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Investigation of Antioxidant, Enzyme Inhibition and Antiproliferative Activities of Blackthorn (*Prunus spinosa* L.) Extracts

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ABSTRACT

Natural products have a key role for drug discovery in pharmacology and medicine. *Prunus spinosa* L. (blackthorn) grown in Çanakkale province in western Turkey, is known as a medicinal plant, a rich source of biologically active compounds such as phenolics, flavonoids and anthocyanidins. The flower and fruit extracts of the plant are subjects of many studies, but they usually lack details of its potential for bio-inhibition studies. Thus, this study aimed to investigate the antioxidant, enzyme inhibition and antiproliferative activity studies of the methanol, ethyl acetate, dichloromethane, and *n*-hexane extracts of the plant. The ethyl acetate and methanol extracts showed better antioxidant activity with DPPH, FRAP, CUPRAC, and TEAC assays. Enzyme inhibition studies of the extracts were performed using *β*-lactamase, proteases and tyrosinase. The methanol (FL) and ethyl acetate (FL and L) extracts at the concentration of 10 mg/mL, displayed better inhibition against *α*-chymotrypsin, trypsin, and papain with values of 22.6%, 34.7% and 92.1%, respectively. Furthermore, the methanol and ethyl acetate extracts have displayed higher cytotoxic effect against cancer cells such as Hep3B and HT29 compared to healthy cells (HUVEC) using MTT assay. The findings suggest that *P. spinosa* L. extracts and their components may be potential for further investigations of novel drug candidates from natural sources.

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Introduction

Medicinal plants that are generally used in the health area, are the most important primary sources of naturally occurring bioactive compounds in the pharmaceutical industry [1]. There is an increasing interest to use these plants to prevent and treat chronic diseases like cardiovascular (CVD) [2,3]. Therefore, searches must continue to find valuable lead compounds from medicinal and aromatic plants [4]. Up to now, it was calculated that

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about 40% drugs in the pharmaceutical markets are derived from plant bioactive components [5]. There are numerous medicinal plants that have not been investigated by the researchers. Among those plants, *Prunus spinosa* L. possesses effective and valuable secondary metabolites [5,6] for which limited information is available. *P. spinosa* L. (Blackthorn or sloe, and also its name is "çakal eriği" in Turkish) which belongs to the Rosaceae family, is growing worldwide [6,7]. The taxonomy of the genus is complicated due to polymorphism and wide ecological tolerance of the species. There has been no taxonomic study of genus *Prunus* in Turkey since Browicz's work, so it is indicated that the studies are not enough to understand its genus and species [8,9]. It is widely used as a source of natural bioactives which have an important role to prevent diseases such as cardiac, hypertensive etc. [3]. It has reported that its leaves and flowers are used for constipation and diuretic and also flowers are used as laxatives in Turkey [7]. According to ethnopharmacological sources, the researchers have been interested in the flowers of this plant due to its medicinal usages such as vasoprotective, diuretic, anti-inflammatory and, spasmolytic activities [5,10,11]. The fruits of the plant have also been used for mild inflammation of oral and pharyngeal mucosa [3,5,6]. Based on the literature reports, highly rich biologically active compounds such as flavonoids, a large group of secondary metabolites, have been identified in *P. spinosa* L. extracts. Especially anthocyanins are present in the fruits and flowers of the plant. The active extracts were demonstrated for its importance on human health [10,12,13]. The proanthocyanidins that are known with their antioxidant activity, anti-inflammatory, and enzyme inhibition, have been investigated in blackthorns [14]. Furthermore, total phenolics and antioxidant activities of the fruit and flower extracts from some *Prunus* species including *P. spinosa* L. have been investigated in several studies by Folin-Ciocalteu (FC) method and DPPH, ABTS and, FRAP assays.

The enzyme inhibitory potentials of the compounds in the extracts can be determined with the therapeutically important enzymes to find the new inhibitors for drug discovery program. For instance, proteases can be given as an example due to their over activity in diseases such as cancer and relationship with neurodegenerative disorders, inflammatory, cardiovascular, and viral diseases [15-17]. The cellular roles of proteases make them prime targets in drug developments. Such important roles have been highlighted in previous works [15-17]. Inhibitors of serine proteases derived from various plants have

been reported for their roles as repressors in tumor cell growth [18-20]. Another example is tyrosinase that possesses a role in melanin synthesis. In hyperpigmentation, hydroxylation from *L*-tyrosine to 3,4-dihydroxyphenylalanine (*L*-DOPA) is catalyzed by tyrosinase and tyrosinase like enzymes [21]. The overproduction of melanin results in discoloration. Therefore, finding effective tyrosinase inhibitors is of prime importance for developing skin whitening agents [22,23]. Recently, the researchers have focused on the determination of new tyrosinase inhibitors from natural sources like plants for medicinal, cosmetic and food industries due to their bioavailability and non-toxic properties on human health [24-27]. Different extracts obtained from leaves of *Prunus* species were investigated for their tyrosinase enzyme inhibition activities and compared to other plant extracts [28]. Anti-tyrosinase activities at low doses of valuable plant bioactive components including coumarins, anthocyanidins, chalcones, flavanols etc. were reviewed in the latest study [29]. Lastly, β-lactam antibiotics including penicillin derivatives are well known antibiotic drugs. β-Lactamases that hydrolyzes β-lactam rings are important enzymes whose inhibitors like clavulanic acid, tazobactam and sulbactam are preferred for the studies of drug discovery and development [30,31]. Therefore, identification of natural inhibitors or synthetic derivatives and their combination with β-lactamase inhibitors have gained an interest to prevent infectious diseases. To determine new bioactive inhibitors from medicinal plants such as leaves of *P. africana*, anti β-lactamase activity of the extracts was investigated in the literature [32]. As stated, plant components could be proposed as an important source for enzyme inhibition studies [21]. Consequently, there are much needed novel enzyme inhibitors from medicinal plants for prevention of those diseases and to be used in the food and cosmetic industry and biotechnological applications. Antitumor potential of *P. spinosa* L. was also investigated and associated with the enriched phenolic compounds in the fruit extracts [33,34]. In addition, water extract obtained from the fruit of *P. spinosa* was investigated for its apoptotic effect against human colon carcinoma cell line (HT29) with dose- and time-response studies [33]. It is considered that *P. spinosa* L. extracts especially isolated from flower gains importance for the treatment of cancer due to these limited, but promising *in vitro* results.

In the light of these explanations, this study aimed to investigate the biologically active extracts such as flowers, fruits and leaves of *P. spinosa* L growing in Turkey (Fig. 1). The

content of total phenolics and antioxidant activities of the extracts were evaluated by using Folin Ciocalteu method and DPPH (1,1-diphenyl-2-picrylhydrazyl), CUPRAC (Cupric Reducing Antioxidant Capacity), FRAP (Ferric Reducing Antioxidant Power) and TEAC (Trolox Equivalent Antioxidant Capacity with ABTS radical cation) assays. Enzyme inhibitory potentials of the extracts were performed against selected enzymes including proteases (trypsin, α-chymotrypsin, and papain), tyrosinase and β −lactamase. Antiproliferative activities of the extracts on Hep3B (human hepatoma cell line), HT29 (human colon carcinoma cell line) and HUVEC (primary human umbilical vein endothelial cells) were succesfully carried out. To our knowledge, this is the first study that is based on the combination of antioxidant, enzyme inhibition and antiproliferative activities of valuable *P. spinosa* L extracts.

Fig 1 Flowers, fruits and leaves of *Prunus spinosa* L.

Materials and methods

Chemicals and reagents

HPLC grade solvents including *n*-hexane, dichloromethane, ethyl acetate, methanol, DMSO were purchased from Sigma-Aldrich and Merck. Biological activity reagents were indicated in our previously reported study; the reference was given in the experimental section. Additionally, human hepatoma carcinoma (Hep3B) and human colon carcinoma (HT-29) cell lines were provided from Cardiff University and Animal Cell Culture Collection (HUKUK, Ankara, Turkey), respectively. HUVEC (Human Umbilical Vein Endothelial Cells) was provided from Bilkent University, Ankara, Turkey). Dulbecco's modified Eagle's medium (DMEM), L-Glutamine, trypsin/ethylenediaminetetraacetic acid (EDTA) solution and, Fetal Calf Serum (FCS) were from Gibco (USA). Silica gel plates (0.25 mm, 60GF 254) were used to perform qualitative analysis with DPPH spraying.

Plant material and preparation of the extracts

Prunus spinosa L. was collected from Panayır place-Ayvacık which is a town and district of Çanakkale in Turkey. The geographic coordinates of Ayvacık are 39° 36' 4" North, 26° 24' 17" East. All parts were identified in the Herbarium Application Research Center and Botanic Garden of Çanakkale Onsekiz Mart University. Taxon's name was recorded as *P. spinosa* L. subsp. *dasyphylla* (Schur) Domain and the altitude was defined as 270 m. It spreads in the bushes and under the forest. It scatters up to 1700 m above sea level. To prepare the plant extract, all parts that were dried without sunlight in the air, were grinded by a blinder and stored at +4 ºC in the refrigerator. The extraction was performed with different solvents including *n*-hexane, dichloromethane, ethyl acetate and methanol, respectively, by Soxhlet extraction. The solvents were removed by using a rotary evaporator under vacuum and stored at +4 ºC for further analysis.

Phenolic content determination

To determine the total phenolics, the most known method was applied to plant samples by using Folin-Ciocalteu (F-C) reagent. The details were reported in our previous studies [35,36]. The concentration of the extracts was prepared as 1 mg/mL. Ferulic acid was used for preparing a standard calibration curve. Total phenolic contents of the extracts were measured at 725 nm by spectrophotometrically using the Perkin Elmer lambda 25 UV spectrophotometer. The data was expressed as mg ferulic acid/100 g extract.

Determination of antioxidant activity

Qualitative analysis

Firstly, plant extracts were analyzed qualitatively using thin layer chromatography (TLC). To detect the active extracts, DPPH solution (4 mg/50 mL in methanol) was prepared. Then, each extract was run on silica gel plates in the mix of proper solvents using *n*-hexane/ethyl acetate and dichloromethane/methanol and sprayed by DPPH solution. The changings of color from purple to yellow were observed at 254 nm following 30 min (Camag UV Cabinet 4). Hereby, active extracts were successfully determined with this assay [36,37].

Quantitative analysis

For antioxidant activity studies, we used DPPH, CUPRAC, FRAP and TEAC assays. DPPH radical scavenging activity was carried out with small modifications by the method of Shoeb *et al.* 2007 as described in our previous work [36,38]. For this purpose, the serial

dilutions (1-0.001 mg/mL) were prepared to be used in the assays. Then, sample solution (500 µL) was treated with DPPH solution (500 µL; 8 mg/100 mL in methanol) for half an hour and the absorbance values were determined at 517 nm spectrophotometrically. IC_{50} values for each solution were determined and then, scanning using a DPPH solution was performed in the range of proper concentrations to identify the most active value. Quercetin was used as a positive standard. CUPRAC assay was implemented according to the literature [39]. Briefly, freshly prepared samples at 0.1 mg/mL concentrations were reacted with copper (II) chloride, alcoholic solution of neocuproine and buffer solution with ammonium acetate for half an hour at the room temperature. The measurement of the absorbances was performed at 450 nm. Quercetin was used as a standard. The data was determined as quercetin equivalent flavonoid concentration. FRAP assay was performed with small modifications [40]. In brief, freshly prepared working solution using TPTZ, ferric chloride, acetate buffer was reacted with 500 µL sample (0.1 mg/mL) and stood at 37 ^{o}C for 10 min and then, the absorbance values were determined at 593 nm. To prepare the standard curve, $FeSO_4.7H_2O$ was used. FRAP values were identified as mmol Fe^{+2}/g of sample. Finally, TEAC assay was applied to the prepared solutions of the extracts according to the literature [41]. In this assay, the experiment was performed using ABTS radical cation. The absorbances for different volumes (50, 75 and 100 µL) of the solutions (1 mg/mL) were determined at 734 nm to calculate the percentage of inhibitions.

Determination of enzyme inhibitory potentials of the extracts

To evaluate the enzyme inhibitory potentials with various enzymes containing proteases (α-chymotrypsin, trypsin and, papain), tyrosinase and *β*-lactamase (penicillinase), the experiment was performed by the method of Rahman *et al.* 2001 [42]. Detailed procedure was also given in our reported study [43]. Stock (10 mg/mL) and diluted (1 mg/mL) solutions for all the parts of the plant were used in the assays. The experiment was conducted in a 96-well plate and total volume was determined as 200 µL. The absorbances were measured by using Molecular Devices Spectramax Plus 384 ELISA microplate reader. Control reactions using PMSF as a positive control were run without inhibitors.

Protease inhibition assay was applied to the extracts using *α*-chymotrypsin, trypsin and, papain with their specific chromogenic substrates [43]. The optimized volumes for *α*-

chymotrypsin, trypsin and papain were determined as enzyme (50 µL), Tris-HCl buffer (pH 7.5) (40 µL), sample (10 µL and 30 µL for papain) and substrate (100 µL). The absorbances of the mixtures incubated at 37 ºC were measured at 410 nm. Tyrosinase inhibition assay was applied like protease inhibition assay. Briefly, the experiment was carried out using a dopachrome method in which L-DOPA was used as a substrate. The reaction was held at 25 ºC for 10 min and activity of tyrosinase was measured spectrophotometrically at 475 nm. *β*-Lactamase inhibition assay that was similar to protease assay, was performed by using penicillinase and nitrocefin. The conditions for 1 mg/mL solutions of the plant extracts were optimized as enzyme (10 µL), Tris-HCl buffer (pH 7.5) (160 µL), sample (10 µL) and substrate (20 µL) for this assay. The volume of the sample (10 mg/mL) was increased from 10 µL to 50 µL. After 10 min incubation at 30 ºC, the absorbance was determined at 495 nm.

Cell viability

To investigate the antiproliferative activities of the extracts, the most-known method (MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)) was applied. Firstly, Hep3B, HT29 and, HUVEC cells were used (50.000 cells in each 96-well) and, cultured overnight at 37 ºC in a humidified incubator (5% (v/v) CO_2 in air). Then, the cells were treated with different concentrations (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL in DMSO) of the extracts for 24 h and 48 h. According to our study, the method was carried out [43].

Results

Phenolic contents in the extracts

The total phenolics of *P. spinosa* L. for all extracts was calculated for the first time according to Velioğlu's method as equivalent to ferulic acid [35]. The higher amounts of phenolics were found in the extracts of methanol (FR) (19.00 ± 0.01 mg FAE/100 g extract), ethyl acetate (FL) (15.00 ± 0.01 mg FAE/100 g extract), and methanol (FL) (12.70 ± 0.12 mg FAE/100 g extract). On the other hand, dichloromethane extracts obtained from flowers and leaves of the plant possessed the lower phenolics (Table 2). In this study, total phenolic content possessed the highest value following Soxhlet extraction with methanol due to the efficiency of the solvent polarity. Table 1 shows that the total phenolics in *n*-hexane extracts (FL, FR and L) were significantly higher than

dichloromethane extracts that were found between 0.40 ± 0.03 mg FAE/g and 2.50 ± 0.08 mg FAE/g.

Antioxidant activity

Qualitative analysis

Before investigation of the extracts in biological studies, the method is based on the reduction of 2,2-diphenyl-1-picrylhydrazyl free radical, the active extracts including strong antioxidants give the yellow spots on the TLC plates, was successfully applied to the extract solutions obtained from *P. spinosa* L. and changings of the colors were observed following the spray with DPPH solution. According to our observations, the spots on TLC plates for active extracts such as ethyl acetate and methanol extracts of flower, leaves and fruit and *n*-hexane extract (L) of the plant changed from purple to white and yellow.

Quantitative analysis

Determining the radical scavenging activities of the *P. spinosa* L. extracts, well-known SET based methods such as DPPH, ABTS, FRAP and CUPRAC were used. The alterations of the solvent polarity, extraction method and the type of assay can affect the results of antioxidant activities (Table 1). Following the qualitative analysis of plant extracts using DPPH, inhibitory concentrations (IC_{50}) of the different concentrations prepared from extracts were determined as mg/mL by the measurement of absorbances at 517 nm. According to the results of DPPH assay, the highest antioxidant capacity was provided by ethyl acetate extracts (FR = 327 ± 0.13 µg/mL, FL = 387 ± 0.08 µg/mL, L = 463 ± 0.08 µg/mL) and methanol extracts (FL = 476 ± 0.06 µg/mL, FR = 480 ± 0.05 µg/mL), except methanol extract (L = 830 ± 0.02 µg/mL) that was determined with its highly phenolic content. Then, it was followed by dichloromethane and *n*-hexane extracts. In CUPRAC assay, the highest activity as quercetin equivalent flavonoid concentration was observed in ethyl acetate (FR) (0.36 ± 0.01) and *n*-hexane (FL) (0.34 ± 0.05) extracts, respectively. However, increasing activity between CUPRAC values of methanol flower and fruit extracts have not been determined. As given in the results of FRAP assay, methanol (L) (666.30 ± 0.01 mM Fe^{+2}) and dichloromethane (FR) (651.00 ± 0.06 mM Fe^{+2}) extracts displayed better scavenging activity. According to ABTS assay, the similar results have been determined in methanol (FR) and ethyl acetate extracts (FR). The values were between 151.03 ± 7.90 and 136.87 ± 9.71 mM Tr/g. The highest percentage of

inhibition values for sample volume (100 µL) were obtained with methanol and ethyl acetate extracts, 48.94% and 54.16%, respectively. In conclusion, methanol and ethyl acetate extracts showed higher antioxidant activity as a dose-dependent manner, compared to other extracts. The ability of scavenging free radicals and reduction of metal ions of active extracts has been explained clearly by the synergic effects of the components. These results were shown in Table 1. It should be noted that antioxidant assay may not generally correlate with each other.

Table 1 Total phenolic content and antioxidant activities of *P. spinosa* L. extracts

Extracts	TPC (mg FAE/100 g extract)	DPPH (IC_{50} = µg/mL)	CUPRAC (QERFC)	FRAP (mM Fe^{+2}/g extract)	ABTS/TEAC (mM TR/g extract)
M-FL	12.70 ± 0.12	476 ± 0.06	0.16 ± 0.01	518.20 ± 0.01	142.19 ± 2.39
M-FR	19.00 ± 0.01	480 ± 0.05	0.18 ± 0.02	273.80 ± 0.01	149.46 ± 15.11
M-L	9.00 ± 0.01	830 ± 0.02	0.30 ± 0.01	666.30 ± 0.01	136.87 ± 9.71
EAA-FL	15.00 ± 0.01	387 ± 0.08	0.25 ± 0.02	423.30 ± 0.01	144.57 ± 9.66
EAA-FR	1.25 ± 0.04	327 ± 0.13	0.36 ± 0.01	570.90 ± 0.03	151.03 ± 7.90
EAA-L	12.50 ± 0.07	463 ± 0.08	0.27 ± 0.02	378.50 ± 0.02	144.62 ± 5.16
DCM-FL	0.40 ± 0.02	580 ± 0.01	---	---	142.17 ± 16.81
DCM-FR	2.50 ± 0.08	670 ± 0.04	0.09 ± 0.01	651.00 ± 0.06	138.59 ± 11.45
DCM-L	0.40 ± 0.03	800 ± 0.02	0.17 ± 0.02	320.00 ± 0.01	45.64 ± 10.30
H-FL	0.80 ± 0.00	850 ± 0.01	0.34 ± 0.05	235.50 ± 0.01	73.33 ± 4.89
H-FR	0.80 ± 0.01	1000 ± 0.02	---	---	15.52 ± 3.64
H-L	3.80 ± 0.01	970 ± 0.03	---	---	22.36 ± 3.99

M-FL: Methanol Flower; M-FR: Methanol Fruit; M-L: Methanol Leave; EAA-FL: Ethyl Acetate Flower; EAA-FR: Ethyl Acetate Fruit; EAA-L: Ethyl Acetate Leave; DCM-FL: Dichloromethane Flower; DCM-FR: Dichloromethane Fruit; DCM-L: Dichloromethane Leave; H-FL: n-Hexane Flower; H-FR: n-Hexane Fruit; H-L: n-Hexane Leave. Total Phenolic Content (TPC). Data is expressed as mg of ferulic acid equivalent (FAE)/100 g of extract. [b]Data is expressed as the value of percentage of DPPH inhibition (%). [c]Data is expressed as the IC50 value of DPPH (µg/mL) [d]CUPRAC (QERFC) (Quercetin equivalent of flavonoid concentration). [e]Data is expressed as mM Fe^{+2}/g extract. [f]Data is expressed as mM of Trolox equivalent per gram of sample. The results are expressed as mean ± standard error

Enzyme inhibition studies

In this study, proteases, tyrosinase and *β*-lactamase enzymes inhibitory potentials of the extracts of *P. spinosa* L. were determined via using different reaction temperatures (25 ºC and 37 ºC) and different wavelengths in a microplate reader. Among the reactions, maximal velocity (Vmax) values as milliunits per min for each of extracts were

determined and inhibition values (%) were calculated by using these values. According to protease enzyme inhibition results as given in Table 3, methanol extract (L) demonstrated that the highest inhibition (20.77%) against *α*-chymotrypsin when compared to methanol extract (FL) (0.39%) at 1 mg/mL solutions on the contrary of 10 mg/mL dose of the same extracts. In the reaction rate graph, Vmax values obtained with trypsin enzyme were determined as 74.22 (inhibition of 9.50%) and 183.30 (inhibition of 34.70%) for 1 and 10 mg/mL doses of flower methanol and ethyl acetate extracts, respectively. This correlation was similar to the inhibition of papain enzyme with methanol (FR) and ethyl acetate (FL) extracts in papain enzyme inhibition study.

Table 2 Proteases enzyme inhibition of *P. spinosa* L. extracts

Proteases												
Extracts	**α-Chymotrypsin**				**Trypsin**				**Papain**			
	1 mg/mL		10 mg/mL		1 mg/mL		10 mg/mL		1 mg/mL		10 mg/mL	
	Vmax	% Inh	Vmax	% Inh	Vmax	% Inh	Vmax	% Inh	Vmax	% Inh	Vmax	% Inh
M-FL	38.06	0.39	43.61	22.67	74.22	9.50	359.00	-	32.92	5.15	13.08	77.34
M-FR	35.32	5.34	69.54	14.25	80.64	1.68	285.00	-	21.07	39.29	34.95	39.45
M-L	30.40	20.77	71.55	11.78	76.62	6.58	289.00	-	27.01	22.18	19.29	66.58
EAA-FL	39.32	-	21.66	20.36	77.45	5.57	183.30	34.70	28.29	18.49	-	-
EAA-FR	36.60	4.61	21.15	22.24	94.08	-	220.70	21.34	32.52	6.30	20.67	64.19
EAA-L	38.88	-	21.60	20.58	89.00	-	221.20	21.20	27.55	20.62	4.56	92.10
CT		-		-		-		-	34.82	-	57.74	-

M-FL: Methanol Flower; M-FR: Methanol Fruit; M-L: Methanol Leave; EAA-FL: Ethyl Acetate Flower; EAA-FR: Ethyl Acetate Fruit; EAA-L: Ethyl Acetate Leave; CT-Control; Inh: Inhibition; - (dashed line) indicates no inhibition. Asterisk (*) indicates the volume of sample as 10 µL in the reaction. Control includes (enzyme + substrate + DMSO + buffer solution). Extract includes (enzyme + sample solution + substrate + buffer solution)

In tyrosinase enzyme assay, the major inhibition was determined with methanol (FR and L) extracts for 1 and 10 mg/mL doses, respectively. Although some of the extracts obtained higher Vmax values at 1 mg/mL, there was no inhibition against penicillinase enzyme (*β*-lactamase), the inhibition by all the extracts was provided with changing values from 7.79% to 12.85%. When *n*-hexane extracts (FL, FR and L) were applied to the reaction media containing enzyme and its chromogenic substrate, *n*-hexane extract (FL) showed inhibitory activity against proteases, tyrosinase and penicillinase enzymes at 1 and 10 mg/mL doses (Table 2,3). According to the literature, enzyme inhibitory abilities of blackthorns can be associated with proanthocyanidins [31].

Table 3 Tyrosinase and penicillinase enzyme inhibition of *P. spinosa* L. extracts

Extracts	Tyrosinase				*β*-Lactamase (Penicillinase)			
	1 mg/mL		10 mg/mL		1 mg/mL		10 mg/mL	
	Vmax	% Inh.	Vmax	% Inh.	Vmax	% Inh.	Vmax	% Inh.
M-FL	73.01	-	44.15	16.14	68.63	3.66	68.59	8.35
M-FR	57.87	13.53	43.36	17.64	80.99	-	67.68	9.56
M-L	61.33	8.36	33.02	37.28	76.19	-	67.86	9.32
EAA-FL	61.42	8.23	36.31	31.03	69.15	2.93	65.22	12.85
EAA-FR	61.55	8.03	39.38	25.20	73.68	-	66.82	10.71
EAA-L	64.67	3.38	36.82	30.06	72.11	-	69.01	7.79
H-FL	62.21	7.05	56.86	-	72.60	-	44.38*	15.20*
CT	66.94	-	65.00	-	71.25	-	71.25	-

M-FL: Methanol Flower; M-FR: Methanol Fruit; M-L: Methanol Leave; EAA-FL: Ethyl Acetate Flower; EAA-FR: Ethyl Acetate Fruit; EAA-L: Ethyl Acetate Leave; Hexane Flower; CT-Control; Inh: Inhibition; - (dashed line) indicates no inhibition. Asterisk (*) indicates the volume of sample as 50 µL in the reaction. Control includes (enzyme + substrate + DMSO + buffer solution). Extract includes (enzyme + sample solution + substrate + buffer solution)

Antiproliferative activity of *P. spinosa* L. extracts

To investigate the antiproliferative activities of *P. spinosa* L. extracts on Hep3B and HT29 cancer cells and HUVEC cells at various concentrations for the treatment of 24 h and 48 h. For this reason, we evaluated *in vitro* cell growth of the cancer cells at increasing doses of each extracts. When the extracts were applied to the cells at five different concentrations that were changing from 62.5 to 1000 µg/mL, the cell growth was inhibited at proper dose of the extracts. For instance, although the cytotoxicity of all methanol extracts on HT29 cancer cells was observed for all doses at two different time points, the components of methanol (FL and L) extracts inhibited the cell growth as a dose-dependent (between 250 and 1000 µg/mL) for 24 h compared to DMSO as a positive control. However, the effect of methanol (FR) extract on HT29 cancer cells significantly reduced the cell viability at 1000 µg/mL for 24 h and 48 h treatments. Also, the percentage of cell viability of methanol extracts (FR) was found as approximately 60% and 50% on HT29 cancer cells at 62.5 and 1000 µg/mL doses in time-response manner study, respectively. Whereas methanol extracts displayed different effects on Hep3B cells. The

antiproliferative effect was observed at an active time points, especially for 48 h. The methanol extract of fruit decreased the cell viability on Hep3B cells nearly 65% at 1000 µg/mL dose for 48 h. Most strikingly, methanol (L) extract showed dramatically inhibition on cell viability of Hep3B cells for all doses for 48 h and the percentage of cell viability was determined as 55% at 1000 µg/mL and 500 µg/mL doses. The methanol extracts did not show any proliferative effect on healthy HUVEC cells. The inhibition profiles of methanol and ethyl acetate extracts on HT29, Hep3B and HUVEC cells were given in Fig 2-4.

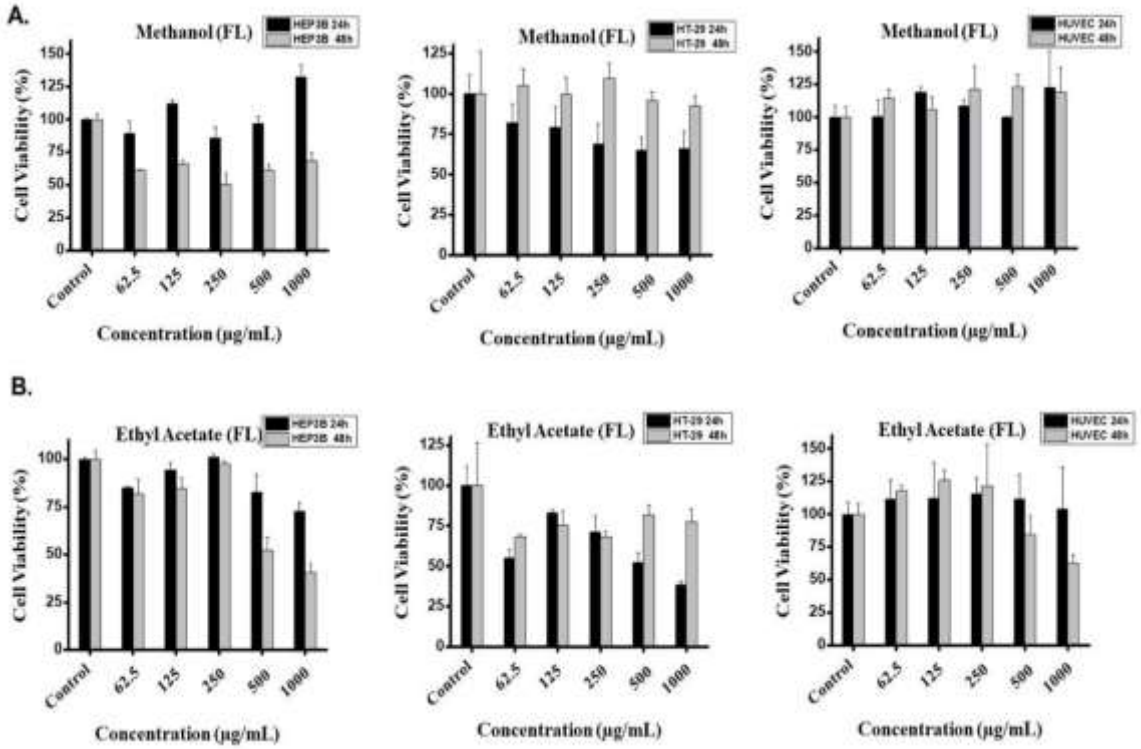

Fig 2 A. Antiproliferative effect of methanol extract (FL) on Hep3B and HT29 cancer cells and HUVEC healthy cells. **B.** Antiproliferative effect of ethyl acetate extract (FL) on Hep3B and HT29 cancer cells and HUVEC healthy cells

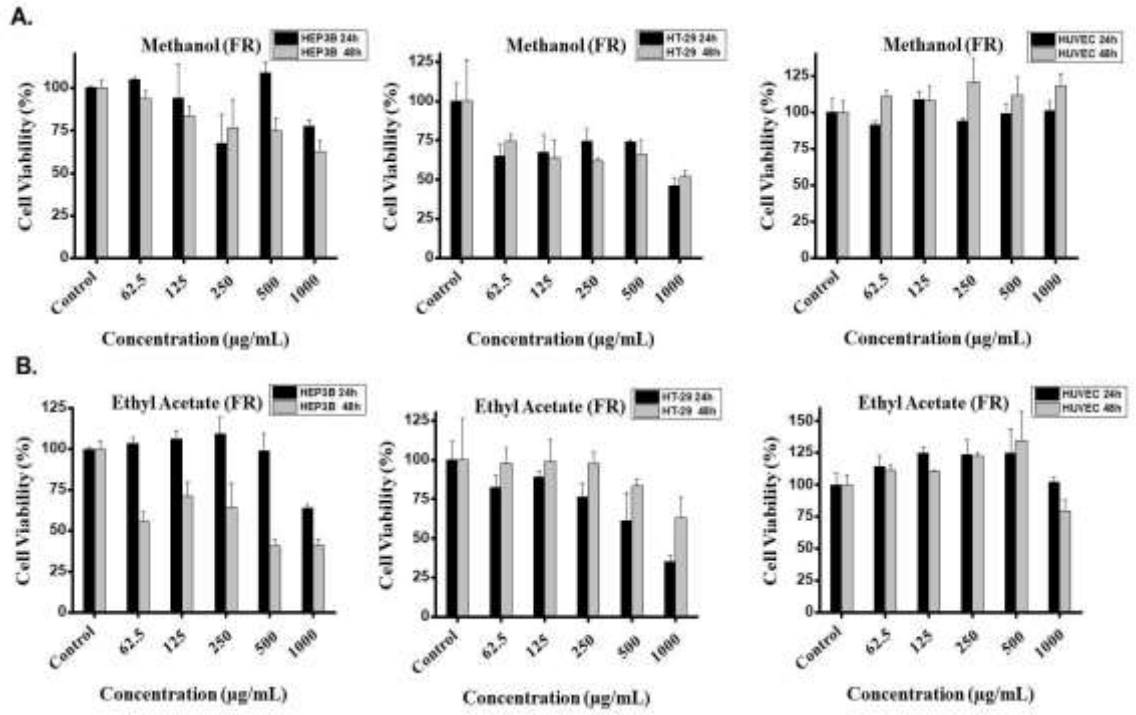

Fig 3 A. Antiproliferative effect of methanol extract (FR) on Hep3B and HT29 cancer cells and HUVEC healthy cells. **B.** Antiproliferative effect of ethyl acetate extract (FR) on Hep3B and HT29 cancer cells and HUVEC healthy cells

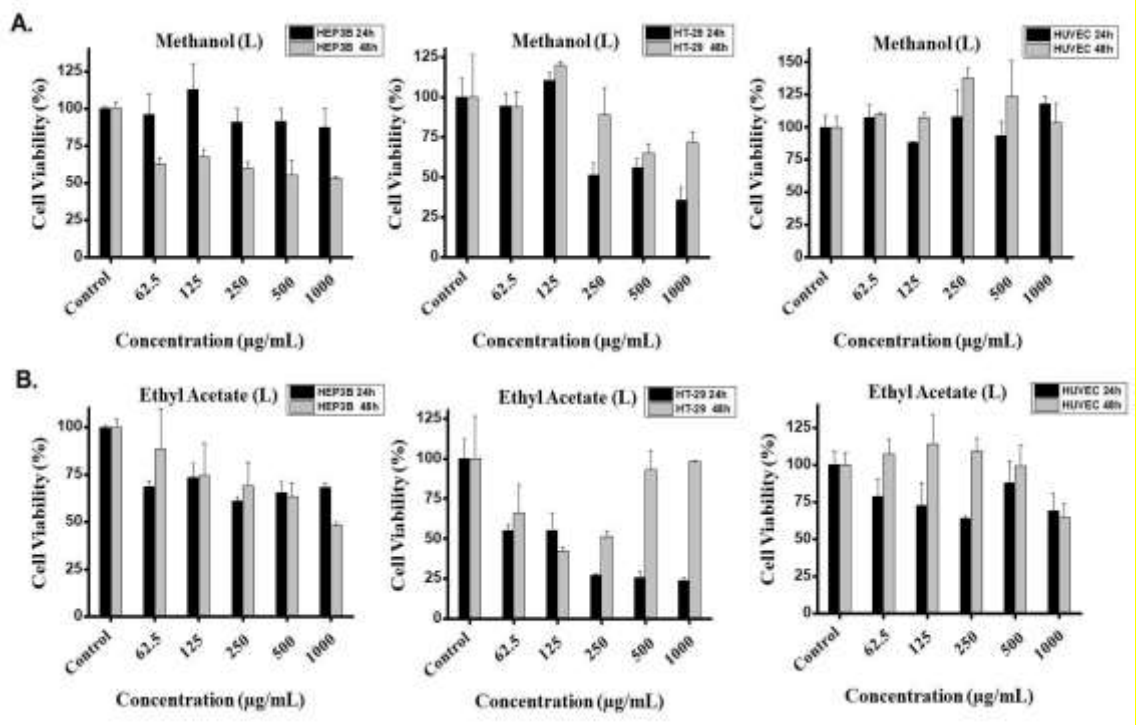

Fig 4 A. Antiproliferative effect of methanol extract (L) on Hep3B and HT29 cancer cells and HUVEC healthy cells. **B.** Antiproliferative effect of ethyl acetate extract (L) on Hep3B and HT29 cancer cells and HUVEC healthy cells

In the cytotoxicity results of ethyl acetate extracts for all parts of *P. spinosa* L., there was a major inhibition on cell viability of cancer cells. Although the ethyl acetate extract (L) displayed linearly inhibition on HT29 cells from changing doses 62.5 to 1000 µg/mL for 24 h, the inhibition correlation was observed at 250, 500 and 1000 µg/mL doses on Hep3B cells for 48 h. The ethyl acetate extract (FR) at 1000 µg/mL verified the cytotoxicity on HT29 cells for 24 h and 48 h. Furthermore, the ethyl acetate extract (FL) showed the antiproliferative effect on HT29 and Hep3B cells at 500 and 1000 µg/mL doses in time response manner study with MTT assay (Fig. 2). When HT29 and Hep3B cells were exposed to dichloromethane extract (FR) of *P. spinosa* L., significant inhibition was determined at 250 µg/mL (approximately 50%) for 24 h (Fig. 5).

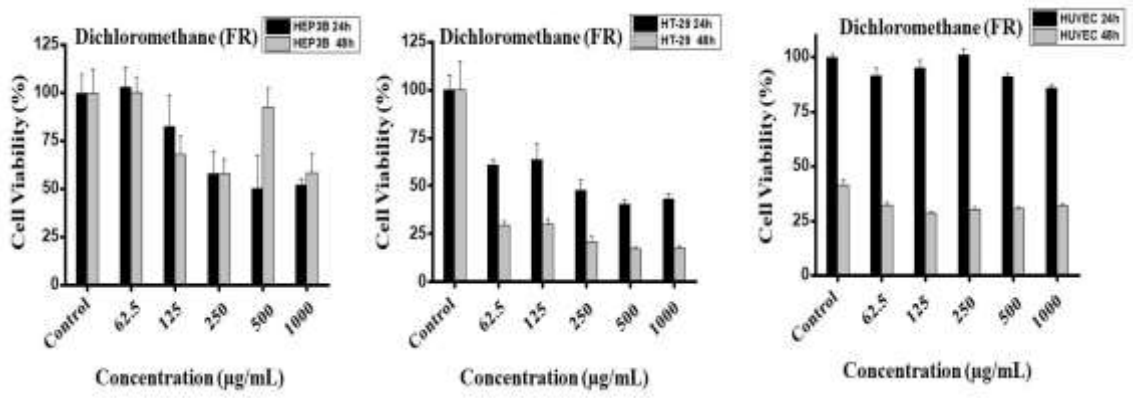

Fig 5. Antiproliferative effect of dichloromethane extract (FR) on Hep3B and HT29 cancer cells and HUVEC healthy cells. The decrease of cell viability on HT29 cancer cells was observed at both 24 h and 48 h treatments between 62.5 and 1000 µg/mL doses. Whereas dichloromethane extract displayed toxicity on healthy cells for 48 h. The cell viability on HUVEC cells at related doses was remarkably decreased. These results may contribute to short time treatment like 24 h

Additionally, although *n*-hexane extracts (FL and L) reduced the cell viability of both cancer cells at different doses, we observed the toxic effect on healthy cells. The methanol and ethyl acetate extracts have a greater cytotoxic potential on studied cancer cells than dichloromethane extracts (FL and L) and *n*-hexane extracts. Consequently, the decreasing of cell viability on cancer cells were observed with dose- and time-dependent studies.

Discussion

Plant phenolics are known with their benefits to human health. They play an important role for antioxidant activity. F-C reagent is used for determining the amount of phenolics [44]. According to the literature findings, total phenol contents of *P. spinosa* L. extracts

(FR and FL) were investigated by several researchers and the results were generally expressed as equivalent to gallic acid [34,45-47]. In the literature, the total amounts of phenolics in the extracts of *P. spinosa* L. have been indicated in alternative equivalents. For instance, in one of the studies, the researchers reported the content of phenolics of methanol extract prepared from fruits of the plant as equivalent to mg gallic acid (2548 ± 18 mg GA/100 g extract) [47]. In the another study, the extracts including dichloromethane, ethyl acetate, ethanol and water obtained from branches, leaves and fruits of *P. spinosa* L. have been studied for determining antioxidant capacities and the amount of total phenolics. The findings showed that the ethanol and ethyl acetate extracts of branches (732.24 ± 6.41 mg GA/g and 499.23 ± 1.99 mg GA/g) and ethanol extract of fruit (359.11 ± 2.54 mg GA/g) had rich phenolic components. Some of the researchers reported that the total phenolic amount of *P. spinosa* L. fruit samples obtained from different solvents such as acetone, ethanol and water determined as changing values from 26.78 ± 4.44 to 19.98 ± 1.28 GAE/g dry weight [34]. Although the results were calculated at different equivalents in this study, the phenolic content of methanol extract (FR) was determined as 19.00 ± 0.01 FAE/100 g that was nearly similar to literature found expressed as GAE [34]. However, synergism in the components of the extract may affect results in the increasing values. Based on the obtained data, active extracts were investigated with *in vitro* enzyme inhibition and cytotoxicity studies on cancer cells to continue analyzing phytochemical behavior and anticancer potentials. To identify qualitative analysis of antioxidant activity in the extracts, TLC that is a simple, easy, and known method was carried out [36,37]. Indeed, the activity depends on the solvent extraction changing polarity of the solvents and it is related with the studied parts of the plant. When the different types of solvents were used, increasing activity was observed on the results in *in vitro* applications. In conclusion, as it is understood from the qualitative analysis, the methanol and ethyl acetate extracts of *P. spinosa* L. might contribute to the major radical scavenging capacities with DPPH. In the previously reported study, it was indicated that the extracts obtained from *P. spinosa* L. showed changing antioxidant activity from low to moderate in the qualitative analysis with DPPH [48]. Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) have a role in deactivation of radicals by antioxidants. To obtain more comparison results, the antioxidant capacity was measured by using SET based methods in this study [49,50]. In

one of the studies, the ethyl acetate extract (FL) of *P. spinosa* L. was identified with its highly antioxidant activity in DPPH and FRAP assays compared to methanol extract [3]. Whereas the ethanol extract (FR) of *P. spinosa* L. possessed the good antioxidant activity in DPPH (257.84 ± 6.57 μg/mL), FRAP (0.10 ± 0.01 Fe^{+2}) and ABTS (184.43 ± 3.88 μg/mL) assays than water and acetone extracts in a reported study [34]. The phenolic compounds and DPPH scavenging activity correlation have been confirmed in one of the reported studies [45]. Additionally, although blackthorn fruits have not been preferred to be used as food products due to their properties such as acidity and bitter taste, rich components having antioxidant properties have been determined in the studies [51]. These results may be led for further studies of *P. spinosa* L. extracts and/or its constituents.

Several studies have been performed to identify the naturally occurring active compounds from the plants and to be used for biological applications. For instance, coumarin based two compounds isolated from the plants have been reported for its tyrosinase inhibitory activity [52,53]. Therefore, showing of *in vitro* enzyme inhibition studies performed with plant extracts may lead to the new concepts for the discovery of plant-based therapeutics. In the literature, there are several studies performed with the medicinal plants to inhibit these selected enzymes [29]. One of the studies suggested that the highest tyrosinase inhibitory activity had been provided by white tea extract compared to green and black tea extracts [21]. Most of the plant protease inhibitors have been determined in preclinical studies. For instance, *Solanum tuberosum* L. inhibited proteases such as trypsin, α-chymotrypsin, and papain at 0-20 mg/mL dose. In addition, *Vigna unguicalata* L. had been determined as a potent inhibitor for trypsin at 2 μg/mL [17]. Valuable proteases inhibitors isolated from plants like *Bauhinia rufa* trypsin inhibitor that inhibited trypsin at 2.9 nM, tested for various diseases [17]. The antioxidant activity of the plant samples can specify the tyrosinase inhibition potential [25,54]. However, in our study, methanol extracts (FR and L) of *P. spinosa* L. exhibited remarkable inhibition against tyrosinase enzyme (13.53% and 37.48%) compared to other studied extracts. These *in vitro* results of methanol fruit extract could be related with its higher total phenolic content (19.00 ± 0.01 mg FAE).

Our preliminary data from *P. spinosa* L. extracts could be shown potential anticancer effects for further studies. Thus, in addition to antioxidant activity and enzyme inhibition

studies, cell viability results were evaluated with time- and dose-response manner studies. We were interested in studying liver and colon cancers, because the liver is a detoxifying organ, colon cancer is also chosen because it is a part of the digestive system when taken as a supplement. Herein, these obtained preliminary results may contribute to determine the anticancer potential of *P. spinosa* L. active components. The extracts including phenolics have gained an interest because of some activities including antioxidant, anticancer etc. According to previously reported study, *P. spinosa* Trigno ecotype extract investigated on human colon cancer cells such as HCT116 and SW480 cells for its cytotoxic and apoptotic activities, the promising results were obtained [55]. According to our study, the results demonstrate a higher cytotoxicity against HT29 cancer cells with methanol extracts (FL and FR) than ethanol extract (L) at the decreasing doses. It was reported that chlorogenic and caffeic acids decreased the cell proliferation on colon cancer cell lines [56,57]. In the literature, the results generally were associated with the bioactive components of the plant. Therefore, it is pointed out the contents of the extracts and synergic effects of the compounds. *P. spinosa* L. active extracts may be potential candidates for further pharmaceutical research.

Conclusion

The findings reveal that methanol and ethyl acetate extracts of *P. spinosa* L. growing in Çanakkale/Turkey seem to have antioxidant activity, enzyme inhibition and antiproliferative properties. The workflow was given in Fig. 6. Solvent extraction affects the isolation of the compounds from the plant. Therefore, the active extracts may have potential for use by related industries for the preparation of natural-based products. We hope that this study may lead to new investigations of *P. spinosa* L. to be used in pharmaceutical applications.

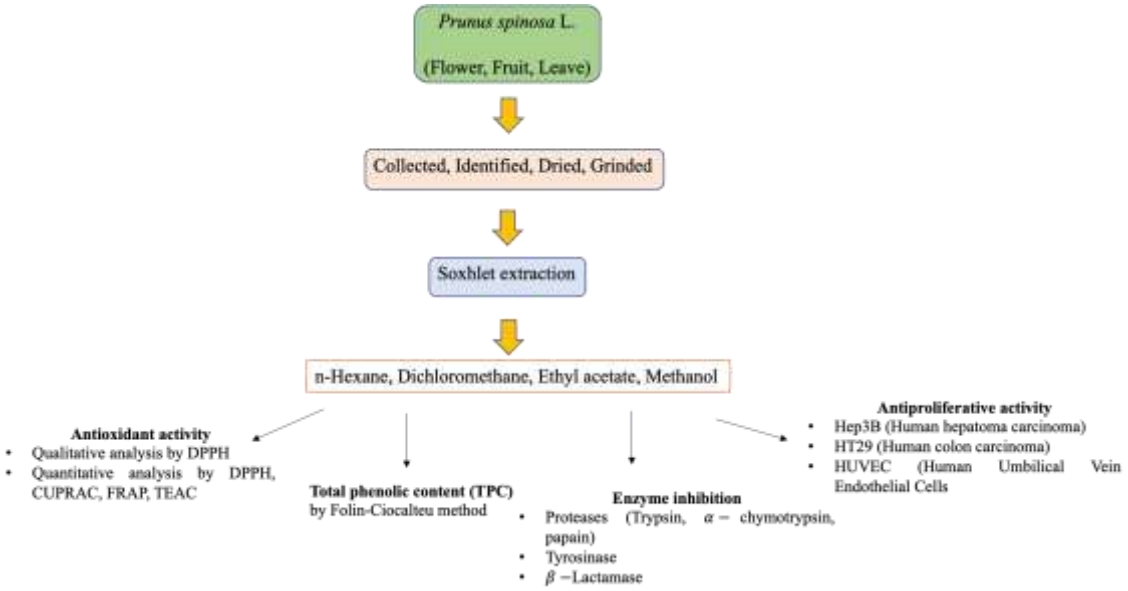

Fig 6 The workflow applied to our study

As stated above, the studies on this medicinal plant have been increasing day by day. Thus, a future prospect of *P. spinosa* plant is the isolation of active components that can be used in the treatment of various cancers and other diseases. Thus, the studies will show a new avenue as well as synergic effect of the components in the extracts. We also thought that the further investigations of the plant against different types of therapeutic enzymes and on human cancer cells may keep light to determination of effect mechanism.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Abbreviations

1,1-diphenyl-2-picrylhydrazyl (DPPH), Quercetin equivalent of flavonoid concentration (QERFC), FAE (ferulic acid equivalent), Total Phenolic Content (TPC), Cupric Reducing Antioxidant Capacity (CUPRAC), Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent Antioxidant Capacity with ABTS radical cation (TEAC), Hydrogen Atom Transfer (HAT), Single Electron Transfer (SET)

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