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Research Article/Araştırma Makalesi

CYTOTOXIC ACTIVITY OF ROSMARINIC ACID ISOLATED FROM *Prunella vulgaris* L. AND *Prunella grandiflora* L. IN DIFFERENT TUMOR CELLS

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Abstract: Rosmarinic acid was isolated from ethanol extractions obtained from *Prunella grandiflora* L. and *P. vulgaris* L. The total phenolic content of ethanol fractions during isolation was determined by the Folin-Ciocateu method. The cytotoxic doses of the isolated rosmarinic acid were determined by WST-1 (Roche Applied Sciences, Mannheim, Germany) cell proliferation assay. A 10-60ng cytotoxic dose was determined for pancreas (PANC-1), prostate (PC-3), colon (HT-29) and breast (MDA-MB 436) cancers and GBM (T98G) cell lines and lymphatic tissues. 24 and 48h incubation periods were applied during dose determinations. An antiproliferative effect was observed at the end of 48h incubation period with 50ng rosmaniric acid treatment in PC3 cell line and with 60ng treatment in PANC-1, HT-29, MDA-MB 436 and T98G cell lines. No cytotoxic activity of rosmarinic acid was observed in non-tumor cells.

Key words: *Prunella* L., isolation, rosmarinic acid, antiproliferative, cytotoxic activity.

Prunella vulgaris L. ve *Prunella grandiflora* L.'den Saflaştırılan Rosmarinik Asitin Farklı Tümör Hücreleri Üzerindeki Sitotoksik Aktivitesi

Özet: Bu çalışmada rosmarinik asit bileşiği, *Prunella grandiflora* L. ve *P. vulgaris* L. türlerinden elde edilen etanol ekstraktlarından saflaştırılmıştır. Saflaştırma işlemi sırasında elde edilen metanol fraksiyonlarının toplam fenol içeriği Folin-Ciocalteu yöntemi ile belirlenmiştir. *Prunella* L. türlerinden saflaştırılan rosmarinik asitin farklı kanser hücreleri üzerinde WST-1 (Roche Applied Sciences, Mannheim, Almanya) yöntemiyle sitotoksik doz çalışmaları yapılmıştır. Buna göre pankreas (PANC-1), prostat (PC-3), kolon (HT-29) ve meme (MDA-MB 436) kanserleri ile GBM (T98G) hücre hatları ve lenf dokularında 10-60ng arasında sitotoksik doz belirlenmiştir. Sitotoksik doz belirleme çalışmaları için 24 ve 48 saat inkübasyon süreleri çalışılmıştır. 48 saat inkübasyon süresi sonunda PC3 hücre hattı için 50ng ve PANC-1, HT-29, MDA-MB 436 ve T98G hücre hatları için 60ng rosmarinik asit uygulamasında antiproliferatif etki gözlenmiştir. Sağlıklı hücrelerde rosmarinik asitin sitotoksik etkisi gözlenmemiştir.

Anahtar kelimeler: *Prunella* L., izolasyon, rosmarinic asit, antiproliferatif, sitotoksik aktivite.

Introduction

Phenolic compounds are natural compounds in aromatic and herbal plants. The main properties of such compounds are their antioxidant and radical scavenging activities (Baricevic *et al.* 2001, Petersen & Simmonds 2003). These compounds also have biological properties such as fungistatic, cytotoxic, antibacterial and antiviral activities.

Prunella L. has been used as a herbal in traditional medicine applications for several years. It has many biological effects such as anti-inflammatory and anti-microbial activities (Zdařilová *et al.* 2009). *Prunella* species also exhibit anti-growth effects on different cancer types (Feng *et al.* 2010, Woo *et al.* 2011). The main source of antioxidant activity in *Lamiaceae* is rosmarinic acid (Kim & Lee 2004). For instance, Yeşil-Çeliktaş et al. (2010) demonstrated that rosmarinic acid derived from the leaves of *Rosmarinus officinalis* L. showed anticancer activity of n lung, prostate, hepatocellular, myeloid leukemia and breast cancers. The antiproliferative effect of phenolics may differ depending on the species (Feng *et al.* 2010), i.e. although the inhibitor effect of rosmarinic acid from *R. officinalis* was demonstrated in different cancer cell lines, it has not been determined in *Prunella* species.

We aimed in the present study to isolate rosmarinic acid in ethanolic extracts of *P. vulgaris* and *P. grandiflora*

in order to demonstrate its antiproliferative effects on pancreas, prostate, colorectal and breast cancers and GBM cell lines.

Materials and Methods

Rosmarinic acid, Folin-Ciocalteu reagent and Sephadex LH-20 were supplied from Sigma–Aldrich. Methanol, ethanol, acetonitrile and formic acid were supplied from Merck. All cell lines were provided by the American Type Culture Collection (ATCC; Rockville, USA). Penicillin, Fetal Bovine Serum (FBS), Streptomycin, RPMI 1640, Sodium Pyruvate (BIOCHROME, Berlin, Germany), Dulbecco's Modified Eagle's Medium-F12 containing L-glutamin (DMEM-F12, HyClone, Utah, USA), and Hitopaque−1077 (Sigma-Aldrich, Chemiegmbh, Steinheim, Germany) and Phytohemagglutinin (PHA; Gibco-Invitrogen, Denmark) were used for maintenance of cancer cell lines and lymphocytes.

Isolation of rosmarinic acid

Prunella species were collected from in Turkey (Bursa, Balıkesir, Eskişehir and Antalya) from June to July in 2009. After dried at room temperature, the samples were stored at 4°C. The parts of *P. grandiflora* and *P. vulgaris* samples (10g) were separately mixed with ethanol at room temperature in dark for 5h under magnetic stirrer. The separated ethanolic fraction (15mL) was isolation using Sephadex LH-20 column chromatography. Standard rosmarinic acid was dissolved in methanol for chromatographic analysis. Because of this reason, the extracts were eluted with methanol from column.

Folin-Ciocalteu method

Folin method (Şahin *et al.* 2014, Singleton *et al.* 1999) was used for determining the total phenolic contents of *Prunella* fractions by UV/vis spectrometer (Varian Cary 50 Conc, Australia) equipped with 10mm quartz cuvettes.

Chromatographic analysis

Rosmarinic acid was determined in *Prunella* samples by HPLC-DAD. The results of our previous study were followed for chromatographic analysis of rosmarinic acid in *Prunella* fractions (Şahin *et al.* 2014).

Determination of the effect of fractions on cytotoxicity and cell viability of cancer cell lines

Five human cancer cell lines, T98G; GBM, PANC-1; pancreas, PC-3; prostate, HT-29; colorectal and MDA-MB 436; breast were grown in DMEM-F12 containing L-glutamine supplemented with 10% FBS, 100µgmL^{-1} streptomycin, 1mM sodium pyruvate and 100Uml^{-1} penicillin in a humidified 5% CO_2 incubator at 37ºC.

Human peripheral blood lymphocytes were used for analyzing the cytotoxic effect of rosmarinic acid fractions in non-tumor cells. Five milliliter of heparinized total blood was obtained from a healthy, 30 years old non-smoking female volunteer with her complete informed consent. Human mononuclear lymphocytes were isolated with density gradient centrifugation using Hitopaque−1077 reagent and washed twice. Lymphocytes (3 x 10^5/well) were added to 5ml of medium containing 78% RPMI 1640, 20% FBS, penicillin and streptomycin, and 2% phytohemagglutinin for stimulation in a humidified 5% CO_2 incubator at 37ºC. The negative control group was obtained from an untreated culture of each cell lines and lymphocytes and the positive control from cells treated with 30mM H_2O_2.

The cytotoxicity of rosmarinic acid derived from *Prunella grandiflora* L. and *Prunella vulgaris* L. with doses ranging from 10 to 60ng in T98G, PANC-1, PC-3, HT-29 and MDA-MB 436 cancer cell lines and lymphocytes were assayed using a cell proliferation kit according to our previous study (Tezcan *et al.* 2015).

Results

Determination of rosmarinic acid in fractions

The ethanol extracts of *Prunella* L. species were purified by column chromatography and eluating in two fractions for *P. grandiflora* and *P. vulgaris* (Fig. 1a, b). The methanolic fractions were monitored at 280nm and analyzed by HPLC-DAD.

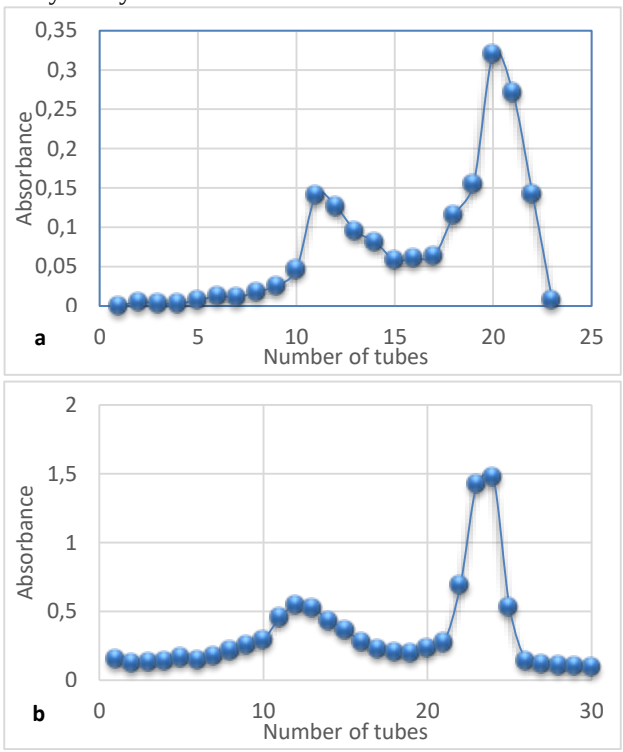

Fig. 1. Eluates following Sephadex LH-20 column chromatography of diluted fraction of **(a)** *P. grandiflora* at 280nm (Fraction 1: 1-15 tube, fraction 2: 16-23 tube), **(b)** *P. vulgaris* at 280nm (Fraction 1: 1-17 tube, fraction 2: 18-30 tube).

Rosmarinic acid was only determined in methanolic fraction 2 of *Prunella* species. There was only one fraction for *P. grandiflora* L. (Fig. 2a) and *P. vulgaris* L. (Fig. 2b) at 280nm in Fraction 2. The results showed that 8.7 and 15.3mg of rosmarinic acid were isolated from *P. grandiflora* and *P. vulgaris*, respectively.

The total phenolic contents of *P. grandiflora* and *P. vulgaris* were found as 24.11 and 24.10mg/g as deduced from dried plants in fraction 2 (Table 1).

Table 1. Total phenolic content of fractions (mg GAE g^{-1} dried plant)

Sample		*P. grandiflora*	*P. vulgaris*
Total phenolic content	Fraction 1	9.03±0.18	8.21±0.01
	Fraction 2	24.11±1.33	24.10±2.72

Cytotoxic effect of rosmarinic acid on cancer cell lines

T98G, HT-29, PANC-1, PC-3, MDA-MB 436 cells and lymphocytes were seeded at a density of $2x10^4$ cells $well^{-1}$ in 96-well plates. Cell proliferation was assessed using the WST-1 assay after 24 and 48h of exposure to rosmarinic acid doses ranging from 10 to 60ng. After treatment with rosmarinic acid of *P. grandiflora*, very low cytotoxic effects were noted in all five cancer cell lines. The percentage decrease was 12.2% in the proliferation of T98G, 17.5% in PANC-1, 11.2% in PC-3, 11.5% in HT-29 and 18.4% in MDA-MB 436 at 60ng rosmarinic acid of *P. grandiflora* extract (Fig. 3). When these cells were treated with H_2O_2, 93.2, 86.9, 89.2, 86.9 and 82.8% reduction in proliferation was observed at 48h, respectively.

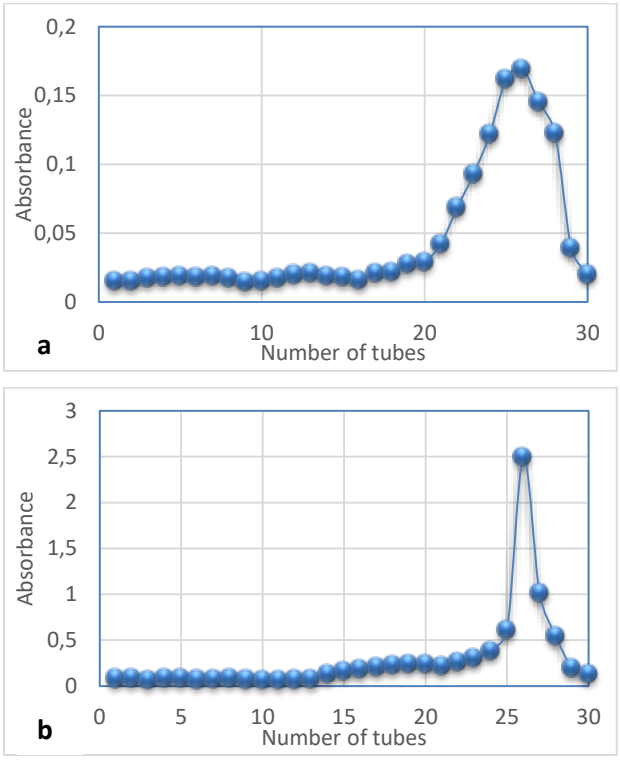

Fig. 2. Eluates following Sephadex LH-20 column chromatography of diluted fraction 2 of **(a)** *P. grandiflora*, **(b)** *P. vulgaris* at 280nm.

After treatment with rosmarinic acid of *P. vulgaris*, dose- and time-dependent reductions were observed in all cell lines (Fig. 4).

The inhibitory concentrations were identified within 48h for all cell lines. After treatment with 60ng rosmarinic acid of *P. vulgaris*, while 27.4% of PANC1 cells and 41.3% of T98G cells were inhibited, the inhibition rate was up to approximately 50% for HT-29 and MDA-MB 436 cells. HT-29, MDA-MB 436 and PC3 are adenocarcinoma cells of colon, breast and prostate which are defined as neoplasia of epithelial tissues. Thus, 60ng rosmarinic acid of *P. vulgaris* caused similar inhibitory effects on these cell lines. However, the pancreatic epithelioid carcinomas and GBM are more aggressive tumor types than adenocarcinomas. So, the inhibiton rate of 60ng rosmarinic acid derived from *P. vulgaris* was lower in PANC1 and GBM cells than the other tumor cell lines. The percentage decreases in the proliferation of cells are shown in Table 2. In addition, negligible cytotoxic effects were determined when the concentrations were tested on activated fresh human mononuclear lymphocytes, indicating that rosmarinic acid of *P. vulgaris* preferentially inhibits tumor cells. When lymphocytes were treated with 60ng rosmarinic acid of *P. vulgaris* for 48h, 5.9% reduction was observed in proliferation, the reduction in proliferation was 73.6%.

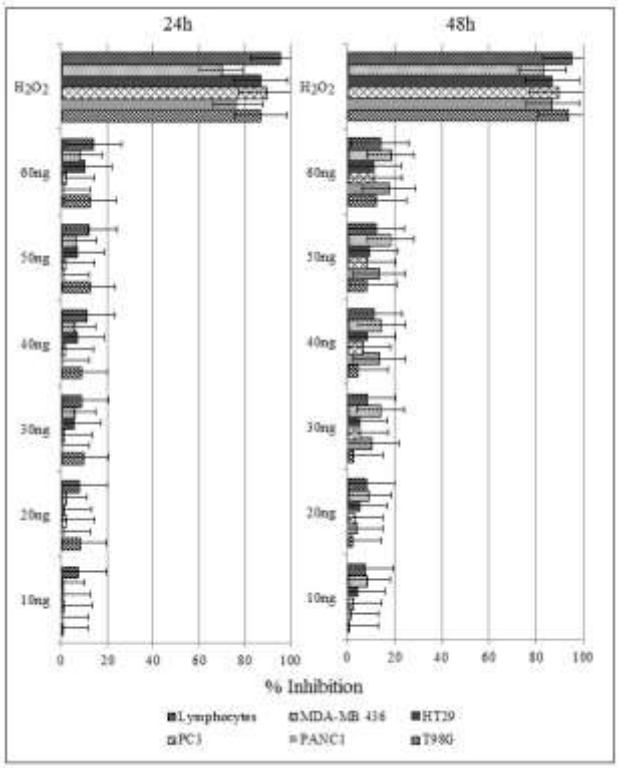

Fig. 3. Inhibition of cell viability at different rosmarinic acid of *P. grandiflora* concentrations in 24 and 48h.

Discussion

Rosmarinic acid is the main phenolic compound in *Prunella* L. species. Therefore, it is also better to demonstrate the total phenolic contents of *Prunella* extracts. It can be seen that most of the rosmarinic acid is isolated in first fraction and other phenolic compounds were eluted in second fraction. HPLC analysis showed that the amount of rosmarinic acid in *P. vulgaris* was higher than the methanol extract of *Nepeta menthoides* Boiss. & Buhse (Hadi *et al.* 2017).

The efficacy of *Prunella* extracts from different regions in China in prevention and treatment of lung

cancer was evaluated (Feng *et al.* 2010). The researchers defined the highest antiproliferative activities in *P. vulgaris* from Bozhou and *P. asiatica* Nakai from Nanjing. Rosmarinic acid content in *P. vulgaris* from Bozhou was 1.32mg/g (Feng *et al.* 2010). While 8.7mg of rosmarinic acid was isolated from *P. grandiflora*, a 15.3mg was extracted from *P. vulgaris*. The efficacy of rosmarinic acid derived from these two species on cancer cell lines was different due to the different collecting localities. Similar results were obtained as Feng *et al.* (2010) for the inhibitory effect of rosmarinic acid derived from *P. vulgaris* on cancer cell proliferation. The variations in the efficacy of *Prunella* species could be related to the existence of differences in genes and rosmarinic acid content.

The inhibitory effect of rosmarinic acid derived from the leaves of *Rosmarinus officinalis* on DU-145 (human, prostate, carcinoma), K-562 (human chronic myeloid leukemia), NCI-H82 (human, small cell lung, carcinoma), Hep-3B (liver, hepatocellular, human, carcinoma, black), MDA-MB-231 (human, breast, adenocarcinoma, breast, human), MCF-7 (adenocarcinoma, breast, human) and PC-3 (human, adenocarcinoma, prostate) was investigated (Yeşil-Çeliktaş *et al.* 2010). Proliferative effect of rosmarinic acid was observed rather than cytotoxic activity in almost all cell lines at 50µg/mL (140µM) in MTT assay. In this study, inhibitor activity of rosmarinic acid derived from *P. vulgaris* was observed in cell proliferation, but the same effect was not observed in rosmarinic acid of *P. grandiflora*. The variation of inhibitory effect of rosmarinic acid could be explained by the plant variation and the contents of other phenolic compounds. Similar results have been observed for quercetin from onions, apples and tea as different phenolic compound sources (Hollman *et al.* 1997). The antiproliferative effect of rosmarinic acid on cancer cells might vary with the variation of plant origin. The cytotoxic effect of rosmarinic acid was determined in Leukemia ARH-77 cell line by MTT assay (Canturk *et al.* 2016). Rosmarinic acid showed cytotoxic effects at 50mM concentration. Also the cytotoxic effect of rosmarinic acid was showed at a concentration of 1000µg/mL on human tumor cells (Júnior *et al.* 2016). According to the results reported so far within similar studies concerning rosmaniric acid, the cytotoxic activities of rosmarinic acid are higher than the values reported in our present study.

Conclusion

Our results showed that *Prunella* fractions can be used as antioxidative and pharmaceutical supplement. Rosmarinic acid derived from *P. vulgaris* could be a good candidate to cause cell death in prostate, pancreas, colorectal, breast cancers and GBM cells. No cytotoxic effect was observed for rosmarinic acid of *P. grandiflora*. On the other hand, 60ng of rosmarinic acid derived from *P. vulgaris* treatment caused antiproliferative effect on prostate cancer cell line (PC3) and 50ng of rosmarinic acid derived from *P. vulgaris* on pancreas (PANC-1), colorectal (HT-29), breast (MDA-MB 436) cancer and GBM (T98G) cell lines. The molecular mechanism of the cytotoxic activity of rosmarinic acid of *P. vulgaris* requires future clinical applications. The present findings could be used as a guidance to formulate a product from these species and also to serve as a reference point for future research.

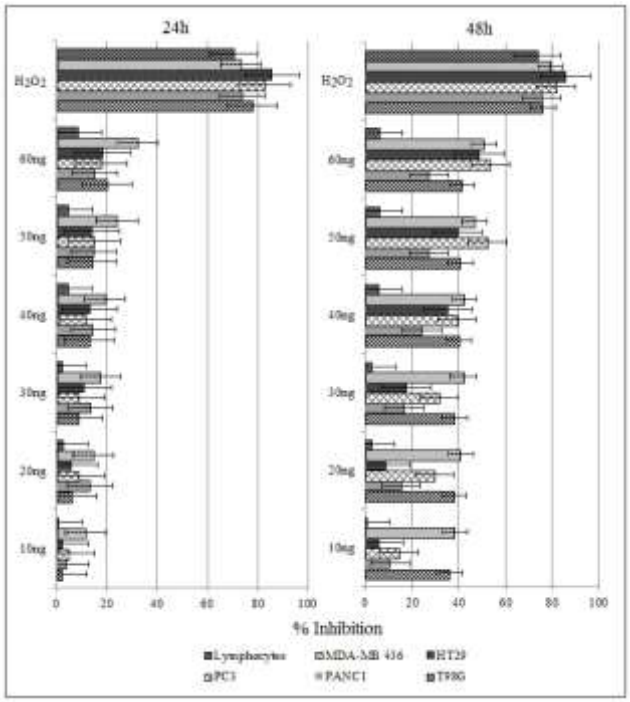

Fig. 4. Inhibition of cell viability at different rosmarinic acid of *P. vulgaris* concentrations in 24 and 48h.

Table 2. The percentage decreases in proliferation of cancer cell lines and lymphocytes after treatment with doses of rosmarinic acid isolated from *P. vulgaris.*

Cell Lines	Cell Type	Doses (ng)	% Inhibition	
			24h	48h
T98G	Glioblastoma multiforme	60	20.1	41.3
PANC1	Pancreas epithelioid carcinoma		15.1	27.4
PC3	Prostate grade IV. adenocarcinoma	50	14.8	52.4
HT-29	Colorectal adenocarcinoma	60	18.3	48.7
MDA-MB 436	Breast adenocarcinoma		32.1	50.8
Lymphocytes	Non-tumor cells		8.2	5.9

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