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Research Article

In vitro EVALUATIONS OF ANTIOXIDANT, ANTIMICROBIAL AND ANTICANCER POTENTIAL OF *Phytolacca americana* L. (POKEWEED) SEED EXTRACT

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Abstract: In this study, different parts of *Phytolacca americana* L. (Pokeweed) fruit from Türkiye were investigated for their antioxidant, antibacterial, antibiofilm and anticancer potentials. The radical scavenging activities, reducing power and total phenolic content were determined to appraise of the antioxidant potentials. The antibacterial and antibiofilm activities of the extracts against *Enterococcus faecalis, Yersinia enterocolitica, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium* were evaluated by using agar-well diffusion, minimum inhibitory concentration (MIC) and biofilm inhibitory concentration (BIC) assays. In addition of latening the onset of apoptosis depending on dose, the potential of the anti-proliferative effects was investigated on MDA-MB-231 and MCF-7 cells. The highest free radical scavenging activity and phenolic content were found in the seed extract. Seed extract showed the highest inhibition zones and significant antibacterial activity at 2.5-5 mg/mL MIC concentrations against tested bacterial strains. More significantly, seed extract was found effective on inhibition of early phase biofilm formation at 2.5-10 mg/mL. BIC concentrations against tested bacterial strains. Next, the main mechanisms of cell death of the seed extract in MDA-MB-231 and MCF-7 cells were investigated. Accordingly, when apoptosis was evaluated morphologically, late apoptosis was observed in cells that showed both Hoechst 33342 and Propidium Iodide (PI) positivity in a dose-dependent manner. This study showed that *P. americana* seed extract can contribute to alternative medicine studies and have potential power in pharmaceutical industry.

Özet: Bu çalışmada, Türkiye'de yetişen *Phytolacca americana* L. (Pokeweed) meyvesinin farklı kısımlarının antioksidan, antibakteriyel, antibiyofilm ve antikanser potansiyelleri araştırılmıştır. Antioksidan potansiyellerini değerlendirmek için radikal süpürücü aktiviteleri, indirgeme aktivitesi ve toplam fenolik içerik belirlendi. Ekstraktın *Enterococcus faecalis, Yersinia enterocolitica, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium*'a karşı antibakteriyel ve antibiyofilm aktiviteleri, agar-kuyu difüzyon, minimum inhibitör konsantrasyon (MIC) ve biyofilm inhibitör konsantrasyon (BIC) testleri yapılarak değerlendirildi. Doza bağlı olarak apoptoz başlangıcını geciktirmenin yanı sıra, MDA-MB-231 ve MCF-7 hücreleri üzerinde anti-proliferatif etkilerin potansiyeli araştırıldı. En yüksek serbest radikal süpürücü aktivite ve fenolik içerik, çekirdek ekstraktında bulundu. Çekirdek ekstraktı en yüksek inhibisyon zonu ve test edilen bakteri suşlarına karşı da 2.5-5 mg/mL MIC konsantrasyonlarında önemli bir antibakteriyel aktivite gösterdi. Daha da önemlisi, çekirdek ekstraktının test edilen bakteri suşlarına karşı 2.5-10 mg/mL BIC konsantrasyonlarında erken faz biyofilm oluşumunun inhibisyonunda etkili olduğu bulunmuştur. Ek olarak, *P. americana* meyvesinin diğer kısımlarına kıyasla çekirdek ekstraktı, 48 ve 72 saat boyunca farklı konsantrasyonlarda hücreler üzerinde anlamlı anti-büyüme etkileri sergilemiştir. Daha sonra, MDA-MB-231 ve MCF-7 hücrelerinde çekirdek ekstraktının hücre ölümünün ana mekanizmaları araştırıldı. Buna göre apoptoz morfolojik olarak değerlendirildiğinde, doza bağlı olarak hem Hoechst 33342 hem de Propidium Iodide (PI) pozitifliği gösteren hücrelerde geç apoptoz gözlendi. Bu çalışma *P. americana* çekirdek ekstraktının alternatif tıp çalışmalarına katkıda bulunabileceğini ve ilaç endüstrisinde potansiyel bir güce sahip olabileceğini göstermiştir.

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Introduction

Medicinal plants have been used in traditional medicine practices since prehistoric times. *Phytolacca americana* L. (Phytolaccaceae, Pokeweed), one of these plants, has a long medical history. *Phytolacca americana*

is a polycarpic perennial herb common to most parts of eastern North and South America, and has been introduced into many regions of the world, especially, as an invasive alien plant in the Black Sea and the Mediterranean regions (Zheleva-Dimitrova 2019). The species can adapt to different climatic conditions and ecological regions, which provides a basis for a rich genetic diversity as a result of the continuous adaptation (Bossdorf *et al.* 2010). The different genotypes and the resulting phenotypes, on the one hand, give the plant the ability to adapt and on the other hand, they provide the plant superiority in conditions such as stress conditions, resistance to diseases and pests, and competition with local plants.

Phytolacca contains valuable biologically active compounds such as red pigments, polyphenolic compounds, amino acids, mineral salts, pectic substances, carbohydrates and aromatic substances (Mchedlishvili *et al.* 2014). Therefore, its extracts display a range of pharmacological activities, including antibacterial, antibiofilm, antiviral, antifungal, anticancer, antioxidant, anti-inflammatory and antiparasitic activities (Bailly & Vergoten 2020, Liberato *et al.* 2021, Solis-Salas *et al.* 2021).

Phytolacca americana is widely used in Central and South America as a traditional medicine for the treatment of many diseases such as inflammation and fever heat, gland swelling, sore throat, syphilis bone pain, diphtheria and pharyngitis *(*Mchedlishvili *et al.* 2014). Pigments obtained by extraction of its fruit have recently been used in cosmetics(Boo *et al.* 2015). The fruit can also be used for the coloration of foods such as preserved fruit and sweets and as ink, clothing dye and body paint (Balogh & Juhasz 2008).

Antioxidant compounds such as phenolic acids, polyphenols and flavonoids found in plant extracts such as *P. americana* scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl, thereby inhibiting the oxidative mechanisms that lead to degenerative diseases (Nunes *et al.* 2012). The recent interest in antioxidants has importantly increased for use in cosmetic, food and pharmaceutical products, because they are important for their multiuse and magnitude of activity (Wannes *et al.* 2010).

Since the emergence of multiple resistant pathogens around the world poses a threat to the clinical effectiveness of many antibiotics available, knowing the antimicrobial properties of *P. americana* is pharmacologically important. There exist few studies on antimicrobial efficacy of *P. americana* and administration. Plant-based antimicrobials have enormous therapeutic potential compared to synthetic antimicrobials with side effects (Boo *et al.* 2015). In addition, biofilm forming increases the resistance of bacterial cells against antimicrobial agents (Meesilp & Mesil 2019) and some phytochemicals are known to be responsible for inhibition of bacterial adhesion and for repression of genes associated with the formation of biofilm where bacteria live within are protected from varieties environmental stresses, such as desiccation, antimicrobials attack by the immune system and ingestion by protozoa hence this architecture makes the biofilm communities to advance as compared to planktonic one (Sarabhai *et al.* 2013, Wilkins *et al.* 2014).

Breast cancer that causes deaths among women globally is an important health problem. Notably, medicinal plant extracts are potential sources for treatment of breast cancer. The effect of Americanin A compound isolated from *P. americana* seeds on human colon cancer cells has been examined and HCT116 has been determined to cause antiproliferative effect *in vivo* and *in vitro* (Jung *et al.* 2015).

Antimicrobial and antioxidant potantials of *P. americana* sampled from different countries have been determined (Nabavi *et al.* 2009, Patra *et al.* 2014, Gins *et al.* 2017, Zheleva-Dimitrova 2019) but there are few studies on its antibiofilm and anticancer potentials (Liberato *et al.* 2021, Guragac Dereli *et al.* 2022) To examine these potentials, interest in *P. americana* studies has increased. This study was carried out to determine the total phenolic contents, antibacterial, antibiofilm and anticancer potentials of seed extracts of *P. americana* plant grown in Türkiye.

Materials and Methods

Materials

Phytolacca americana fruits were collected in Bursa-Mustafakemalpaşa: Uçtaşlar, Türkiye. It was registered to Bursa Uludağ University Faculty of Arts and Sciences Herbarium with the code BULU37913b. *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 700603) and *Yersinia enterocolitica* (ATCC 9610) obtained from Bursa Uludağ University, Faculty of Medicine were used as test microorganisms.

Preparation of the extracts

Phytolacca americana fruits were washed with distilled water and parts of exocarp, pulp, berry juice and seed were separated kindly. Since it is stated that the highest antioxidant activity of plants was found at the methanolic extract (Miliauskas *et al.* 2004, Antasionasti *et al.* 2017), samples were extracted with methanol. 10 g each of fruit parts was extracted with 200 ml 99.9% methanol by using Soxhlet-extractor (Behr Kex 60F) for 24 hours and methanol was removed by using a rotary evaporator (Bibby RE 100) at 50°C until it dried . The crude extracts were stored in a desiccator at +4°C until analyses.

Antioxidant activity and total phenolic contents

The method proposed by Singleton & Rossi (1965) was used to determine the total phenolic content. 250 µL of 50% Folin-Ciocalteu reagent (Sigma) was added onto 250 µL plant extract and kept at room temperature for 30 minutes. After incubation, absorbance at 725 nm was measured against blank by spectrophotometer (Beckman

Coulter DU 730). Blank was prepared with methanol instead of sample. The results were calculated as gallic acid equivalent from the standart curve (range of 20-400 µg/mL).

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was performed with some modifications of the method proposed by Blois (1958). 2 mL of 0.1 mM DPPH was added onto the extract in different concentrations (25-300 µg/mL) and kept in the dark for 30 minutes. After then, the decreasing absorbance at 517 nm was measured. Control was prepared with methanol instead of plant extract. 99.9% methanol was used as a blank. Percent inhibition rate of DPPH free radical was calculated using the formula below.

$$\%\text{inhibition} = \left(\frac{\left(A_{control} - A_{\text{sample}}\right)}{A_{\text{control}}}\right) \times 100$$

Determination of 2,2'-Azino-bis(3-ethylbenzoathiazoline-6-sulfonic acid) ABTS scavenging activity was performed using the method recommended by Re *et al.* (1999). Equal volumes of 7 mM ABTS and 2.45 mM potassium persulfate were mixed and kept for 16 hours in dark at room temperature. After then, the mixture was diluted with 99.9% methanol until the absorbance gives approximately 700 nm. Plant samples of varying concentrations (25-400 µg/mL) were prepared with 99.9% methanol. 0.1 mL of plant samples were mixed with 5 mL of ABTS solution. Control was prepared with methanol instead of plant extract. Methanol was used as a blank. Absorbance at 734 nm was measured and percent inhibition rate of ABTS free radical was calculated using the formula below.

$$\%\text{inhibition} = \left(\frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{control}}}\right) \times 100$$

The method proposed by Oyaizu (1986) was used to determine the reducing power (Oyaizu 1986). 120 µL of various concentrations of methanolic extracts were mixed with 290 µL of phosphate buffer (0.2 M, pH: 6.6) and 290 µL of 1% stock solution potassium ferricyanide and the mixture was kept in dark for 20 minutes at 50°C. The reaction was terminated by adding 10% Trichloroacetic acid (TCA) and the mixture was - centrifuged for 10 minutes at 3000 g. The supernatant was mixed with 1 mL of distilled water and 0.2 mL of 0.1% ferric chloride and the mixture was left to stand for 15 minutes. Blank was prepared with 99.9% methanol instead of plant extract. After then, absorbance at 700 nm was measured.

Screening for antimicrobial activities

Agar well diffusion assay

The antibacterial potential of plant extracts was determined by agar well diffusion assay (Cheesbrough 2006). The test strains were prepared as 1.5×10^8 colony forming units per milliliters (CFU/mL) for inoculation on nutrient agar. Wells (7 mm in diameter) were made on the agar surface under sterile conditions. The seed extracts were prepared in 20% of DMSO and then filtered (0.2 mm). 100 µL of extract (10-80 mg/mL) were filled into the wells. It was allowed to 1 hour for the extracts to diffuse onto the agar surface. Plates were incubated at 37°C for 24 hours for observation of inhibition zones which were measured in millimeters (mm).

Minimal inhibitor concentration (MIC) assay

The growth inhibitory effect of *P. americana* seed extract was determined by MIC assay in MHB II (Muller Hinton Broth II) according to CLSI guidelines (CLSI 2005). Test bacteria were diluted in phosphate-buffered saline to OD546 0.13 and then diluted 1/100 (1×10^6 CFU/mL) in medium before adding to 96 - well plates. Firstly, each well was filled with 100 µL of medium and then 100 µL of seed extracts (20 mg/mL) in MHB II were added to row A. The seed extract was serially diluted, then 100 µL diluted bacterial culture were placed into plates. In addition, 200 µL MHB II as reference wells (negative/sterility control) and 100 µL bacterial suspensions and 20% of DMSO were used as negative control. Plates were incubated at 37°C for overnight. The MIC values were determined by adding 40 µL of 0.002% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to wells to visualize the growth after incubation.

Antibiofilm assay

The effect of 2 × MIC concentrations of seed extract on biofilm formation was evaluated by the microtiter plate (MTP) assay (Nithya & Pandian 2010). Briefly, 100 µL of inoculated media (adjusted to 1×10^6 CFU/mL) were aliquoted to wells of the plate and cultured in 100 µL of 2 × MIC values of extract in 37°C for 24 hours. Wells containing media without extracts were used as controls. After incubation, plates were emptied by removing the supernatant and wells were gently washed with distilled water to discard free-floating bacterial cells. Then the plates were air-dried for 30 minutes and the biofilm forming bacterial cells were stained with 0.1% crystal violet at 15 minutes. After that, crystal violet solutions were removed by washing sterile distilled water and 250 µL of 95% ethanol to wells was added to solubilize the dye bound cells. After incubation about 15 minutes, colorless wells were determined as biofilm inhibitory concentration (BIC) by observing the color change of 0.1% crystal violet in the microplate wells as described above.

Anticancer activities

Cell lines

Breast cancer cell lines, MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (Rockville, Maryland). Cells were cultured with penicillin G (100 U/mL), streptomycin (100 µg/mL), L-glutamine and 10% fetal bovine serum enriched RPMI 1640 media. All cells were stored in humid atmosphere containing 5% CO_2 at 37°C.

Cytotoxicity assay: the sulforhodamine B (SRB) assay

MCF-7 and MDA-MB-231 cells were cultured in appropriate plates at a density of 5×10^3 cells per well. Then, doses of 1.56, 3.125, 6.25, 12.5, 25, 50 and 100

µg/mL were administered from each of the extracts of *P. americana*. Each experiment was repeated twice in triplicate. At the end of the treatment period, the colorimetric SRB test was performed as previously described (Vichai & Kirtikara 2006).

Determination of Apoptosis; Fluorescence Staining

After examining the results of the viability tests, seed extract of *P. americana* which was determined to be the most cytotoxic on MCF-7 and MDA-MB-231 cells, was selected for further analysis. To detect the presence of apoptosis, cells were seeded at 5×10^3 cells per well, followed by the administration of seed extract doses in the range of 1.56-100 µg/mL for 48 and 72 hours. At the end of the treatment period, cells were stained (Akgun *et al.* 2019) and the morphological changes of the cells and, depending on the dose, both Hoechst 33342 and Propidium Iodide (PI) positivity showed the presence of late apoptosis in the cells.

Statistical analysis

The results were evaluated by taking the average of three independent experiments. Data of antioxidant and antimicrobial activity were presented as the mean ± standard deviation. One-way analysis of variance was used to establish differences among different parts of *P. americana*. Data were tested for normality (Kolmogorov-Smirnov' test) and subjected to Levene's test to verify the homogeneity of variances among different parts of fruit. Post-hoc test (Tukey's HSD) was performed when the data revealed significant differences at a level of $P<0.05$. The analyses were performed using the SPSS 17.0 (IBM Corporation) package.

Results

Phenolic contents and antioxidant activity

Approximately 2 g of the extract remaine after the solvent was completely removed was used in different concentrations for different purposes in the experiments. Total phenolic contents of different parts of *P. americana* was expressed as gallic acid equivalent (Fig. 1a). The highest phenolic content was found in the seed extract, followed by the pulp, fruit exocarp and the berry juice extracts, respectively.

Scavenging activity for DPPH and ABTS radicals are presented in Figs 1b and 1c, respectively. The highest scavenging activity for DPPH radical was found in the seed extract. Similarly, scavenging activity of seed extract for ABTS radical was higher than all fruit parts. However, no difference was found among the fruit exocarp, pulp and fruit juice.

The reducing power method determined the electron transfer ability of plant extracts. High absorbance values indicated high reducing power. As with DPPH and ABTS scavenging activity, seed extract possessed a high level reducing power among the studied fruit parts (Fig. 1d).

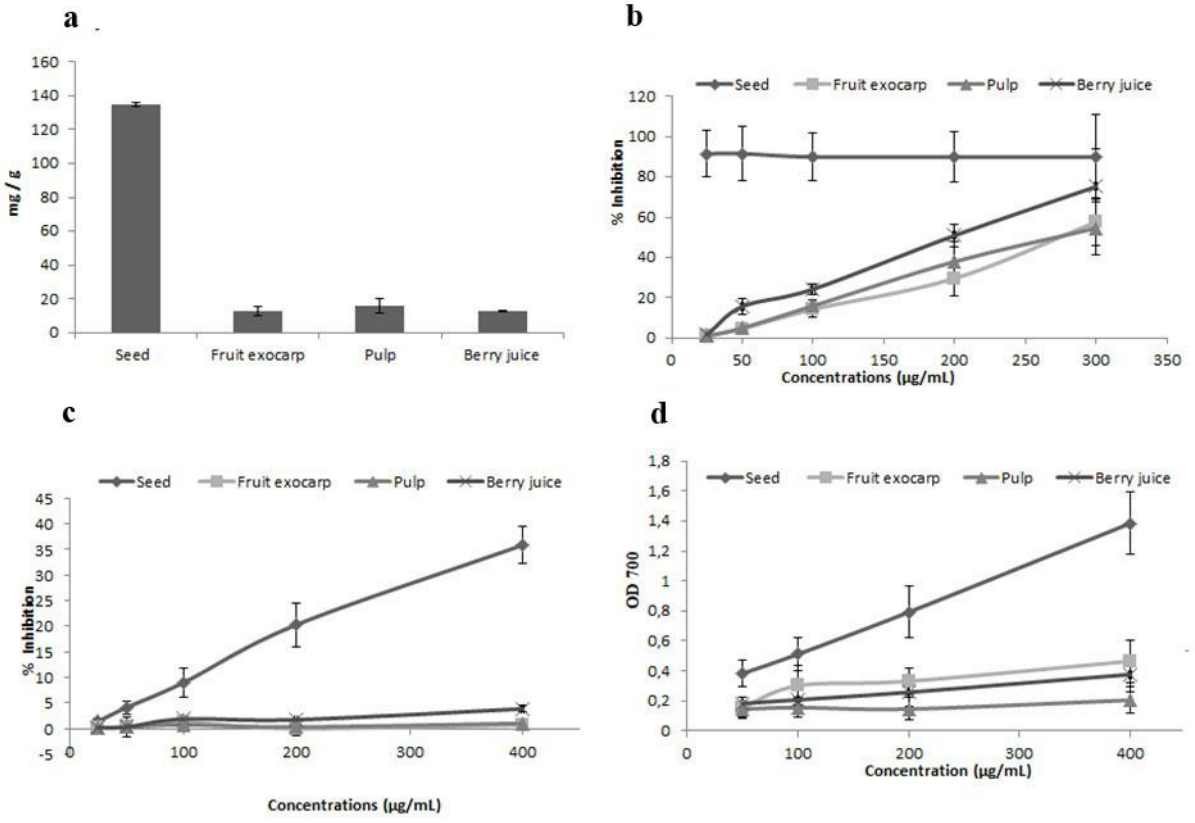

Fig. 1. a. Total phenolic contents, **b.** DPPH scavenging activity, **c.** ABTS scavenging activity, **d.** reducing power of fruit extracts of *P. americana*.

Antibacterial and antibiofilm activity

Antibacterial potential of methanolic extracts different parts of *P. americana* against six test microorganisms were evaluated. The results were analyzed by means of the diameter of inhibition zones expressed as bacterial strains, and the values of the seed, fruit exocarp, pulp were listed in Table 1. For comparison, the antimicrobial activity of the commercial antibiotic bacitracin was assessed against all bacterial strains as positive control. 20% DMSO was added in separate wells and no inhibition zone diameter was observed. Seed extract revealed inhibitions for five bacterial strains, with values ranging from 10 to 80 mg/mL concentrations (Table 1). Both the fruit exocarp and pulp extracts showed zones at highest concentration, but seed extract showed the highest inhibition zones for all concentrations (10 to 80 mg/mL).

The seed extract was selected to determine the MICs against test bacteria considering its strong inhibition zones. The MICs of seed extract were determined at the concentrations between 2.5-5 mg/mL. *E. coli, S. typhimurium* and *Y. enterocolitica* were found more sensitive at 2.5 mg/mL of MICs.

The activity of the seed extract was tested against the biofilms of the tested bacterial strains. The performed biofilm assay showed that seed extract could inhibit biofilm formation at concentrations of 2.5-10 mg/mL (Table 2).

Table 2. MIC and BIC values of seed extract of *P. americana* against the tested pathogen bacterial strains.

Test bacteria	MIC values (mg/mL)	BIC values (mg/mL)
E. faecalis	5	10 - 5
E. coli	2.5	5 - 2.5
S. typhimurium	2.5	5
S. aureus	5	10
K. pneumoniae	5	5 - 2.5
Y. enterocolitica	2.5	5 - 2.5

Anticancer activity

Effects of the *P. americana* on cell proliferation

Only seed extract significantly reduced cell viability as time and dose dependent manner in MCF-7 cells at 48 and 72 hours ($P<0.001$) (Figs 2a, b). However, cell viability was found to be below 50% at only 100 µg/mL dose for 48 hours in MDA-MB-231 cells ($P<0.001$) (Fig. 2c). Similarly, at this dose and 50 µg/ml, the seed extract have inhibitory effect on MDA-MB-231 cells about 50% at 72 hours ($P<0.001$) (Fig. 2d). As a result, particularly high doses of the seed extract of *P. americana* exhibited statistically significant potent anti-growth effects.

Effects of P. americana seed extract on apoptosis in breast cancer cells

SRB assay results showed that the seed extract displayed a potent cytotoxic activity (particularly at 12.5 - 100 µg/mL for 48 and 72 hours) against MCF-7 and (particularly at 50 - 100 µg/mL for 48 and 72 hours) MDA-MB-231 cells. The cell death mode induced by seed extract. Hoechst 33342 and PI positivity showed late stage of apoptosis in cells (Figs 3 and 4, respectively).

MCF-7 staining cells with Hoechst 33342 (blue) and PI (red) after treatment with seed extracts at different concentrations for 48 and 72 hours. Untreated cells were used as control.

Discussion

Phytolacca americana, is a plant with antioxidant, antimutagenic, anti-inflammatory, antimicrobial and anticancer activities based on the active compounds it possesses (Mchedlishvili *et al.* 2014). In this study, methanol extracts obtained of *P. americana* fruit (exocarp, pulp, berry juice) and seed were evaluated in terms of phenolic contents, antibacterial, antibiofilm and anticancer potentials.

The highest levels of phenolic compounds were found in seed extracts. Flavonoids, phenolic acid and tannins are important phenolic compounds that increase the antioxidant potential of plants. There is a correlation between phenolic compounds and antioxidative activity of plants (Karker *et al.* 2016). DPPH is a free radical that easily damages the cell membrane. Therefore, DPPH radical scavenging activity has been determined so far for many herbal extracts (Giovanelli & Buratti 2019). ABTS, which is a chromogenic redox radical, is a stable radical. The seed extract of *P. americana* with the highest phenolic content also showed higher DPPH / ABTS radical scavenging activity and reducing power compared to other parts of the fruit. We therefore conclude that methanolic extracts of *P. americana* seed can be used as a natural resource in medicine, pharmacy and food industry due to its high antioxidant potential and being an easily accessible plant.

The seed extract also showed the highest antibacterial activity. Boo *et al.* (2015) found the best results in their antimicrobial study with the root, stem, leaf and fruit of *P. americana*. Patra *et al.* (2014) reported that soft and stem leaf extracts of *P. americana* stopped the growth of *P. gingivalis* by 100% and *S. mutans* by 44% and very weak against *E. coli*. Results of this study concluded that the seed extract of *P. americana* with MICs (2.5-5 mg/mL) and inhibition zones (16-23 mm) exhibited the highest antibacterial activity among the other parts of fruit. The seed extract was tested against bacterial biofilms for further study and this test appears to be the first study as the antibiofilm activity of *P. americana* seed extract. Bacteria inside biofilms are more resistant to antimicrobial agents than planktonic forms. So, to determine the BIC, higher concentrations than MIC of antibacterial agent is used (Nithya & Pandian 2010).

Table 1. The inhibitory zones of fruit extracts of *P. americana.*

Test bacteria	Diameter of inhibition zone (mm)																		
	Seed (mg/mL)					Fruit exocarp (mg/mL)					Pulp (mg/mL)					Bacitracin (µg/mL)			
	80	60	40	20	10	80	60	40	20	10	80	60	40	20	10	6.25	3.13	1.56	0.78
E. coli	14±0.1	12±0.8	11±1	11±1	11±2	11± 2.4	-	-	-	-	-	-	-	-	-	18±0.2	12	12	15±2
E. faecalis	15±0.3	15	13± 0.7	13	11	11± 2	-	-	-	-	-	-	-	-	-	-	-	-	-
S. typhimurium	16±0.5	15±1	12± 0.3	12	11±2	11± 3	-	-	-	-	11±0.7	10± 3	-	-	-	20±1	19±0.8	18±1	17
S. aureus	15±1	13±0.6	13±1	13	10±1	13± 1	-	-	-	-	-	-	-	-	-	20±0.4	18±0.2	17±0.8	17±0.5
Y. enterocolitica	19±2	18	14±1.4	12±1.2	11±0.4	-	-	-	-	-	-	-	-	-	-	20±1.5	18±1	15±2	12±0.7
K. pneumoniae	23±0.7	20±0.2	12±0.7	12±1	-	10±1	10± 3	-	-	-	11±2	11± 4	-	-	-	12±3	12±2	11±2.5	11±1

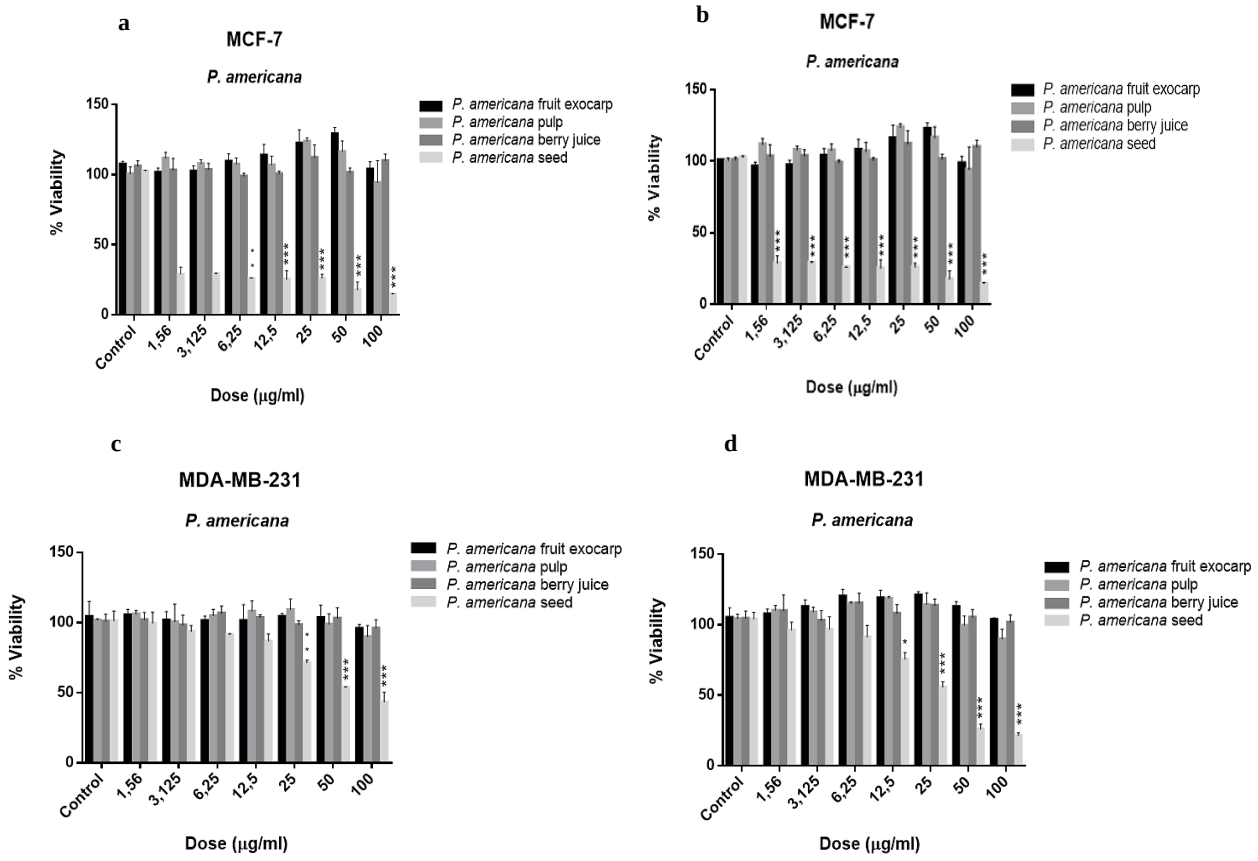

Fig. 2. Viability of MCF-7 (**a** and **b**) and MDA-MB-231 (**c** and **d**) cells after treatment with *P. americana* extracts. Seed extract of *P. americana* (1.56 - 100 µg/mL) decreased cell viability of MCF-7 (a and b) and MDA-MB-231 (c and d) cells in a dose and time-dependent manner after 48 and 72 hours, respectively. *Denotes statistically significant differences in comparison with control: *($p < 0.05$) or [a]($p < 0.05$); **($p < 0.01$) or [b]($p < 0.01$); ***($p < 0.001$) or [c]($p < 0.001$). Data are presented as mean± SD (n=3).

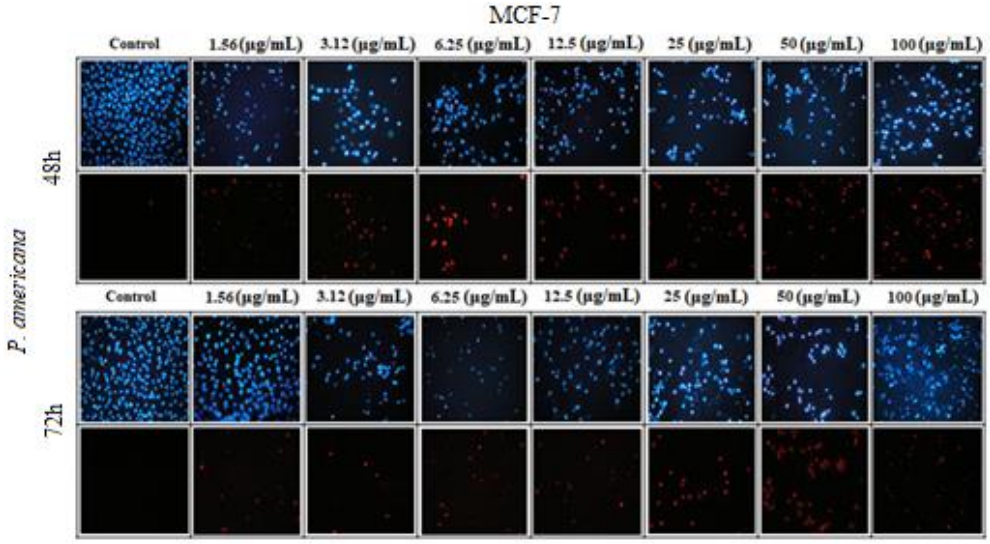

Fig. 3. Fluorescence staining for cell death mode determination on MCF-7 cells.

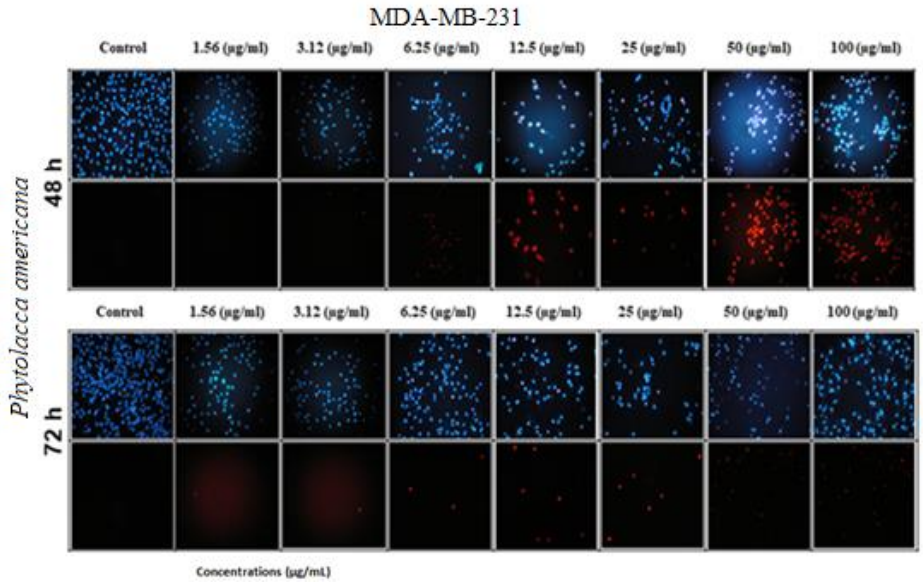

Fig. 4. Fluorescence staining for cell death mode determination on MDA-MB-231 cells. MDA-MB-231 staining cells with Hoechst 33342 (blue) and PI (red) after treatment with seed extracts at different concentrations for 48 and 72 hours. Untreated cells were used as control.

In a previous study, it was stated that plant extracts such as *P. americana* seed extract may have reduced biofilm formation by interrupting the cell-to-cell communication strategies of bacteria (Merghni *et al.* 2018).

Seed extract of *P. americana* was found to exert a growth inhibitory effect over time and in a dose-dependent manner (doses of 12.5, 25, 50 and 100 µg/mL at 48 and 72 hours) on MCF-7 and MDA-MB-231 cells. But, in a previous study, it was determined that none of the extracts from *P. americana* (ethanol, methanol, and water) showed anti-proliferative effect in MCF-7 cells (Maness *et al.* 2012). Also, in HCT-116 human colon cancer cells, ethanol extract was found to have higher antiproliferative activity compared to others. As a result, the ethanol extract and its water fraction showed strong anti-proliferative and apoptotic activities in HCT-116 cells (Maness *et al.* 2012). In another study by the same researchers, changes in gene expression were examined in HCT-116 cells after exposure to *P. americana* ethanol and water extracts. Changes were detected in the expression of the MYC, PLAU and TEK genes in HCT-116 cells exposed to ethanol extract and in the expression of NME4, TEK and THBS1 genes in HCT-116 cells exposed to the water extract. Thus, it was stated that more studies are needed to understand the effect of these changes on colon cancer treatment (Maness *et al.* 2014). On the other hand, in another study it was investigated that the antiproliferative and antitumor activities of Americanin A isolated from *P. americana* seeds in HCT-116 cells (Jung *et al.* 2015). Americanin A inhibited the proliferation of HCT-116 cells both in vitro and in vivo. Also, long-term

treatment with Americanin A induced apoptosis by producing an excess of ROS (Jung *et al.* 2015). Consequently, it was concluded that ATM/ATR and Skp2-p27 modulation may be suitable mechanisms of action for the antiproliferative and antitumor activities of Americanin A in HCT-116 cells (Jung *et al.* 2015).

Conclusion

In present study, phenotypic differences of *P. americana* growing in Türkiye were revealed. The antioxidant, antibacterial, antibiofilm and anticancer activities of fruit extracts were investigated and it was found that the seed extract had potent biological activities. Seed extract with the highest phenolic content also showed the highest free radical scavenging activity and reducing power. The seed extract of *P. americana* showed more antibacterial activity than other parts and exhibited strong anti-growth effects on MCF-7 and MDA-MB-231 cells. It led to late stage of apoptosis in the cells. Therefore, *P. americana* seed extract can be used as a source for further research to identify and characterize the active molecules and to determine its potential for use in the pharmaceutical industry for the health field.

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