Evaluating antimicrobial and antioxidant capacity of endemic *Phlomis russeliana* from Turkey and its antiproliferative effect on Human Caco-2 Cell Lines

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Abstract: In this study, the antimicrobial, antioxidant and antitumor activity of ethanol extracts obtained from *Phlomis russeliana* (Sims.) Lag. ex Benth. (Lamiaceae) were evaluated. Disc diffusion and microdilution methods were used to test the extracts for antimicrobial activity against seven bacteria strains (*Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 10538, *Proteus vulgaris* ATCC 6899, *Salmonella typhimurium* CCM 5445 and *Pseudomonas aeruginosa* ATCC 27853) and four yeast strains (*Kluyveromyces fragilis* ATCC 8608, *Rhodotorula rubra* ATCC 70403, *Debaryomyces hansenii* DSM 70238 and *Candida albicans* ATCC 10239). Notably, they were more effective against the yeast strains than the bacterial strains. Of the yeast cultures, *D. hanseii* was among the most susceptible, having an inhibition zone of 16.2 mm with minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of 64(128)µg/ml, respectively. For cytotoxic determination, Caco-2 cells were cultured as per ATCC protocol, and were treated with log concentrations (5–80 mg/ml) of *P. russeliana*. The potency of cell growth inhibition for each extract was expressed as an IC50 value. Moreover, oxidant capacity was evaluated via TOC assay. This product induced antiproliferative activity of 31.33% at 40 mg/ml and 20.96% at 80 mg/ml, without toxic effects on cells, although the oxidant capacity was decreased to 27.06 ± 0.7 nm in the 80 mg/ml-applied group compared to 47.9 ± 1.8 nm in the untreated one. Advanced pharmacological studies are needed to further evaluate *P. russeliana* for distinctive features.

Key words: *Phlomis russeliana*, antioxidant activity, cytotoxic effect, antimicrobial activity, endemic plant.

INTRODUCTION

The use of plants in the treatment of various diseases is an ancient practice that is widespread in many parts of the World (Masika et al. 2000). More than 100 species of the genus *Phlomis* are distributed throughout Eurasia and North Africa, and 34 of the 52 taxa are endemic to Turkey (Cali 2016). The *Phlomis* species found in Turkey are used as herbal teas, tonics, carminatives, appetizers and stimulants in traditional folk medicine and recognized by local names as ballık otu, calba or şalba (Baytop 1999). *Phlomis russeliana* (Sims.) Lag.ex Benth. (Lamiaceae), commonly called

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Turkish sage or Jerusalem sage, is endemic to Turkey (Dadandi 2002). During routine field excursions, it was found that this plant is used in the treatment of stomach disorders. The present study aimed to investigate the activity of this endemic plant on the cells, and especially determining the effect of cell proliferation at the beginning cancer mechanism deeply. Colon cancer was selected for the study because of its high incidence. Indications are that further detailed studies are need to clarify the efficacy of this plant.

MATERIALS AND METHODS

PLANTS EXTRACTION PREPARATION

Aerial parts of the plant were collected from Golyaka (Duzce Province), Turkey, during the flowering stage in July, 2017. The extraction was performed as described previously by Dulger et al. with some modifications (Dulger et al. 2017). The extract was filtered and the filtrate solvent was evaporated under vacuum using a rotary evaporator at 55 °C (yeild: 14.12% for ethanol). The resulting dried extract was labelled and stored in sterile screw-capped bottles at -20 °C. Around 2 g of the extract was dissolved in 0.1 ml of dimethyl sulfoxide (DMSO) (5 mg/ml) before testing.

TEST MICROORGANISMS

The *in vitro* antimicrobial studies were carried out with seven bacteria strains (*Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 10538, *Proteus vulgaris* ATCC 6899, *Salmonella typhimurium* CCM 5445 and *Pseudomonas aeruginosa* ATCC 27853) and four yeast strains (*Kluyveromyces fragilis* ATCC 8608, *Rhodotorula rubra* ATCC 70403, *Debaryomyces hansenii* DSM 70238 and *Candida albicans* ATCC 10239) obtained from the Microbiology Research Laboratory of the Duzce University Department of Biology.

DISC DIFFUSION METHOD

The disc diffusion method was employed (Collins et al. 1987). Sterile 6-mm discs were impregnated with 50 µl of the plant extracts. The bacterial cultures were inoculated into Nutrient Broth (Oxoid) and incubated for 24 h at 37 ± 0.1 °C, while the yeast cultures were inoculated into Malt Extract Broth (Oxoid) and incubated for 48 h at 28 ± 0.1 °C. The counts of bacterial cultures and yeast cultures were adjusted to 0.5 McFarland standard. Adequate amounts of Mueller Hinton Agar (Oxoid) were dispensed onto sterile plates under aseptic conditions. The test microorganisms (0.1 ml) were inoculated onto the surface of the appropriate solid media on the plates. The agar plates inoculated with the test microorganisms were incubated for 1 h before placing the extract-impregnated paper discs on the plates. The bacterial plates were then incubated at 37 ± 0.1 °C for 24 h and the yeast plates were incubated at 28 ± 0.1 °C for 48 h. After incubation, the inhibition zones formed on the plates were evaluated in mm. Penicillin, Tobramycin, Ampicillin, Nystatin, Clotrimazole and Ketoconazole were used as positive controls.

MICRODILUTION METHOD

Determination of the minimum inhibitory concentration (MIC) was carried out according to the methods described by Zgoda and Porter with some modifications (Zgoda and Porter 2001). Dilution series (10–0.5 mg/ml) of the extracts were prepared in test tubes and then transferred to the broth in 96–well microtiter plates. Final concentrations in the medium ranged from 1000 to 50 µg/ml. Before inoculation, the bacteria strains and yeast strains were adjusted to 0.5 McFarland and diluted 1:1000 in Mueller Hinton Broth (Oxoid) and Malt Extract Broth (Oxoid), respectively. The plates were incubated at 35 °C for 18–24 h for bacteria and at 30 °C for 48 h for the yeast cultures. The MIC values of the extracts were defined as the

lowest concentration that showed no growth, and the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by seeding the samples from the clear wells onto Mueller Hinton agar and Malt Extract Agar, respectively. The MBC and MFC were defined as the lowest concentrations yielding negative subcultures. Ampicillin and streptomycin were used as standard antibacterial agents, whereas nystatin was used as the standard antifungal agent. Their dilutions in the microtiter plates were prepared in concentrations of 128–0.25 µg/ml.

CELL CULTURE

The human colon cancer cell line Caco-2 (ATCC number: HTB37) used for the study is well to moderately differentiated, aggressive, microsatellite unstable and epithelial growth factor-dependent. The cells were maintained according to standard cell culture protocol. The colon cancer line was cultured in confluent clusters for application.

CYTOTOXIC ACTIVITY AND MEASUREMENT OF OXIDANT CAPACITY

In order to assess cellular function, the cells were seeded in equal volumes of 5×10^4 cells/100 µl in each well of 96-well plates. The MTT cell proliferation assay was conducted after the Caco-2 cells were plated for 24 h. The cultures were treated with *P. russeliana* dissolved in (5mg/ml) EtOH (ethanol) at concentrations of 5, 10, 20, 40 and 80 mg/ml and the control (EtOH in DMEM) were prepared with 0.5% of Ethanol and Dulbecco's Modified Eagle's Medium (DMEM). They were analysed via colorimetric MTT assay to determine the IC50 (Alpay 2017). Metabolically active cells were determined according to MTT procedure at 540 nm density (Alpay 2017). At the same time, trypan blue assay was performed for each group in order to corroborate the MTT data (Bioresearch, Lonza). The total oxidant status (TOS) was then measured, respectively (Rel Assay Diagnostics,Turkey) (Erel 2005). This method uses xylenol orange to measure ferric ions, which is an oxidized form of ferrous ions in an acidic medium. Briefly, reagents were mixed with 35 µl samples of *P. russeliana* + 80 mg/ml application and with samples of the untreated group. The results of the assay are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 Equiv/L). The total oxidant capacity was analysed for comparison of the *P. russeliana* cell oxidant levels.

STATISTICAL ANALYSIS

Statistical analysis of results were evaluated by using version 2.03 of the Sigma Stat software. Quantitative data were presented as mean and standard error (SE). The statistical difference between the control and the *P. russeliana* groups was determined by the Student's t test, with $p<0.05$ considered as significant. However, this significant difference was coincided with the microscopic analysis of cell proliferation rate.

RESULTS AND DISCUSSION

Herbal medicine has provided a valuable guide to drug discovery throughout the years (Sahranavard et al. 2009). Almost one-third of prescription drugs in the world are derived from plants, and anticancer properties have been indicated in over 3000 plant species (Ghafari 2015). The objective of this study was to evaluate the antimicrobial, antioxidant and antitumor properties of *Phlomis russeliana*. Investigations on the antimicrobial activity of *Phlomis* are limited. In one previously study, ethanol and methanol *Phlomis* extracts exhibited moderate antibacterial activity against *S. epidermidis* (9 and 8.5 mm, respectively) and *S. pyogenes* (9.5 mm) (Yildirim 2013). In another study, *P. russeliana* essential oil has tested against common food-borne bacterial pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus* and *S. typhimurium* (Demirci 2008). The antimicrobial activities of the *P. russeliana* extract against the test

microorganisms examined in the present study were qualitatively and quantitatively assessed by the presence of inhibition zones, MIC, MBC and MFC (Tables I and II). The ethanol extract of the plant exhibited moderate antimicrobial effects against the test microorganisms, with inhibition zones of 10.0–16.2 mm. Notably, among the bacterial strains, *S. typhimurium,* with an inhibition zone of 13.2 mm, was more susceptible to the plant extract compared to the standard antibiotic tobramycin. Similarly, with an inhibition zone of 12.4 mm, the

extract also showed higher antibacterial activity for *S. aureus* than tobramycin. The antibacterial effects of the extract against the other bacterial strains were far below those of the antibacterial levels of all the standard antibiotics. *D. hansenii* was more susceptible to the antifungal effects of the extract compared to the standard antifungal antibiotic clotrimazole, and the effects of the extract were equivalent to those of the antifungal antibiotic nystatin. The other yeast strains were more resistant to the antifungal effects of the extract, which

TABLE I
Disc diffusion results of *Phlomis russeliana*.

Microorganisms	Plant extract	P	AMP	TOB	NYS	CLT	KETO
Bacillus cereus ATCC 7064	10.8	14.0	17.4	16.4	NT	NT	NT
Bacillus subtilis ATCC 6633	11.0	14.8	14.2	20.2	NT	NT	NT
Staphylococcus aureus ATCC 6538P	12.4	20.4	16.8	12.0	NT	NT	NT
Escherichia coli ATCC 10538	10.0	18.2	14.4	11.4	NT	NT	NT
Proteus vulgaris ATCC 6899	12.8	10.8	16.2	14.8	NT	NT	NT
Salmonella typhimurium CCM 5445	13.2	14.8	14.6	11.0	NT	NT	NT
Pseudomonas aeruginosa ATCC 27853	11.2	10.0	12.0	13.2	NT	NT	NT
Kluyveromyces fragilis ATCC 8608	14.2	NT	NT	NT	17.6	16.4	19.2
Rhodotorula rubra ATCC 70403	15.8	NT	NT	NT	18.4	21.8	16.8
Debaryomyces hansenii DSM 70238	16.2	NT	NT	NT	16.2	15.2	22.2
Candida albicans ATCC 10239	14.4	NT	NT	NT	18.2	20.2	24.0

Inhibition zones (mm)*

NT: Not tested; P: Penicillin (10 mcg); AMP: Ampicillin (20 mcg); TOB: Tobramycin (10 mcg); NYS: Nystatin (30 mcg); KETO: Ketocanazole: (20mcg); CLT: Clotrimazole (30 mcg).

TABLE II
Minimum inhibitory concentration (MIC) of *Phlomis russeliana*.

Microorganisms	EtOH extract (µg/ml)	ST	AMP	NYS
Bacillus cereus ATCC 7064	1000(>1000)	4.0 (4.0)	8.0 (8.0)	NT
Bacillus subtilis ATCC 6633	1000(>1000)	0.5 (1.0)	1.0 (2.0)	NT
Staphylococcus aureus ATCC 6538P	500(>1000)	2.0 (4.0)	0.25 (0.35)	NT
Escherichia coli ATCC 10538	1000(>1000)	4.0 (4.0)	32 (64.0)	NT
Proteus vulgaris ATCC 6899	500(>1000)	8.0 (16.0)	0.5 (1.0)	NT
Salmonella typhimurium CCM 5445	500(>1000)	16 (32)	2.0 (4.0)	NT
Pseudomonas aeruginosa ATCC 27853	1000(>1000)	1.0 (2.0)	16 .0 (32.0)	NT
Kluyveromyces fragilis ATCC 8608	250(>500)	NT	NT	16 (16)
Rhodotorula rubra ATCC 70403	128(>500)	NT	NT	16 (32)
Debaryomyces hansenii DSM 70238	64(128)	NT	NT	16 (32)
Candida albicans ATCC 10239	128(>250)	NT	NT	8 (16)

MIC (MBC or MFC) — Standards

NT: Not tested; ST: Streptomycin; AMP: Amphicillin; NYS: Nystatin.

exhibited levels far below those of all the standard antifungal antibiotics.

The ethanoic extract was further tested by microdilution in order to determination the MIC, MBC and MFC values. The lowest MIC and MFC values of the extract were 64 (128) μg/ml against *D. hansenii,* followed by *C. albicans* and *R. rubra,* which ranged between 128(>250) and 128(>500) μg/ml, respectively. Notably, the extract showed a potential effect against *K. fragilis* with MIC and MFC values of 250(>500) μg/ml. The MIC and MBC values against bacterial strains were very far below those of the standard antibiotics. These findings showed that the extract was more effective against the yeast strains than the bacterial strains. A weak to moderate MIC (125– >1000 μg/ml) was observed in the study mentioned earlier, and the *P. armeniaca* extracts showed no antimicrobial effects against the tested microorganisms (Yildirim 2013). In another study, the ethanol extract of *P. fruticasa* L. was evaluated against seven bacterial and seven fungal species (Ristic et al. 2000). The essential oils displayed antibacterial activity against *S. aureus*, *E. coli*, *B. subtilis*, *K. pneumoniae* and *M. luteus* and the antifungal activity of the extract was positive against *A. niger*, *A. ochraceus*, *C. cladosporoides*, *F. tricinctum* and *P. helianthi*. Several researchers investigated the antimicrobial activity of methanol, ethanol and ethyl acetate extracts and essential oil of *P. oppositiflora* was investigated against eight bacteria and three fungi via disc diffusion. The methanol, ethanol and ethyl acetate extracts of *P. oppositiflora* showed antibacterial activity with 8–15 mm, 7–11 mm and 9–12 mm inhibition zones, respectively (Toroglu and Cenet 2013). Other researchers revealed that methanol extracts of some *Phlomis* species (*P. bruguieri*, *P. herba-venti* and *P. olivieri*) exhibited antibacterial activity against *E. coli*, *K. pneumoniae*, *S. aureus*, *S. sanguis* and *P. aeruginosa* (Amor et al. 2009). Likewise, in that study, the extract exhibited moderate antimicrobial activity against both bacterial and yeast strains,

while being more effective against yeast strains than bacterial strains. Consequently, all these studies partially support the present findings. Some studies have found a relationship between the antimicrobial activity and the chemical structures of the most plentiful compounds in the tested plant extracts or essential oils and the researchers have stated that the antimicrobial activity of *P. russeliana* could be based on these compounds (Farag 1989, Deans and Svoboda 1989).

The present results revealed that an aqueous extract of this plant possessed moderate antitumor effects *in vitro* against Caco-2 cells. A previous study stated that cytotoxic activity of different fractions of *P. lanceolata* showed that the petroleum ether fraction exhibited high cytotoxic activity on HT29, Caco-2, T47D and NIH3T3 cancer cell lines. In all the present experiments, the MTT assay was used to determine the viability of the Caco-2 cells incubated for 24 and 48 h with the positive control and *Phlomis* at 5, 10, 20, 40 and 80 mg/ml. The control was taken as 100% for all time periods, and the other lysates were calculated from the percent values shown in Figure 1. The cell viability results verified the synergistic effect
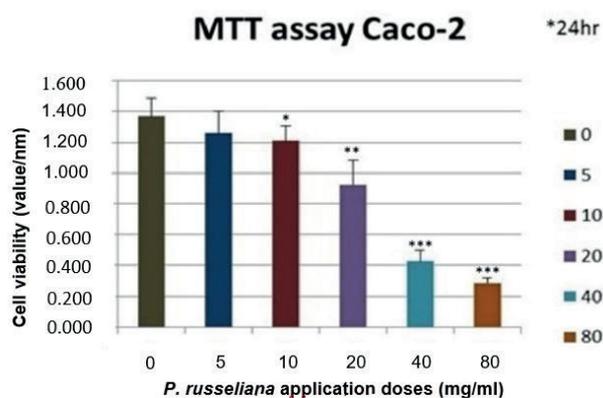

Figure 1 - The effects of different dose *P. russeliana* treatments with 5; 10; 20; 40; 80 mg/ml on cell viability of colon cancer (Caco-2) cell line. *p < 0.001 is 10 mg/ml concentration through **p < 0.001 20; 40 mg/ml concentration and ***P < 0.001 is 80 mg/ml treatment concentration as compared to the negative control group up to 24 h incubation.

of *P. russeliana* on the proliferation inhibition of the colon cancer. The morphologic survival is shown in Figure 2. The oxidant status of the experimental and control groups was measured by TOS assay. The oxidant status was significantly decreased in the treatment groups, while the antiproliferative effect was observed to be at a significant level (Figure 3). The oxidant capacity was indicated as 27.06 ± 0.7 nm at 80 mg/ml for the treatment group compared to 47.9 ± 1.8 nm for the untreated one. The effect of the combined oxidant capacity of *P. russeliana* on cancer cells to counteract reactive oxygen species was analysed. Research studies on some *Phlomis* species have shown cytotoxic activity against tumour cell lines (Soltani-Nasab et al. 2014). Previously reported findings when correlated with the present results

confirmed that many current chemotherapy cancer treatments affect cancer cells. The specific plant phytochemicals responsible for the observed antiproliferative effects are not precisely known at the present time. This encouraging preliminary data on the antiproliferative effects of *P. russeliana* may lead to the development of potential anticancer agents based on such natural wild plants.

CONCLUSIONS

The present findings showed that *P. russeliana* exhibited possible antioxidant and antimicrobial activity despite cytotoxic activity in colon cancer. In conclusion, in order to utilize the therapeutic agents from the *Phlomis* species, there is a need for further studies to investigate its safety and molecular mechanism.
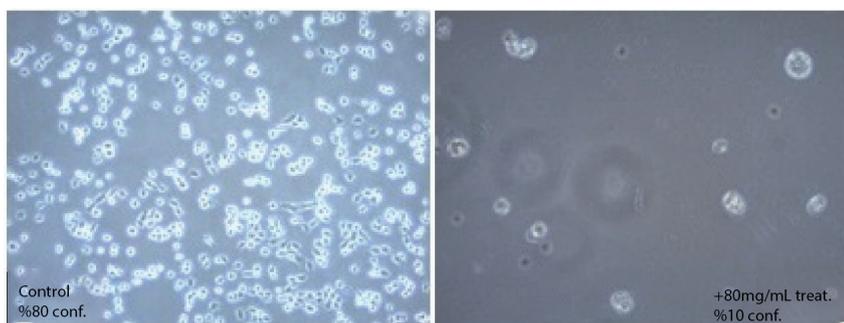

Figure 2 - The role of *P. russeliana* on human colon cancer cells by morphological manner. Cells were compared untreated with 80mg/ml of *P. russeliana* (captured from T-25 flask). The difference growth rate log phase between %80-%10 confluency. (displayed inverted microscope x100).
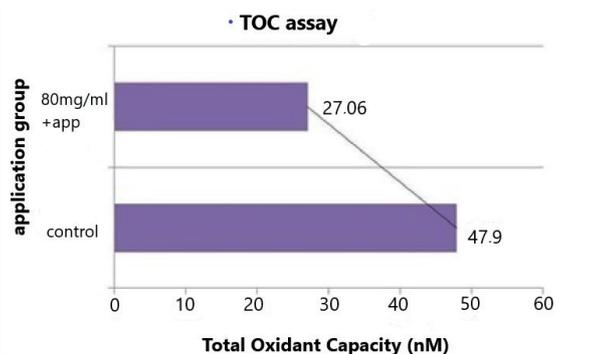

Figure 3 - Quantitative analysis of total oxidant status (TOS) of Caco-2 cells treated with 80 mg/ml of *P. russeliana* crosscheck with untreated group up to 48 h incubation.

AUTHOR CONTRIBUTIONS

G.D. and M.A. conceived of the presented idea. B.D. developed the theory and performed the computations. G.D and I.E.S. verified the analytical methods. M.A. supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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