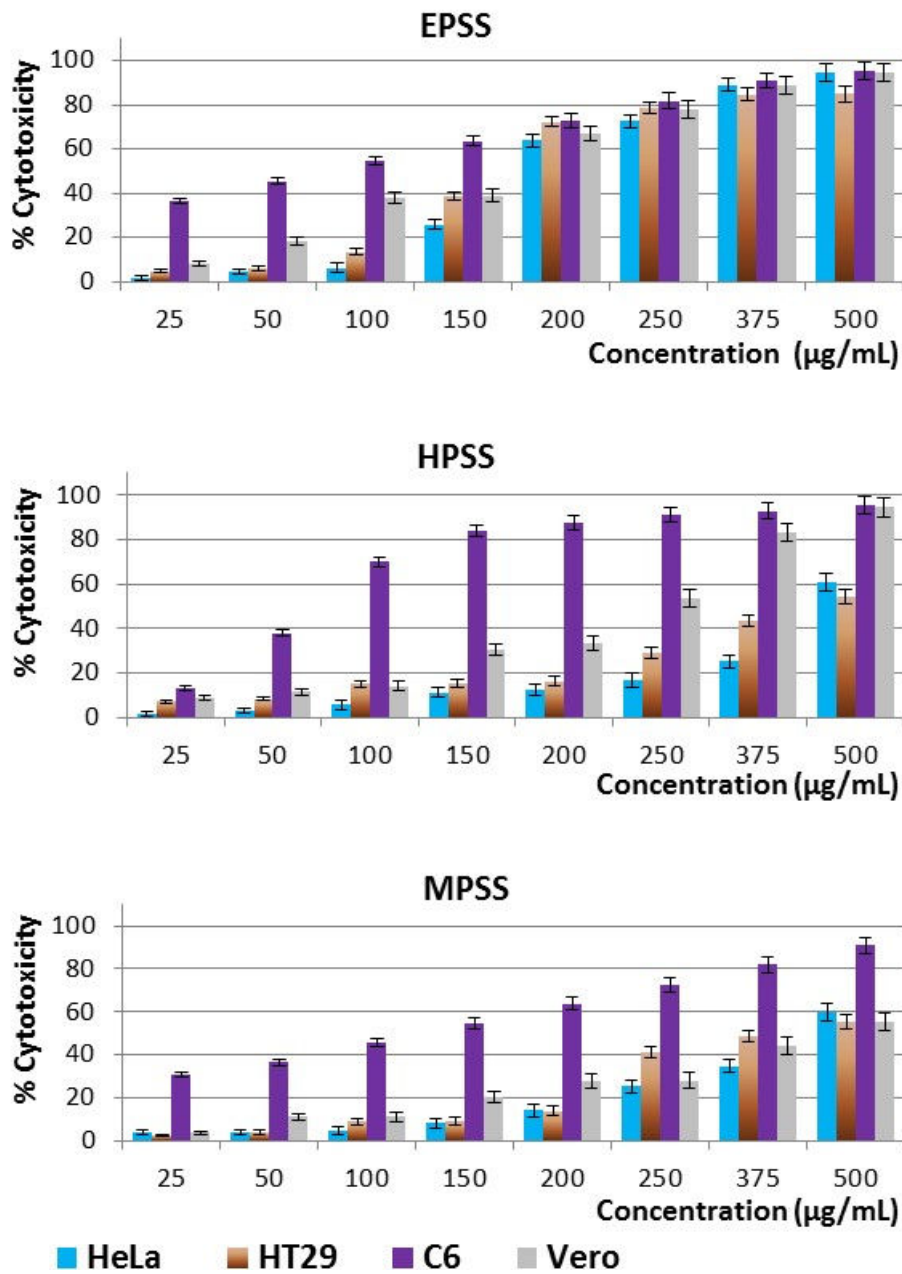


Figure 3. Cytotoxic effects of opium stem extracts on HeLa, HT29, C6 and Vero cell lines. Cell lines were incubated with extracts of varying concentrations for 24 hrs and cytotoxicity was determined using the LDH cytotoxicity kit. Inhibition percentage was reported as \pm SEM value of three independent measurements ($P < 0.05$).

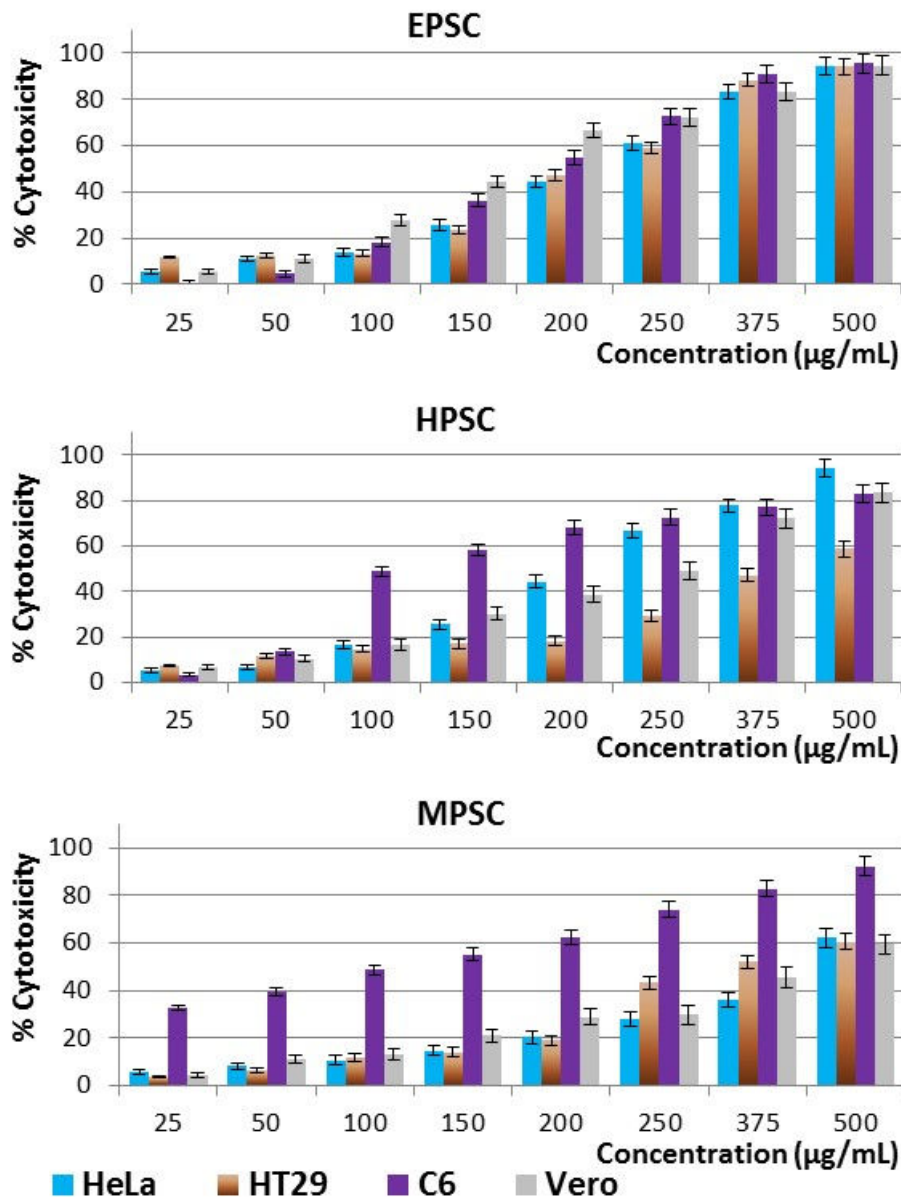


Figure 4. The cytotoxic effects of opium capsule extracts on HeLa, HT29, C6 and Vero cell lines. Cell lines were incubated with extracts of varying concentrations for 24 h and cytotoxicity was determined using the LDH cytotoxicity kit. Inhibition percentage was reported as \pm SEM value of three independent measurements ($P < 0.05$).

It is known that the alkaloid contents of the opium poppy are very highly dynamic due to growth stages, environmental conditions and locations, plant diseases by fungi or bacteria, and parts and ages of plants. Our results showed that the opium extracts had high antiproliferative effects but caused toxic effects towards the cells (Figure 3-6). So it is not possible to evaluate opium extracts as a medical purpose. However, in the future, high noscapine (anticancer agent) and total antioxidant and phenolic content (protective agents) lines of opium poppy will be likely developed commercially to eliminate the alkaloid induced toxic effects.

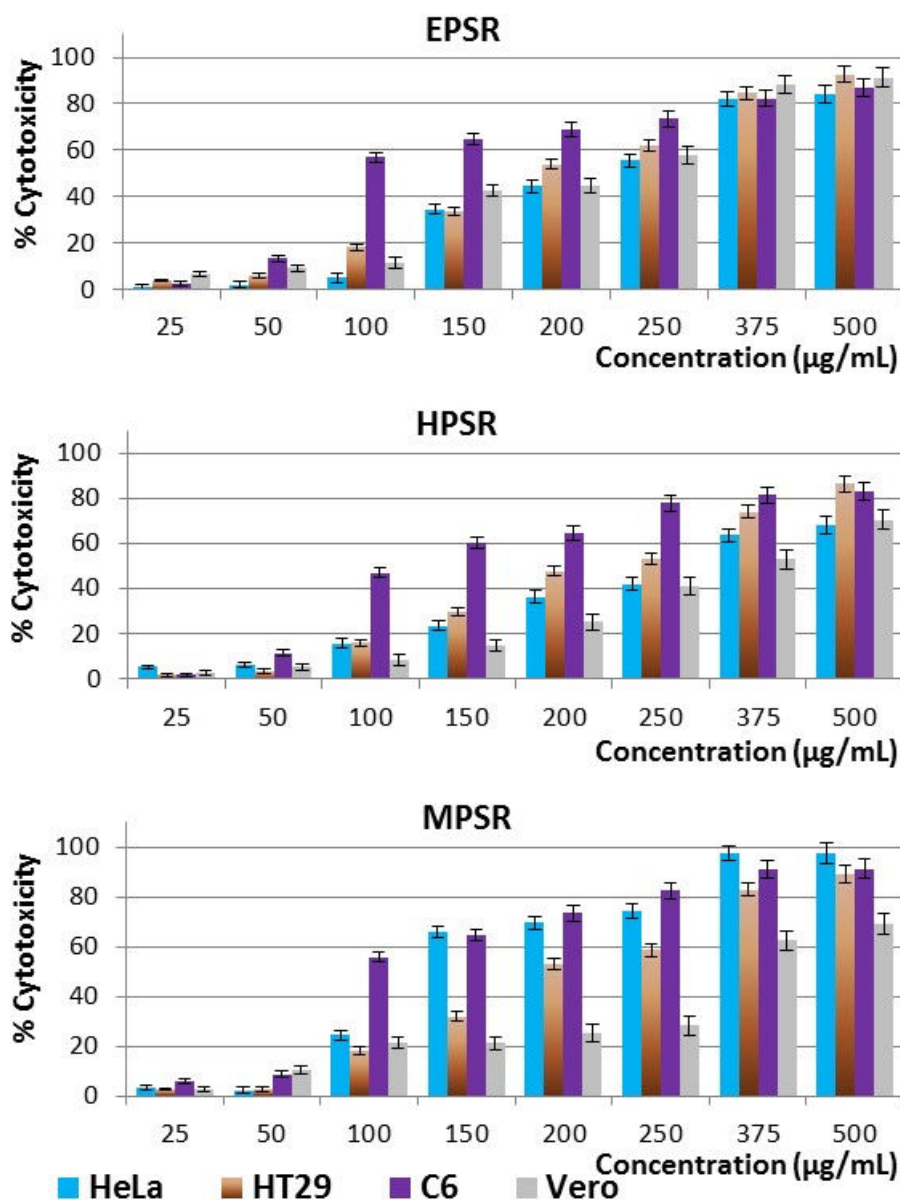


Figure 5. The cytotoxic effects of opium root extracts on HeLa, HT29, C6 and Vero cell lines. Cell lines were incubated with extracts of varying concentrations for 24 h and cytotoxicity was determined using the LDH cytotoxicity kit. Inhibition percentage was reported as \pm SEM value of three independent measurements ($P < 0.05$).

Opium leaf ethyl acetate and methanolic extracts (EPSL, MPSL) were cytotoxic generally at 200 $\mu\text{g/mL}$ and above concentrations, whereas the opium leaf hexane extract (HPSL) was safe up to 375 $\mu\text{g/mL}$. C6 cell line was the most effected from leaf extracts (50-150 $\mu\text{g/mL}$) while Vero and HT29 cell lines were the least effected (Figure 6). The fact that opium extracts displayed not very high cytotoxic activities while showing quite high antiproliferative properties indicates that these extracts could be used for further studies such as bioactivity-guided isolation and identification of active metabolites, preclinical and nonclinical studies.

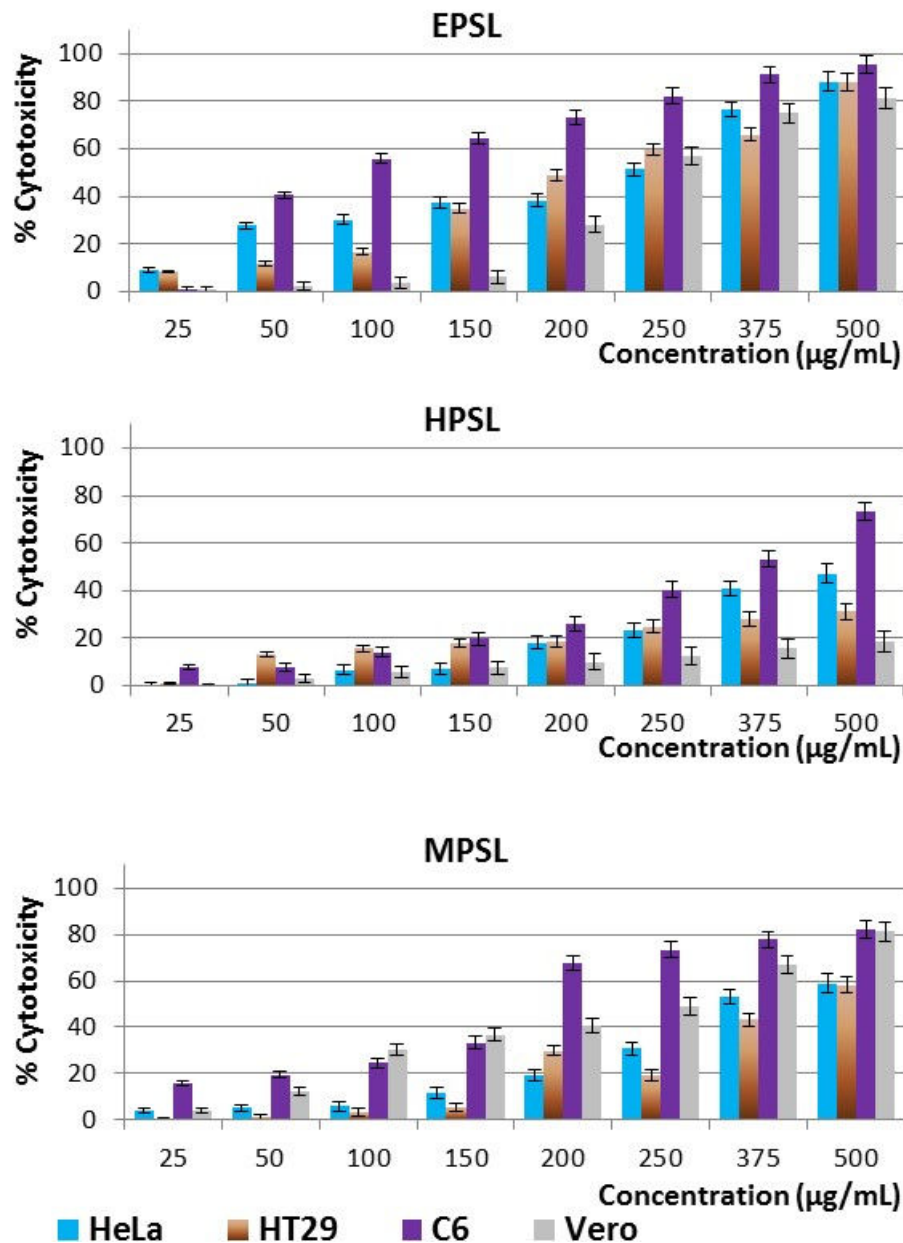


Figure 6. The cytotoxic effects of opium leaf extracts on HeLa, HT29, C6 and Vero cell lines. Cell lines were incubated with extracts of varying concentrations for 24 h and cytotoxicity was determined using LDH the cytotoxicity kit. Inhibition percentage was reported as \pm SEM value of three independent measurements ($P < 0.05$).

Morphological assessment of the cytotoxic activity of the *Papaver somniferum* L. extracts

Effects of opium extracts on cell morphology were determined with a phase-contrast microscope with a digital camera attachment. The images show phase-contrast pictures of cell morphologies treated with various opium extracts compared to negative controls. Cell morphologies treated with opium extracts especially at high concentrations ($>375 \mu\text{g/mL}$) include cell rounding, mixing of cells with medium and then floating (indicates that the cells are dead), cytoplasmic bubbles, contraction, abnormal globular structures and apoptotic particles. Opium extracts could not inhibit cell growth at low concentrations ($<75 \mu\text{g/mL}$). At

these concentrations, normally growing cells and control cells display a confluent image with intact membrane integrity (Figure 7). Similar to control cells, a big majority of cells treated with opium extracts kept their starry or normal fibroblast looks. In general, 250 µg/mL and above doses caused separation of cells, smaller looking cells and decreasing cell numbers. In cells treated with opium extracts, medium and high concentrations resulted in cell morphologies similar to apoptosis and cellular damage seen in affected growth (Figure 7).

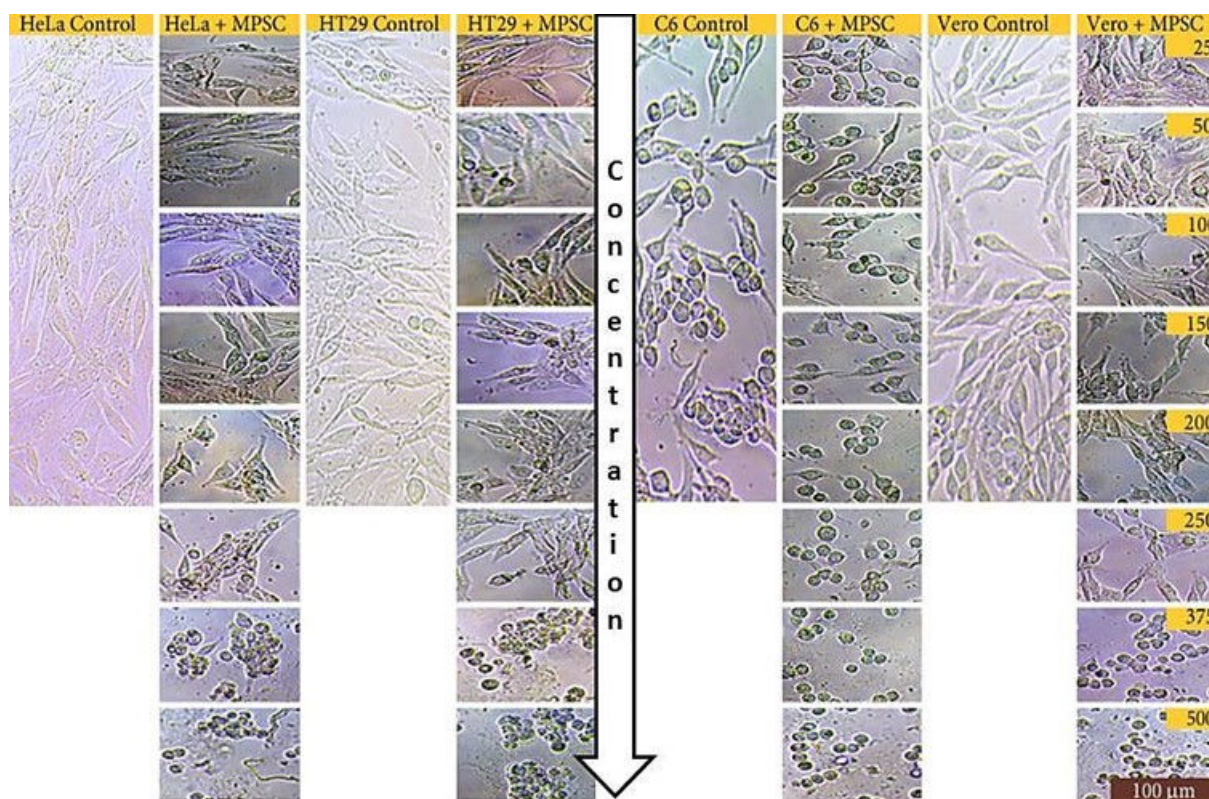


Figure 7. Effects of opium capsule's methanolic extracts on the morphologies of HeLa, HT29, C6 and Vero cell lines. Exponentially growing cells were incubated overnight with various concentrations of opium extracts at 37 °C. Control cells were treated with only DMSO. All measurements were 100 µm.

In this study, we have evaluated the biological activity of *P. somniferum* L. extracts. The findings showed that these extracts can inhibit cell proliferation and thus may create a new opportunity for the treatment of cancer diseases. Similar to other reports using various methods [18, 19], *P. somniferum* L. extracts generally exhibited the outstanding antiproliferative effect. Necrosis is an unprogrammed cell death and is the undesirable increased LDH leakage of plasma membrane. Hence, LDH assay results may imply that opium extracts show a low necrotic effect on the cells at therapeutic concentrations. Our findings showed that opium extracts caused high antiproliferative and low cytotoxic activities towards cell lines at IC₅₀ concentrations; indicating they have significant potential as a useful medicine. Previous studies reported that these extracts have a similar effect with current chemotherapeutics in various cell lines [20, 21]. These extracts induced decrease in cell growth in culture through causing cell shrinkage and changing their astrocyte-like and

fibroblast-like structures to globular shape. Results show that different extraction methods applied to different plant parts yield different antiproliferative and cytotoxic effects on cells. However, it is possible to say that the extract containing the most effective metabolites in general is methanol extract. If the extraction method is disregarded, then the plant part with the most active ingredients is roots, while capsule contains the least active substances. The extract HPSL, EPSC and HPSC showed a potent antiproliferative activity and low cytotoxic effects against HeLa cancer cells, compared with that of Vero normal cells. However, the effects could differ depending on the cancer cell line, due to metabolic differences. In addition, growth conditions, maturity levels, and presence of various stresses may affect the amount and content of alkaloids and therefore the pharmacological potency of Papaver species [22-29]. Results of the present study exhibit extensive similarities with the results of similar studies in terms of statistics [30-32]. Other possible effects of these extracts are currently being investigated in our laboratory. Overall, we have demonstrated that these extracts may be used as chemotherapeutic and potentially valuable pharmacological agents.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DAG AA IP. Performed the experiments: AA DAG. Analyzed the data: AA IP MK ŞT. Contributed reagents/materials/analysis tools: AA IP.

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Türkçe Öz ve Anahtar Kelimeler

***Papaver somniferum* L.'nin Antikanser Aktivitesi**

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Öz: Bu çalışmada *Papaver somniferum* L. (bir haşhaş türü) ekstraktlarının farmakolojik aktivitesi tarif edilmektedir. *P. somniferum* L. ürünleri hâlâ pek çok hastalık için benzersiz bir ilaç kaynağı olarak düşünülmektedir. Bu çalışmada *P. somniferum* L. ekstraktlarının HeLa (İnsan Rahim Ağzı Kanseri), HT29 (İnsan Kolorektal Adenokarsinoma), C6 (Sıçan Beyni Tümör Hücreleri) ve Vero (Afrika Yeşil Maymun Böbreği) hücre dizilerinde sitotoksik etkisi ve antiproliferatif etkisinin belirlenmesi için tasarımlar yapılmıştır. Alkaloidçe zengin *P. somniferum* L. ekstraktları çeşitli kanser hücre dizilerinde, özellikle yüksek derişimlerde antiproliferatif etkiler göstermiştir. Hücre zarlarına zarar vermek için *P. somniferum* L. ekstraktlarının yeteneklerini inceledik. Sonuçlara göre *P. somniferum* L. ekstraktları yüksek derişimde tümör hücre dizilerinde hücre zararını yok etmektedir. Dikkat çekici şekilde, LDH test sonuçları *P. somniferum* L.'nin hücreler üzerindeki sitotoksitesinin orta düzey derişimlerde düşük olduğu bulunmuştur. Bu da ekstraktların sitostatik potansiyelini gösteriyor olabilir. Bu çalışmanın sonuçları *P. somniferum* L. ekstraktlarının antikanser araç olarak etkinliğini desteklemektedir.

Anahtar kelimeler: *Papaver somniferum* L., HT29, HeLa, Antikanser aktivitesi, Sitotoksik aktivite.

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