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Anticancer Activity of Endemic Phlomis Extracts in HCT116 Human Colon Cancer Cells

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ABSTRACT

Aim: Previous studies have reported that *Phlomis russeliana* shows cytotoxic effects against several cancer cell lines; however, its anti cancer activity on HCT-116 cancer cells has not yet been investigated. Therefore, the present study is designed to explore anti-cancer properties of Phlomis russeliana against HCT-116 human colon cancer cell line and HUVEC normal cell line.

Material and Methods: HCT-116 cells and HUVECs treated with different concentrations of *Phlomis russeliana* (2, 4, 6, 8 and 10 mg/ml) and cell viability evaluated by the MTT assay. Anti-migratory and anti-colonigenic effects of Phlomis russeliana were assessed with wound healing and colony formation assays respectively. Quantitative determination of total antioxidant status (TAS), total oxidant status (TOS) and caspase-3 activation were performed with colorimetric Elisa kits.

Results: *Phlomis russeliana* significantly decreased cell viability of HCT-116 cells in a concentration dependent and showed weaker toxicity against normal HUVECs. *Pholomis russeliana* significantly inhibited migration and colony formation potential of HCT-116 cells. A significant increase in caspase-3 activation was observed after treatment with Phlomis russeliana. Phlomis russeliana did not significantly affect the TAS and TOS level in HCT-116 cells.

Conclusion: These results revealed that *Phlomis russeliana* showed anti-cancer activity in human colon cancer cells, through the suppression of colony formation, inhibition of migration and induction of caspase-3 activation. *Phlomis russeliana*, could be a promising source for the development of new anti-cancer agents against cancer.

Keywords: Phlomis russeliana; HCT-116 cells; migration; caspase-3 activation; colony formation.

HCT116 İnsan Kolon Kanseri Hücrelerinde Endemik Phlomis Ekstraktlarının Antikanser Aktivitesi

ÖZ

Amaç: Önceki çalışmalar, *Phlomis russeliana*'nın çeşitli kanser hücre hatlarına karşı sitotoksik etkiler gösterdiğini bildirmiştir; ancak, HCT-116 kanser hücreleri üzerindeki anti kanser aktivitesi henüz araştırılmamıştır. Bu nedenle, bu çalışma *Phlomis russeliana*'nın HCT-116 insan kolon kanseri hücre dizisi ve HUVEC normal hücre dizisine karşı anti-kanser özelliklerini araştırmak üzere tasarlanmıştır.

Gereç ve Yöntemler: HCT-116 ve HUVEC hücreleri *Phlomis russeliana*'nın farklı konsantrasyonları (2, 4, 6, 8 ve 10 mg/ml) ile muamele edildi ve hücre canlılığı MTT yöntemi ile değerlendirildi. *Phlomis russeliana*'nın anti-migrasyon ve anti-kolonijenik etkileri, sırasıyla yara iyileşmesi ve koloni oluşumu analizleri ile değerlendirildi. Kolorimetrik Elisa kitleri ile toplam antioksidan durumunun (TAS), toplam oksidan durumunun (TOS) ve kaspaz-3 aktivasyonunun kantitatif tespiti yapıldı.

Bulgular: *Phlomis russeliana*, konsantrasyona bağlı olarak HCT-116 hücrelerinin canlılığını önemli ölçüde azalttı ve normal HUVEC'lere karşı daha zayıf toksisite gösterdi. *Phlomis russeliana*, HCT-116 hücrelerinin göçünü ve koloni oluşturma potansiyelini önemli ölçüde engelledi. Phlomis russeliana ile tedaviden sonra kaspaz-3 aktivasyonunda önemli bir artış gözlendi. *Phlomis russeliana*, HCT-116 hücrelerinde TAS ve TOS seviyesini önemli ölçüde etkilemedi.

Sonuç: Bu sonuçlar; *Phlomis russeliana*'nın koloni oluşumunun baskılanması, migrasyonun engellenmesi ve kaspaz-3 aktivasyonunun indüklenmesi yoluyla insan kolon kanseri hücrelerinde anti-kanser aktivitesi gösterdiğini ortaya koydu. *Phlomis russeliana*, kansere karşı yeni anti-kanser ajanlarının geliştirilmesi için umut verici bir kaynak olabilir.

Anahtar Kelimeler: *Phlomis russeliana;* HCT-116 hücreleri; migrasyon; kaspaz-3 aktivasyonu; koloni oluşumu.

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INTRODUCTION

According to Global 2020 data sources, colorectal cancer is the 3rd most frequently diagnosed cancer type in the world and the 2nd cause of cancer-related death among cancer types (1). Colorectal cancer is a major disease that results from the progressive accumulation of genetic and epigenetic changes (2). Chemotherapy and surgery are the most common existing treatment options for colon cancer but effects of these treatments are still unsatisfactory because development of drug resistance and terrible side effects (3). Therefore, the discovery and identification of new anti-tumor agents are urgently needed. Many studies stated the anti-cancer effects of plants against cancer (3). For centuries, herbal products have been used in the treatment of various human diseases (3). Numerous anti-cancer drugs derived from plants have demonstrated good pharmacological effects and currently have been administered around the world (3,4).

Phlomis L. species of Lamiaceae family generally have aromatic properties. The *Phlomis* species are used for several treatment as immunosuppressive, anti-inflammatory, antimicrobial, and free radical scavenging activities, ulcers and haemorrhoids in Anatolian folk medicine. (5-7). Studies have stated that cytotoxic, cytostatic and antioxidant activities most of the phenylethanoid glycosides isolated from *phlomis* species (8-9)**.** In a study, various phenylethanoid glycosides such as Forsythoside B, verbascoside, samioside, alyssonoside, isoverbascoside, martynoside in methanolic extracts of various *Phlomis* species which include *Phlomis russeliana* were identified by using HPLC method (5).

One of these *Phlomis* species is endemic Turkish plant *Phlomis russeliana* commonly named Turkish sage or Jerusalem sage (10). A previous study conducted by Alpay and co-workers showed cytotoxic effects of *Phlomis russeliana* methanol extracts against Caco-2 (10). Ghaffari and co-workers reported anti-proliferative effects of *Phlomis russeliana* leaf extracts on human breast cancer cell line (MCF-7) (11). To the best of our knowledge, the effects of *Phlomis russeliana* on HCT-116 cancer cells have not yet been investigated. In the present study, we aimed to investigate the antioxidant and antitumor activities of ethanol extracts obtained from *Phlomis russeliana* on colon cancer cell line HCT-116 in vitro.

MATERIAL AND METHODS

Reagents

RPMI 1640 medium, DMEM high glucose medium, fetal bovine serum (FBS), penicillin–streptomycin, phosphate-buffered solution (PBS), and 0.25% trypsin were purchased from Capricorn Scientific (Germany), MTT assay kit bought from Biotium (San Francisco, USA), caspase-3 assay kit purchased from Bt Lab (Shanghai, China).

Collection and Ethanol Extraction of Pholomis *russeliana*

Aerial parts of the plant were collected from Bolu Province, Turkey, in August, 2021. The extraction was performed as described previously by Alpay et al. with some modifications (10). About 20g of dried aerial parts of plant were powdered and extracted with 150 ml ethanol (95%) extraction by a Soxhlet extractor. After, the resultant dried extract was stored in sterile screw-capped bottles at 4 °C for further use (10).

Cell Lines and Cell Culture

Colon cancer cell line HCT-116 was kindly supplied by Prof. Dr. Ferda Arı (Bursa Uludag University, Department of Biology) Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. C. Verda Bitirim (Ankara University Stem Cell Institute). HUVECs were cultured in 10% FBS-containing DMEM high glucose medium (Capricorn Scientific, Germany) and HCT-116 cells were cultured with RPMI-1640 medium (Capricorn Scientific, Germany) supplemented with L-glutamine, 10% inactivated fetal bovine serum (FBS, Capricorn Scientific, Germany), 1% penicillin–streptomycin (Capricorn Scientific, Germany). Cells were maintained an incubator supply humidified atmosphere with 5% CO_2 at 37^0C.

Cell Viability

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) method was investigate the effects of *Phlomis russeliana* on HCT-116 and HUVEC cell viability. Cultured cells (HCT-116 and HUVEC) were seeded into 96-well cell culture plates at $5x10^3$ cells per well and incubated for 24 h at 37°C and 5% CO_2. After the incubation period, medium was removed and cells treated with 100 µL of the dilutions of the *Phlomis russeliana* concentrations (2, 4, 6, 8 mg/ml) were added to each well. Cells incubated with medium containing ethanol solvent (0.5%) were used as a control. Treated cells further incubated for 24h. After 24 hour of incubation, 10 µL of MTT solution (Biotium, San Francisco USA) was added and cells were incubated for 4 h at 37 °C. Then 200 µL DMSO was added to each well and formazan crystals were dissolved. Finally the absorbance of samples was measured spectrophotometrically at 570 nm wavelength using a microplate reader (BioRad, USA). Each experiment was tested in triplicate.

Microscopic Observation of HCT-116 Cell Morphology

Morphological changes in response to different concentrations of *Phlomis russeliana* were observed by an inverted microscope (Euromex Arnhem, The Netherlands). Cells were cultured in a 6-well plate and incubated with different *Phlomis Russeliana* concentrations (4 mg/ml and 6 mg/ml). After an incubation period, morphological alterations were examined under an inverted microscope.

Caspase- 3 Assay

Caspase-3 ELISA Kit (Bt-lab, China) was used the detect caspase-3 levels in HCT- 116 cells treated with *Pholomis russeliana* extracts. Caspase-3 activity was measured as per the manufacturer's protocol. Hct-116 cells treated with *Pholomis russeliana* extracts (4 mg/ml and 6 mg/ml) for 24 h. After treatment cells were harvested and transferred into the 15 mL conical tubes. Thereafter cells were centrifuged at 2,000 x g for 5 minutes at 4°C. The supernatant was discarded and cell pellet was dissolved in chilled RIPA lysis buffer. Cell suspension incubated on ice for 30 minutes and then centrifuged at 14000xg for 10 minutes. Supernatants were transferred to a new microcentrifuge and stored at -20^0C. Optical density (OD value) of each well was measured with a microplate reader

(Epoch BioTeK, Agilent Technologies, U.S.A.) at 450 nm.

Wound Healing

The influence of *Pholomis russeliana* extract on the cell migration of HCT-116 cells was analyzed by wound healing assay. HCT-116 cells (2×10^5) were plated in 6-well culture with RPMI-1640 medium and cells were incubated until they formed a confluent cell monolayer. A wound was generated with a sterile yellow pipette tip on the midline of the confluent cell monolayer in each well. And then cell debris was removed by PBS washing. Thereafter, the cells in each well were incubated with fresh medium containing different doses of *Pholomis russeliana* extracts (4 mg/ml and 6 mg/ml) incubated for 24 hours. Images of the migrating cells were photographed using an inverted microscope. The wound size was analyzed by ImageJ software.

Colony Formation Assay

HCT-116 cells were seeded into 6-well plates at (~300 cells/well) and incubated for 24 hours to allow the cells to adhere to the surface. After that cells were treated with different concentrations of *Pholomis russeliana* extracts and incubated at 5% CO_2 and 37°C for 7 days. After incubation, the medium was removed and the cells were washed with 1X PBS. After washing, colonies were fixed with cold methanol solution for 30 minutes and stained with crystal violet (0.5%) for 30 minutes. Colonies containing more than 50 cells were counted and photographed under a light microscope.

Measurement of intracellular TAS and TOS levels

TAS levels were analyzed with a commercial Elisa kit (Rel Assay Diagnostics, Turkey) and results were expressed as mmol Trolox Eq/L. TOS levels were measured by using a commercially available Elisa kit (BT Lab, Chine) and results were given as μmol H_2O_2 Eq/L. TOS and TAS levels in cell lysed were measured spectrophotometrically (Epoch, BioTek) as per manufacturer's directions.

Statistical Analyses

Descriptive statistics of the quantitative variables in the study are given as mean and standard deviation. The conformity of the variables to the normal distribution was examined using the Shapiro Wilk test. One way analysis of variance (one way anova) was used in the mean comparison of the groups with more than two categories, and the comparisons with the control group for the variables that differed were examined using the Dunnett test. Two way anova was used to evaluate the comparison of means for more than two periods and groups. $P<0.05$ was accepted as the statistical significance level. SPSS (version 28) and GraphPad Prism 9.4.1 were used in the analysis.

RESULTS

***Phlomis russeliana* inhibited proliferation of HCT116 colon cancer cells and showed lower cytotoxicity against normal HUVEC cells**

Anti-proliferative and cytotoxic effects of ethanol extract of *Pholomis russeliana* were investigated on HCT-116 colon cancer cells and normal HUVEC cells by using the MTT assay. The cells were treated with various doses of *Pholomis russeliana* extract (2, 4, 6, 8,10 mg/ml) for 24h. *Pholomis russeliana* significantly inhibited the cell viability of HCT-116 cells in a dose-dependent manner as compared to control group. HCT-116 cell viability was detected %53 for 4 mg/ml administration dose. The highest inhibition of proliferation was observed at 10mg/ml (Figure 1(a)). There was no significant difference in cell viability among HUVEC cells treated with 2 and 4 mg/ml of *Pholomis russeliana* ethanol extract compared to the control group (Figure 1(b)). Although HUVEC cell viability was significantly reduced at higher doses of *Phlomis russeliana* extract, the cell viability of HUVECs was not decreased below 43%. These results indicated that *Pholomis russeliana* has low cytotoxic activity against normal HUVEC cells and selectively inhibited HCT-116 cancer cells proliferation.

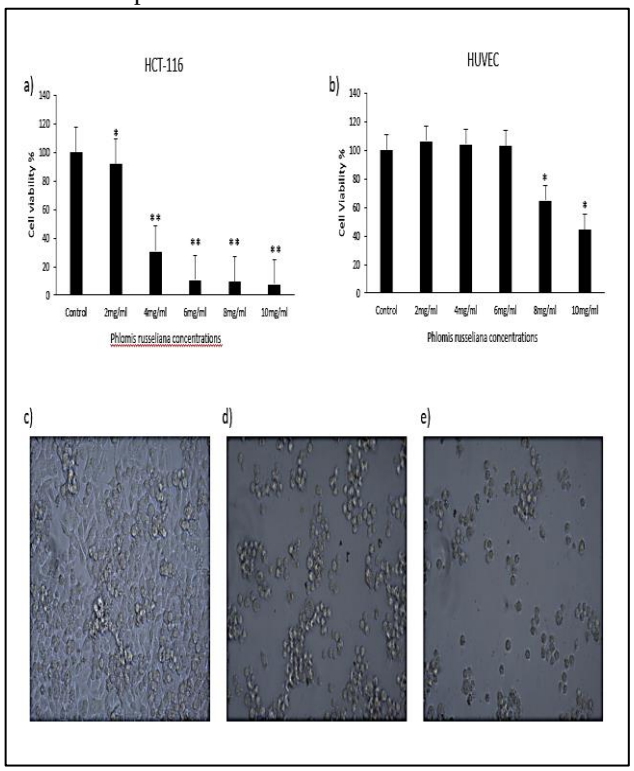

Figure 1. *Phlomis russeliana* inhibits the viability of HCT-116 cells. HUVEC (a) and HCT-116 (b) cells were incubated with different concentrations of *Phlomis russeliana* for 24 h, and then analysed by the MTT assay. P.*russeliana* exhibited little cytotoxicity on HUVECs compared with HCT-116 cells. Representative macroscopic images of morphological alterations in HCT16 cells following treatments of DMSO (c), 4mg/ml (d) and 6 mg/ml (e) doses of P. *Russeliana*. P. *Russeliana* decreased the HCT-116 cell density in a concentration-dependent manner and caused rounded cell shape compared to untreated control cells. *p=0,007 **p<0,001 vs. control group. Data were expressed as mean standard deviation (SD) of three independent experiments.

***Phlomis russeliana* caused morphological alterations in HCT-116 cells**

Following treatment with *Phlomis russeliana*, morphological changes were analyzed by an inverted microscope. As seen in the inverted microscope images (Figure 1(c) to (e)), *Phlomis russeliana* treatment reduced the number of HCT-116 cells and led more rounded cell shapes compared with the cells in the vehicle (control) treated group.

***Phlomis russeliana* induce Caspase-3 activation in HCT116 cells**

A colorimetric sandwich ELISA kit (E4804Hu) was used for the quantitative detection of human caspase-3 concentration in the HCT-116 cell lysates. Based on MTT data, two major cytotoxic concentrations of P.russelina were selected for caspase-3 activity. Caspase-3 activity was measured after treated cells with 4 mg/ml and 6 mg/ml of P.russelina extracts for 24h. Caspase-3 activity was significantly increased by the treatment of HCT116 cells with *Pholomis russeliana* extracts. Compared with control, caspase-3 activity was increased by 1,45 and 5,16 fold in HCT116 cells treated with 4 mg/ml and 6 mg/ml doses of *Pholomis russeliana* respectively. The changes found in caspase-3 activities after P.russelina administration is presented in Figure 2.

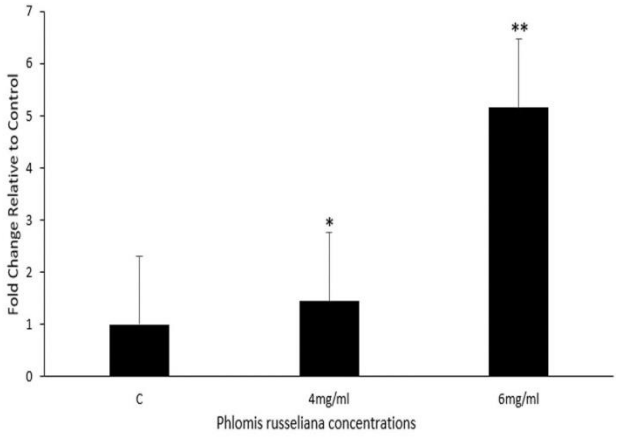

Figure 2. Relative expression levels of caspase 3 in the HCT-116 cells treated with two concentrations (4 mg/ml and 6 mg/ml) of Pholomis *russeliana*. Treatment of HCT-116 cells with P. *russeliana* extracts led to a significant increased caspase-3 activity. Each treatment replicated in three times and the data were presented as the mean + standard deviation (SD) of three independent experiments, *p=0,020, **p < 0,001.

***Phlomis russeliana* inhibited the HCT-116 cell migration**

To investigate the effect of *Pholomis russeliana* on the migration potential of HCT-116 cancer cells we performed wound healing assay. After a wound was generated, HCT-116 cells were incubated with 4 mg/ml and 6 mg/ml of *Pholomis russeliana* extracts for 12 and 24 h. Images of scratch areas were photographed with an inverted microscope (magnification, x100) at 0, 12 and 24 h of *Phlomis russeliana*-treatment and results are shown in Figure 3. *Pholomis Russelina*-treated cells covered less of the scratch area compared to untreated control cells. After 12 h, covered of the wound area in 4mg/ml and 6 mg/ml treated HCT116 wells were % 18,63 and % 20,67 respectively and %32,29 in the control group At 24 hours after treatment, these rates were found % 28,86 and %21,55 respectively in 4 mg/ml and 6 mg/ml treated groups and 45,84% in the control group. Our results showed that *Pholomis russeliana* significantly inhibited migration ability of HCT-116 cells.

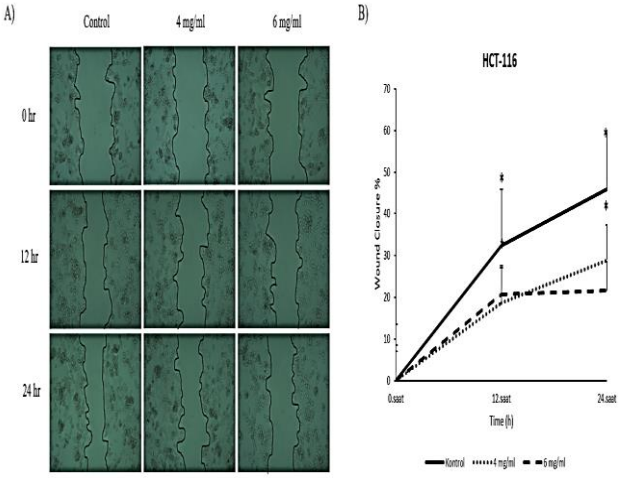

Figure 3. Lower migration rate was observed in treated HCT-116 wells compared to untreated cells. HCT-116 cells (2×10^5 cells/well) were seeded in a 6 well plate and incubated with denoted concentrations of P. Russelina for 24h. Images of the relative wound closures at 0,12 and 24 h of P. Russelina treatment were illustrated (magnification, x100) (A). The percentage of wound closure area at 0, 12 and 24 h of P. Russelina treatment presented as graphs (B). Three repetitions of each treatment were made and results from each experiment were presented as mean standard deviation (SD) of three independent experiments. * p<0,05 vs. control group.

***Phlomis russeliana* inhibit the colony formation in HCT-116 cells**

The colony formation ability of *Phlomis russeliana*-treated HCT 116 cells was determined by colony formation assay. *Pholomis russeliana* suppressed colony formation activity of HCT-116 cells in a dose dependent manner (Figure 4). *Phlomis russeliana* dropped the colony number to 23 at 4 mg/ml and to 8 at 6 mg/ml concentration significantly. The results revealed that *Phlomis russeliana* significantly inhibited colony formation of HCT-116 cells.

***Pholomis russeliana* did not affect TAS and Tos levels in HCT-116 cells**

TAS and TOS levels were investigated in HCT-116 cells after exposed to two different concentrations (4 mg/ml and 6 mg/ml) of *Phlomis russeliana* extracts. Compared with the control group, there was a slight increase in TAS levels after treatment with 4 mg/ml P. *Russelina*, while a decrease was observed in TAS levels in HCT116 cells after treatment with 6mg/ml of *Phlomis Russeliana*. When we analyzed TOS levels, treatment of HCT116 cells with P.r*usselina* extracts caused a dose-dependent increase in TOS levels compared to the control group (Figure 5). However, these results were not statistically significant (all $p > 0.05$).

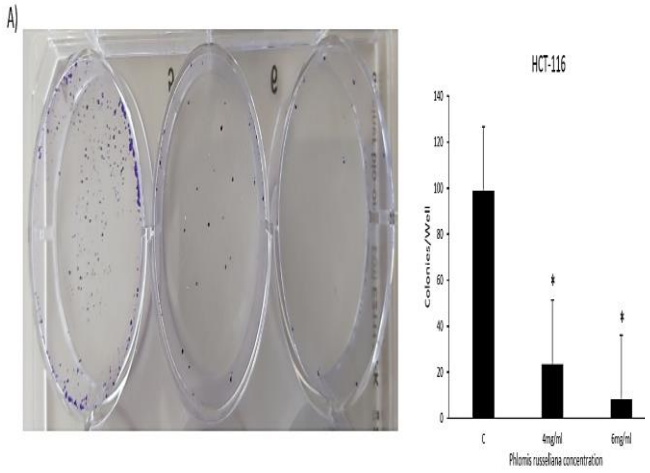

Figure 4. Effect of Pholomis *russeliana* on the colony formation ability of HCT-116 cells. Pholomis *russeliana* significantly decreased the number of colonies of HCT-116 cells. The number of colonies of Pholomis *russeliana*-treated HCT-116 cells were significantly lower than non treated control group (A). Experiment were repeated three times and data are presented as the mean ± SD.*p < 0,001 vs. control group.

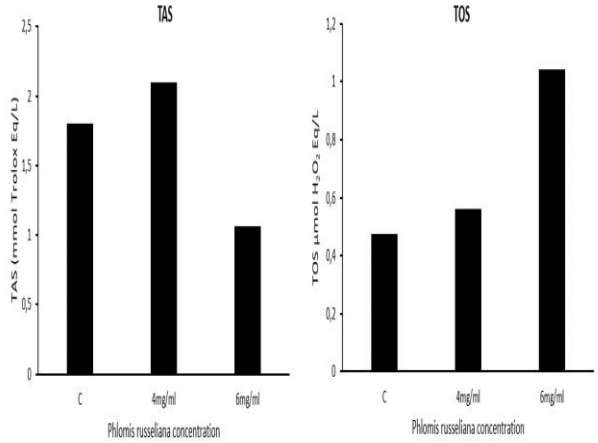

Figure 5. TAS and TOS levels in HCT-116 cells after treatment with *Phlomis russeliana*.

DISCUSSION

For centuries, medicinal herbs have been used in traditional medicine for the treatment of many diseases (12). Herbal medicine has been an important source of drug discovery used in cancer therapy over the course of many years (12,13). One of the sources of these herbal medicinal products is the genus *Phlomis* has been used for many years in the treatment of various diseases such as diabetes (14) gastritis (15) and dermal infections (6). Cytotoxic activity of *Phlomis* species against different cancer cell lines has shown in previous studies (8,10-12). Saracoglu et al. found that herb compounds isolated from *Phlomis* species such as phenyl propanoid caffeic acid, phenyl ethyl alcohol show cytotoxic effects against different cancer cell lines (8). Ghaffari et al. investigated the anti-proliferative effects of P.russelian in MCF-7 breast cancer cells (11). Our results are in agreement with previous studies which showed that *Phlomis russeliana* induced cytotoxic effects on human cancer cell lines. Our findings revealed that *Phlomis russeliana* at lower doses showed a strong cytotoxic effect against colon cancer cells. However, *Phlomis russeliana* showed a lower cytotoxicity against normal HUVEC cells compared with HCT-116 cells. *Phlomis russeliana* extracts did not exhibit a cytotoxic effect on the tested cells at 2-6 mg/mL concentrations. Even though, inhibitory effect of *Phlomis Russeliana* significantly increased at higher concentrations (8 mg/ml and 10 mg/ml), the cell viability of HUVECs did not decrease below 44%. The obtained data revealed that *Phlomis Russeliana* exhibits a selective toxicity against HCT-116 cells. Ghaffari et al. investigated the anti-proliferative effects of P.russelian in MCF-7 breast cancer cells (11). They tested *Phlomis Russeliana* on MCF-7 cancer cells at low but over a wider range of doses (0.001, 0.01, 0.1, 1 and 10 µg/ml). They found that 0.001 and 0.01 mg/ml of *Phlomis russeliana* leaf extracts induced proliferation of MCF-7 cells but 1 and 10 mg/ml of doses led to a significant decrease in viability of MCF-7 cells (11). Alpay et al.,investigated the antimicrobial, antioxidant and antitumor activity of *Phlomis russeliana* ethanol extracts against Caco-2 colon cancer cell line (10). They used higher doses of *Phlomis russeliana* extracts (5, 10, 20, 40 and 80 mg/ml) on cancer cells compared to our study. They also found proliferation inhibition effect of the *Phlomis russeliana* on colon cancer cells (10). They also showed that proliferation inhibitory effect of the *Phlomis russeliana* on Caco-2 colon cancer cells. Unlike Alpay's study, in this study we investigated the caspase-3 activity, clonogenic and migratory potential of HCT-116 colon cancer cells treated with *Phlomis russeliana* extracts. Also, we didn't reach any study that examined these anti-cancer properties of the *Phlomis russeliana* on colon cancer cells in the literature.

Caspases are promoter of apoptosis and activated by intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) apoptotic signaling pathways (16). Caspase-3 is well known effector caspase and it is at the center of both signaling pathways. When caspase-3 is activated a series of substrates in cells are specifically cleaved and finally cell death occurs (17). It is a key regulator of chemotherapy, radiotherapy, and immunotherapy-mediated apoptosis induction (18). Cancer therapy efficacy is generally evaluated according to increased caspase-3 activation (18).

In our study, *Phlomis Russeliana* significantly increased the caspase-3 activation in HCT-116 cells in a dose dependent manner. Our results suggest that *Pholomis russeliana* could induce caspase dependent cell death pathways in HCT-116 cells.

Cell migration is a tightly regulated process involved in many physiological events such as cell differentiation and proliferation. (19,20). Uncontrollable cell migration promotes anti-cancerogenic events such as cancer initiation, relapse and metastasis, leading to tumorigenesis (20). *Therefore inhibiting the migration process is a substantial treatment strategy.* According to our results, migration of HCT-116 cells across the wound area was significantly suppressed by *Phlomis russeliana* treatment. In this study, we demonstrated for the first time the

migration inhibition potential of *Phlomis russeliana* on HCT-116 cells.

Colony formation is an alternative in vitro technique used to evaluate survival and proliferation ability of cancer cells (21). This assay is helpful to evaluate survival and proliferation of cancer cells after treatment with cytotoxic agents (21, 22). In other words, it is the ability of cancer cells to form a colony from a single cell. (23). In our study we found that treatment with *Phlomis russeliana* significantly inhibited colony formation in HCT-116 cells. Oxidative stress is an important factor in cancer development (24) The total oxidant status (TOS) and the total antioxidant status (TAS) are used as biomarkers to assess oxidative stress status. (25). Alpay et al. reported that TOS levels were decreased in Caco-2 cells treated with *Phlomis Russeliana* extracts (10). In our study, decreases in TAS and increases in TOS levels according to different *Phlomis Russeliana* concentrations in HCT-116 cells were statistically insignificant. This result suggested that oxidative stress state is not an anti-cancer mechanism exerted by *Phlomis Russeliana* against HCT-116 cells.

CONCLUSION

This is the first study to show anti-cancer activities (caspase-3 activation, anti-migration and anti-clonogenic) of *Phlomis russeliana* against HCT-116 colon cancer cells. Our findings revealed that *Phlomis* russeliana exhibited a strong anti-cancer effect against HCT-116 cells and a lower cytotoxic effect on normal cells. Our results might be important contributions to the development of new treatment approaches that can support further clinical research. Further studies are required to identify new molecular mechanisms underlying the anti-tumor effects of *Phlomis russeliana*.

Authors's Contributions: Idea/Concept: Y.K.; Design: Y.K.; Data Collection and/or Processing: Y.K.; Analysis and/or Interpretation: Y.K., Ö.P.; Literature Review: Y.K.; Writing the Article: Y.K.; Critical Review: Y.K., Ö.P.

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