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Evaluation of the antioxidant and cytotoxic potency of *Euphorbia rigida* and *Arbutus andrachne* methanol extracts in human hepatocellular carcinoma cell lines in vitro

Özlem Sultan Aslantürk¹, Ebru Şebnem Yılmaz², Tülay Aşkın Çelik^{1*}  and Yelda Güzel²

Abstract

Background: Ethnobotanical studies on plants and their active compounds take a great interest in traditional medicine. After pharmacological and toxicological studies, there will be a possibility to be used in therapy. This study aimed to examine the in vitro antioxidant and cytotoxic activity of the methanol extracts of *Arbutus andrachne* L. and *Euphorbia rigida* M.Bieb. 10, 25, 50, 75, 100 and 150 $\mu\text{g mL}^{-1}$ concentrations of *A. andrachne* and *E. rigida* were tested for antioxidant activity by using DPPH radical scavenging assays, total antioxidant capacity (phosphomolybdate assay) and metal ion chelating activity. In addition, in vitro cytotoxic effects of these plants' methanol extracts on Hep3B and HepG2 human hepatocellular carcinoma cell lines were evaluated at 24, 48 and 72 h. The cytotoxicity test was carried using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.

Results: Methanol extract obtained from both plants showed increased antioxidant activity depending on the increase in concentration. When *A. andrachne* and *E. rigida* methanol extracts were compared in free DPPH scavenging activity, total antioxidant capacity and metal ion chelating activity, *A. andrachne* methanol extract was found more effective than *E. rigida*. Results from MTT assay revealed that except for 72 h treatment of HepG2 cells with 400 and 500 $\mu\text{g mL}^{-1}$ extract concentrations, *A. andrachne* methanol extract did not show significant cytotoxic effects on either Hep3B or HepG2 cells at any concentration and treatment time. On the contrary, it significantly increased proliferation in Hep3B cells from 48 h and at a concentration of 100 $\mu\text{g mL}^{-1}$. *E. rigida* methanol extract exhibited statistically significant cytotoxic activity on HepG2 cells after 48 and 72 h treatment. However, the treatment concentrations of *E. rigida* methanol extract were not as effective on Hep3B cells as on HepG2 cells.

Conclusions: According to our findings, it was determined that *A. andrachne* methanol extract did not have cytotoxic activity on neither Hep3B nor HepG2 cells, while *E. rigida* methanol extract had cytotoxic activity especially on HepG2 hepatocellular carcinoma cells. Further research is needed to identify and purify the active ingredients in *E. rigida* extracts.

Keywords: *Arbutus andrachne* L., *Euphorbia rigida*, Antioxidant activity, Cytotoxic activity, Hep3B and HepG2 cancer cells

1 Background

Medicinal plants are widely used in developing countries because of their advantages such as dissimilarity, flexibility, easy to approach, relatively low cost, low levels of technological input and increasing economic importance.

*Correspondence: tcelik@edu.tr

¹ Department of Biology, Faculty of Art and Science, Aydın Adnan Menderes University, 09010 Aydın, Turkey

Full list of author information is available at the end of the article

WHO estimates that 80% of the population around the world use medicinal plants for primary health care. Usually, plant seeds, berries, roots, leaves, bark, or flowers are used for medicinal purposes [1]. There are very many reports on the use of herbal medicines for the treatment of individual health conditions or multiple health conditions. Several plant parts used for herbal medicines have been extensively studied.

Cancer, an abnormal malignant growth of body tissue or cell, is main health problem in both developed and developing countries. Hepatocellular carcinoma is the sixth most widespread cancer and the third most common cause of death from cancer [2]. Today, the active substances of many drugs used in cancer treatment are obtained from medicinal plants [3]. Medicinal plants have to be screened for anticancer activity for more of use. Herbal drugs show their anticancer effects by mechanisms such as carcinogen inactivation, antiproliferation, cell cycle suspension, induction of apoptosis and differentiation, suppression of angiogenesis, antioxidation and reduction of multiple drug resistance [4]. The growing cost for conventional treatments of cancer has prompted people to depend more on traditional medicine [5, 6]. As a therapeutic alternative and a safe choice, herbal medicine might even increase the success rate of most cancer treatments by having a lower systemic toxicity indicated in chemotherapy [7]. Despite the afore-mentioned advantages, little is known about the possible medicinal application of medicinal plants or their cytotoxicity [8].

Arbutus andrachne is belonging to the Ericaceae family and distributed in the Eastern Mediterranean, mainly in Greece and Turkey [9]. The local name in Turkey is “sandalağacı” [10]. Fruits of this plant contain tannin, anthocyanin and carotenoids, and fruits are used widely in food products such as marmalade, fruit jelly and jam, and also in alcoholic beverages like wine and liquor [11]. Whole plant is used for various ethno medicinal purposes in the Mediterranean countries [12]. The antioxidant activities of methanol extracts from roots, leaves and fruits of the *A. andrachne* were investigated. Also, the effects of the extracts on the cardiodynamics of isolated perfused rabbit hearts. It was reported that methanol extract of the roots possesses high antioxidant activity and antihypertensive effect [13].

The genus *Euphorbia* is the largest in the plant family Euphorbiaceae, comprising about 2000.

known species and ranging from annuals to trees. 91. *Euphorbia* species are growing in Turkey [14]. All contain latex and have unique flower structures *Euphorbia* genus is known to contain a wide variety of terpenoids, ranging from mono-, sesqui-, and diterpenes to triterpenoids and steroids. Many of these compounds have been investigated for their toxicity or their potential

therapeutic activity, promising anticancer activity and some have been used as medicines since ancient times [15]. This *Euphorbia* species have been used in folk medicine to treat skin diseases, migraines, inflammatory infections, respiratory infections, body pain, intestinal parasites and warts [12, 16, 17]. The biological activities of the genus, including antitumor, antiviral, cytotoxic properties [18–26]. In this study, we investigated the antioxidant activities of the methanol extracts from *Arbutus andrachne* and *Euphorbia rigida* by measuring the free radical scavenging, total antioxidant capacity and metal chelating activity. Also, we evaluated the in vitro cytotoxic effects of the extracts on Hep3B and HepG2 human hepatocellular carcinoma cells by MTT assay. To the knowledge, there is no comparative study has been published to evaluate the cytotoxic properties of *A. andrachne* stem (wood and bark) methanol extract. Also, there are studies on the biological effects of various *Euphorbia* and *Arbutus* species but, we did not find any studies on the cytotoxic effects of plants in the literature review.

2 Methods

2.1 Plant material and methanol extraction

The branches (anatomically stem that consists of wood and bark) of *A. andrachne* and aerial parts of the *E. rigida* were used in this study. Collections of the branches and identifications were done by Assoc. Prof. Dr. Yelda Güzel in 2018 from the natural habitats from Hatay, TURKEY. Voucher specimens were deposited in the Herbarium of the Biology Department of Hatay Mustafa Kemal University. Voucher numbers are; Y. Güzel-1104 for *A. andrachne* and Y. Güzel-1105 for *E. rigida*. Plant samples air dried. Then, stems of *A. andrachne* and aerial parts of *E. rigida* (air-dried) samples were chopped and grounded into powder using an electric blender (HR2118 Philips, Netherlands). These plant powders (20 g) were extracted with 500 mL methanol at room temperature for 24–48 h. After filtration, the extract was evaporated at 40 °C under reduced pressure (Laborota 4002, Heidolph). The crude extracts were kept at +4 °C until the experimental studies.

2.2 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging potential assay is based on the stable DPPH scavenging activity. Quantitative measurements of radical scavenging assay were carried out according to the method described by Brand-Williams et al. [27]. One milliliter of 0, 1 mM DPPH methanol solution was added to 3 mL of different concentrations (10, 25, 50, 75, 100 and 150 µg mL⁻¹) of extracts in methanol. The mixture was vigorously shaken, then left at room temperature

to stand. Using a microplate reader (Elisa Reader, Biotek Co, USA) the absorbance of the mixture was measured at $\lambda=517$ nm after 30 min. 25 and 50 $\mu\text{g mL}^{-1}$ ascorbic acid, the commercial known antioxidant was used as a positive control. All experiments were performed in triplicate.

The percentage of the DPPH free radical was calculated using the following equation:

$$\text{DPPH Scavenging capacity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the *A. andrachne* and *E. rigida* methanol extracts. The actual decrease in absorption induced by the test was compared with the positive controls.

2.3 Phosphomolybdate assay (total antioxidant capacity)

The total antioxidant capacity (TAC) assay of samples was carried out by the phosphomolybdenum method [28]. A 0.1-mL aliquot of the extract (10, 25, 50, 75, 100 and 150 $\mu\text{g mL}^{-1}$) solution was shaken with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were covered with aluminum foil and incubated in a water bath at 95 °C for 90 min. After the samples were cooled, the absorbances were measured at 765 nm. Ascorbic acid was used as standard (25 and 50 $\mu\text{g mL}^{-1}$) and the results were expressed as $\mu\text{g mL}^{-1}$ of ascorbic acid equivalents. All experiments were performed in triplicate. The total antioxidant capacity (TAC) of the extracts was estimated using the following formula:

$$\text{Total antioxidant capacity (\%)} = [(\text{Abs. of control} - \text{Abs. of sample}) / (\text{Abs. of control})] \times 100$$

Abs: absorbance.

2.4 Metal ion (Iron II) chelating ability

The chelation of ferrous ions by extracts was estimated by method of Dinis et al. [29]. Briefly, 50 μL of 2 mM FeCl_2 was added to 1 mL of different concentrations of the methanol extracts 10, 25, 50, 75, 100 and 150 $\mu\text{g mL}^{-1}$. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. All experiments were performed in triplicate. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine – Fe^{2+} complex formation was calculated as:

$$[(A_0 - A_s)/A_s] \times 100$$

where A_0 was the absorbance of the control, and A_s was the absorbance of the methanol extracts/standard. Na_2EDTA was used as positive control (standard).

2.5 Cell culture and cytotoxicity assay

In cytotoxicity assays, Hep3B (ATCC HB-8016) and HepG2 (ATCC HB-8065) human hepatocellular carcinoma cells were used, because these cell lines are widely used hepatocellular carcinoma cells in studies about hepatotoxicity and drug metabolism. These cell lines possess well-known similarities each other. However, there are significant differences between these cell lines such as ethnic origins, intrinsic and drug-dependent gene expressions, cell growth inhibition, and signaling pathways associated with differential drug responses [30]. Hep3B and HepG2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS, 1% penicillin-streptomycin and *Amphotericin B* under humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cells were trypsinized and cytotoxicity assays were carried out in 96 well-plates.

Cell viabilities were measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, Germany)] assay. In this assay, viable cells cleave the yellow tetrazolium salt MTT, by the mitochondrial enzyme succinate dehydrogenase [31]. For MTT assay, Hep3B and HepG2 cells were plated 1×10^3 cells/plate in 96- well plate and incubated for 24 h, until monolayer formed. After that 25, 50, 100, 150, 200, 400 and 500 $\mu\text{g mL}^{-1}$ concentrations of *E. rigida* and *A. andrachne* methanol extracts were added for treatment. Control cells received only maintenance medium. Furthermore, anticancer agent Farmorubicin (40 $\mu\text{g mL}^{-1}$) was used for Hep3B and HepG2 cells. As a solvent control, 0.1% DMSO (Dimethyl Sulfoxide) was used. The plates were incubated at 37 °C

in a humidified incubator with 5% CO_2 for 24, 48 and 72 h. At the end of treatment times cellular viabilities were determined by MTT assay [30]. The absorbance was read at 570 nm by using a microplate reader (BioTek Epoch). All experiments were performed in triplicate.

2.6 Statistical analysis

Each experiment was performed in three replicates. Results were expressed as means $\pm$ SD and analyzed by One Way ANOVA (SPSS 20.00 software package program). Statistically significant difference was considered at the level of $p < 0.05$.

3 Results

3.1 Antioxidant activity

3.1.1 DPPH assay

The antioxidant potential of methanol extracts of *A. andrachne* and *E. rigida* was evaluated on the basis of their ability to scavenge stable free DPPH radicals.

This test is based on change in color of DPPH solution from purple to yellow, due to scavenging of stable free DPPH radicals, which from purple to yellow measured at 517 nm [32]. A stronger yellow color indicates a greater ability of the extract to scavenge free DPPH radicals and stronger antioxidant potential. In the present study, the antioxidant potential of the methanol extracts from *A. andrachne* and *E. rigida* was explored

in a dose-dependent ($10\text{--}150\ \mu\text{g mL}^{-1}$) manner as shown in Table 1 and Fig. 1. An increase in DPPH scavenging ability was observed with increase in concentration of extracts. The DPPH scavenging ability of *A. andrachne* methanol extract was higher than that of *E. rigida* methanol extract at each concentration tested. *E. rigida* methanol extract, on the other hand, showed high DPPH scavenging activity from the concentration of $50\ \mu\text{g mL}^{-1}$.

Table 1 Antioxidant activities of *A. andrachne* and *E. rigida* methanol extracts

	Concentrations ($\mu\text{g mL}^{-1}$)	DPPH scavenging activity (% $\pm$ SD)	Total antioxidant capacity (% $\pm$ SD)	Metal chelating activity (% $\pm$ SD)
Ascorbic acid	25	$77.36 \pm 0.008^*$	$76.50 \pm 0.059^*$	—
	50	$79.43 \pm 0.020^*$	$77.92 \pm 0.053^*$	—
EDTA	10	—	—	$97.55 \pm 0.016^*$
<i>Arbutus andrachne</i> methanol extract	10	$52.13 \pm 0.012^*$	19.29 ± 0.291	44.52 ± 0.072
	25	$69.08 \pm 0.002^*$	30.33 ± 0.041	45.19 ± 0.047
	50	$82.24 \pm 0.008^*$	46.77 ± 0.231	48.02 ± 0.051
	75	$83.71 \pm 0.004^*$	$65.57 \pm 0.145^*$	$51.80 \pm 0.142^*$
	100	$84.21 \pm 0.011^*$	$77.68 \pm 0.141^*$	$51.63 \pm 0.144^*$
	150	$84.38 \pm 0.010^*$	$81.30 \pm 0.235^*$	$55.32 \pm 0.055^*$
<i>Euphorbia rigida</i> methanol extract	10	32.40 ± 0.004	24.87 ± 0.112	38.43 ± 0.031
	25	34.21 ± 0.001	36.98 ± 0.058	42.31 ± 0.070
	50	$55.75 \pm 0.001^*$	$52.33 \pm 0.064^*$	45.18 ± 0.017
	75	$69.08 \pm 0.009^*$	$59.27 \pm 0.072^*$	48.19 ± 0.043
	100	$78.13 \pm 0.004^*$	$66.18 \pm 0.126^*$	49.60 ± 0.072
	150	$82.40 \pm 0.006^*$	$67.40 \pm 0.129^*$	$51.44 \pm 0.023^*$

Data are the mean $\pm$ SD of three separate determinations. Means with an asterisk in the same line indicate a significant ($p < 0.05$) difference between the concentrations tested. Means with asterisks in the same column indicate the significant ($p < 0.05$) difference between the solvent used

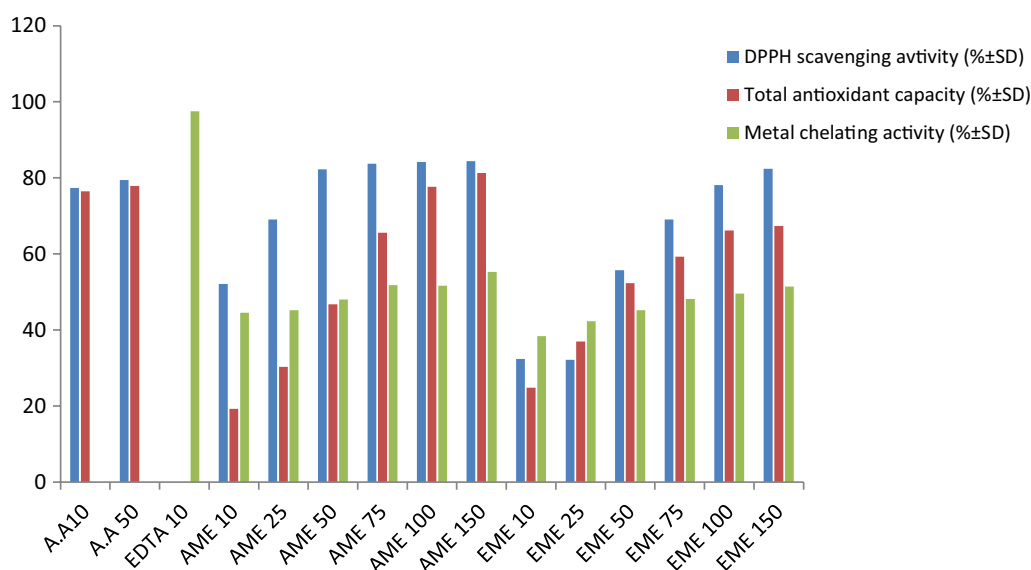


Fig. 1 Antioxidant activities of *A. andrachne* and *E. rigida* methanol extracts. Values are presented as mean $\pm$ standard deviation of three individual determinations

The DPPH scavenging activity of *A. andrachne* stem methanol extract was even higher than the standard antioxidant ascorbic acid from a concentration of $50 \mu\text{g mL}^{-1}$.

3.1.2 Total antioxidant capacity assay (TAC)

The results in Table 1 and Fig. 1 show that *A. andrachne* stem methanol extract possess high TAC starting from $75 \mu\text{g mL}^{-1}$, while the highest TAC was at $150 \mu\text{g mL}^{-1}$ ($81.30 \pm 0.235\%$). These results were even higher than the TAC value of ascorbic acid (76.50 ± 0.059). Although *E. rigida* methanol extract also showed total antioxidant activity, the total antioxidant capacity of *E. rigida* was considerably lower than both ascorbic acid and *A. andrachne* stem methanol extract.

3.1.3 Metal chelating activity

Phosphomolybdate assay is based on the reduction of phosphomolybdate ion in the presence of an antioxidant resulting in the formation of a green phosphate/MoV complex which is measured spectrophotometrically [33, 34]. Metal chelating ability assay is based on the measurement of iron-ferrozine absorbance at 562 nm in presence of an antioxidant compound [29].

A. andrachne stem methanol extract was tested in the concentration range of $10\text{--}150 \mu\text{g mL}^{-1}$, with only three of the six concentrations ($50, 100$ and $150 \mu\text{g mL}^{-1}$) showing concentration-dependent chelating activity (Table 1; Fig. 1). However, this activity is considerably lower than the metal chelating activity of EDTA ($97.55 \pm 0.053\%$) used as a standart. Compared at the concentration of 150 mg/mL *A. andrachne* stem methanol extract ($55.32 \pm 0.055\%$) the strongest activities, while the weakest activities were detected in $10 \mu\text{g mL}^{-1}$ *A. andrachne*

stem methanol extract ($44.52 \pm 0.072\%$). *E. rigida* methanol extract was also tested in the concentration range of 10 to $150 \mu\text{g mL}^{-1}$ and only one of the six concentrations ($150 \mu\text{g mL}^{-1}$) showed chelating activity above 50% ($51.44 \pm 0.023\%$) (Table 1; Fig. 1).

3.2 Cell proliferation assay

To investigate the cytotoxic potential of the *A. andrachne* and *E. rigida* methanol extracts on Hep3B and HepG2 cancer cells, the MTT colorimetric assay was performed. The cytotoxic activity of *A. andrachne* stem methanol extract against two cancer cell lines (Hep3B and HepG2) was evaluated (Table 2; Figs. 2 and 3). Hep3B and HepG2 cancer cells were treated with seven different concentrations of *A. andrachne* stem methanol extract ($25, 50, 100, 150, 200, 400$ and $500 \mu\text{g mL}^{-1}$) for $24, 48$ and 72 h . Three concentrations ($25, 50$ and $100 \mu\text{g mL}^{-1}$) after 24 h of treatment slightly reduced viability in Hep3B cells. The highest decrease in viability was at 100 mg mL^{-1} ($89.76 \pm 0.057\%$). In contrast to these results, treatment with the other four concentrations ($150, 200, 400$ and $500 \mu\text{g mL}^{-1}$) for 24 h increased Hep3B cell viability dependently. The highest viability rate was at $500 \mu\text{g mL}^{-1}$ ($112.31 \pm 0.144\%$). The cell viability obtained with the positive control farmorubicin was only $1.06 \pm 0.014\%$. Farmorubicin ($40 \mu\text{g mL}^{-1}$) showed a very high cytotoxic effect on all Hep3B cells and killed almost all Hep3B cells. The viability and proliferation enhancing effect of *A. andrachne* methanol extract of Hep3B cells at 24 h increased at all concentrations after 48 and 72 h of treatment, and these data were statistically significant when compared to control and solvent control (Table 2; Fig. 2).

Table 2 The effect of *A. andrachne* methanol extract on the viability of Hep3B and HepG2 cells

Concentrations ($\mu\text{g mL}^{-1}$)	Cell viability % $\pm$ SD					
	Hep3B cells			HepG2 cells		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Solvent control (DMSO)	98.96 ± 0.039	97.97 ± 0.063	97.27 ± 0.058	95.89 ± 0.076	95.49 ± 0.052	95.25 ± 0.066
Formorubicin, 40	$1.06 \pm 0.014^*$	$0 \pm 0.000^*$	$0 \pm 0.000^*$	$1.49 \pm 0.013^*$	$0.20 \pm 0.002^*$	$0 \pm 0.000^*$
25	93.61 ± 0.138	102.13 ± 0.189	105.40 ± 0.264	95.61 ± 0.296	94.49 ± 0.115	92.23 ± 0.484
50	93.25 ± 0.155	111.24 ± 0.051	$118.33 \pm 0.071^*$	102.87 ± 0.215	100.09 ± 0.028	96.15 ± 0.089
100	89.76 ± 0.057	$117.39 \pm 0.041^*$	$123.99 \pm 0.123^*$	111.36 ± 0.123	102.71 ± 0.045	94.79 ± 0.121
150	104.14 ± 0.097	$118.08 \pm 0.016^*$	$126.72 \pm 0.040^*$	105.64 ± 0.035	100.34 ± 0.077	91.43 ± 0.037
200	105.80 ± 0.178	$127.00 \pm 0.134^*$	$131.03 \pm 0.061^*$	104.59 ± 0.133	93.19 ± 0.092	86.87 ± 0.521
400	108.03 ± 0.066	$128.01 \pm 0.028^*$	$132.42 \pm 0.100^*$	99.59 ± 0.030	91.77 ± 0.039	$82.53 \pm 0.145^*$
500	112.31 ± 0.144	$128.48 \pm 0.071^*$	$133.43 \pm 0.211^*$	97.90 ± 0.042	88.56 ± 0.216	$67.05 \pm 0.031^*$

Data are the mean $\pm$ SD of three separate determinations. Means with an asterisk in the same line indicate a significant ($p < 0.05$) difference between the concentrations tested. Means with asterisks in the same column indicate the significant ($p < 0.05$) difference between the solvent used

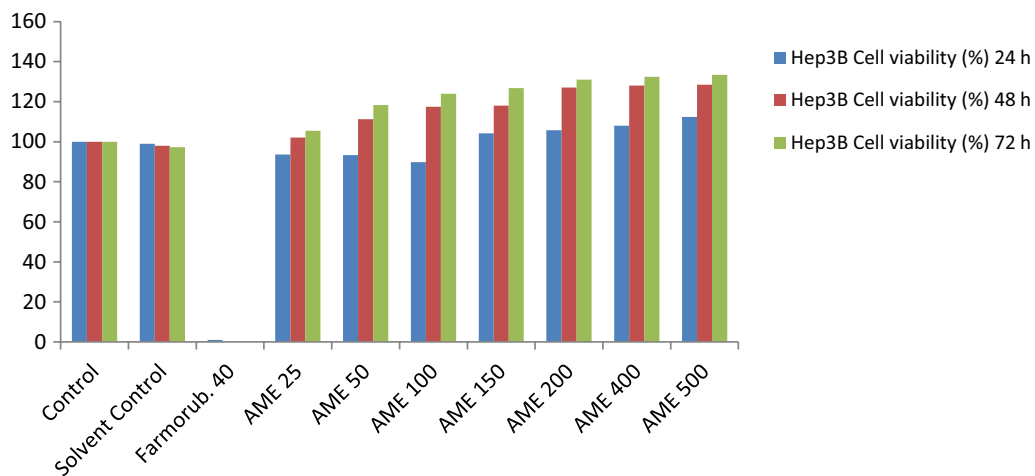


Fig. 2 The effect of *A. andrachne* methanol extract on the viability of Hep3B cells (AME: *A. andrachne* methanol extract)

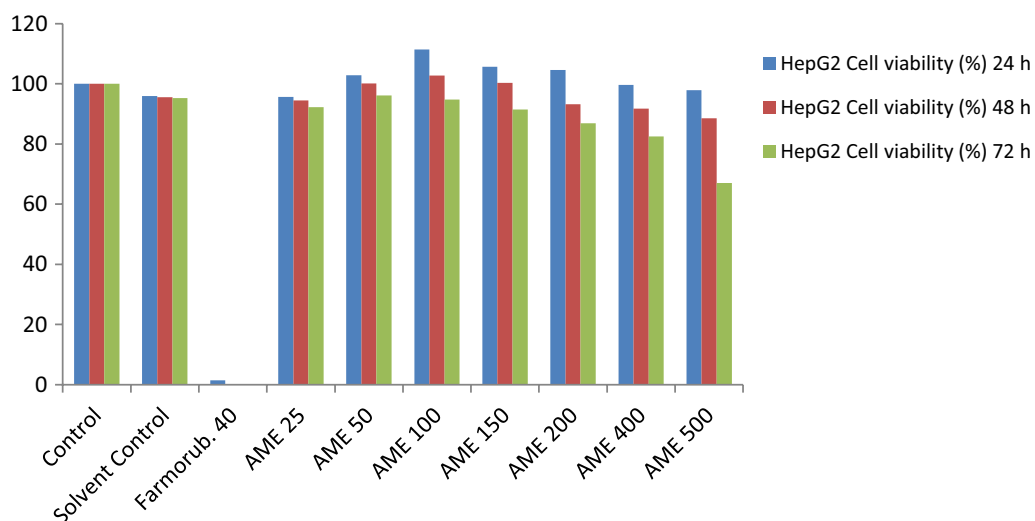


Fig. 3 The effect of *A. andrachne* methanol extract on the viability of HepG2 cells (AME: *A. andrachne* methanol extract)

The cytotoxic effect of *A. andrachne* stem methanol extract on the proliferation and viability of HepG2 cells is presented in Table 2 and Fig. 3. *A. andrachne* methanol extracts at 50, 100, 150, and 200 $\mu\text{g mL}^{-1}$ increased the viability of HepG2 cells somewhat at 24 h of treatment, but slightly decreased at the other three concentrations (25, 400, and 500 $\mu\text{g mL}^{-1}$) (Table 2; Fig. 3). This effect was not statistically significant when compared with solvent control and farmorubicin (40 $\mu\text{g mL}^{-1}$). As a result of 48 h of treatment, cell viability started to decrease due to the increase in concentration with the application of 200, 400 and 500 $\mu\text{g mL}^{-1}$ concentrations in the viability and proliferation of HepG2 cells. The most significant decrease in cell viability occurred after 72 h of treatment

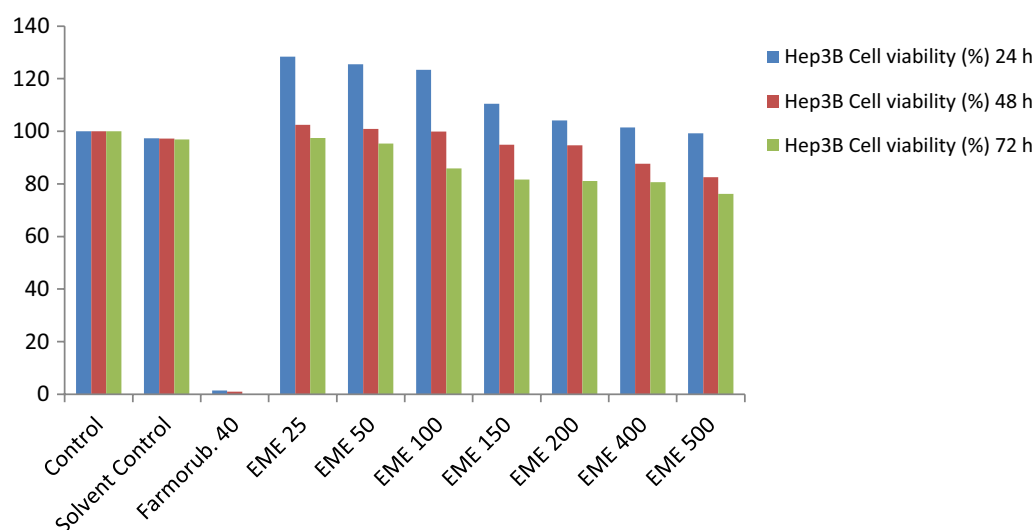
with 500 $\mu\text{g mL}^{-1}$ extract concentration ($67.05 \pm 0.031\%$). Farmorubicin used as control produced a very high cytotoxic effect on HepG2 cells at all three treatment times and killed almost all cells.

Effect of *E. rigida* methanol extract on the proliferation and viability of Hep3B cells are present in Table 3 and Fig. 4. After 24 h of treatment of *E. rigida* methanol extract, all concentrations except 500 $\mu\text{g mL}^{-1}$ increased proliferation in Hep3B cells. This effect was found to be significantly high in 25, 50 and 100 $\mu\text{g mL}^{-1}$ ($128.40 \pm 0.015\%$, $125 \pm 0.033\%$, $123 \pm 0.098\%$, respectively) extract treatments compared to the control and solvent control (DMSO) group ($p < 0.05$). After 48 h of treatment, no significant effect of methanol extract was

Table 3 The effect of *E. rigida* methanol extract on the viability of Hep3B and HepG2 cells

Concentrations ($\mu\text{g mL}^{-1}$)	Cell viability % $\pm$ SD					
	Hep3B cells			HepG2 cells		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	100 $\pm$ 0.000	100 $\pm$ 0.000	100 $\pm$ 0.000	100 $\pm$ 0.000	100 $\pm$ 0.000	100 $\pm$ 0.000
Solvent control (DMSO)	97.32 $\pm$ 0.005	97.19 $\pm$ 0.024	96.88 $\pm$ 0.116	96.30 $\pm$ 0.015	96.59 $\pm$ 0.042	96.49 $\pm$ 0.020
Farmorubicin 40	1.41 $\pm$ 0.134*	1.05 $\pm$ 0.008*	0 $\pm$ 0.000*	1.63 $\pm$ 0.015*	0 $\pm$ 0.000*	0 $\pm$ 0.000*
25	128.40 $\pm$ 0.015*	102.51 $\pm$ 0.088	97.40 $\pm$ 0.004	97.88 $\pm$ 0.166	92.98 $\pm$ 0.140	84.34 $\pm$ 0.142*
50	125.44 $\pm$ 0.033*	100.93 $\pm$ 0.148	95.30 $\pm$ 0.079	94.05 $\pm$ 0.073	90.36 $\pm$ 0.189	83.76 $\pm$ 0.085*
100	123.37 $\pm$ 0.098*	99.88 $\pm$ 0.088	85.89 $\pm$ 0.109	93.20 $\pm$ 0.035	83.49 $\pm$ 0.384*	80.72 $\pm$ 0.184*
150	110.50 $\pm$ 0.233	94.95 $\pm$ 0.113	81.69 $\pm$ 0.065*	89.52 $\pm$ 0.031	82.37 $\pm$ 0.040*	77.62 $\pm$ 0.057*
200	104.10 $\pm$ 0.052	94.72 $\pm$ 0.038	81.10 $\pm$ 0.017*	86.41 $\pm$ 0.038	80.58 $\pm$ 0.153*	67.11 $\pm$ 0.420*
400	101.48 $\pm$ 0.056	87.68 $\pm$ 0.053	80.63 $\pm$ 0.028*	84.00 $\pm$ 0.023*	70.12 $\pm$ 0.029*	59.11 $\pm$ 0.117*
500	99.26 $\pm$ 0.018	82.54 $\pm$ 0.285*	76.17 $\pm$ 0.209*	72.68 $\pm$ 0.009*	58.92 $\pm$ 0.087*	45.91 $\pm$ 0.052*

Data are the mean $\pm$ SD of three separate determinations. Means with an asterisk in the same line indicate a significant ($p < 0.05$) difference between the concentrations tested. Means with asterisks in the same column indicate the significant ($p < 0.05$) difference between the solvent used

**Fig. 4** The effect of *E. rigida* methanol extract on the viability of Hep3B cells (EME: *E. rigida* methanol extract)

observed on the cell viability and proliferation, except for a 500 $\mu\text{g mL}^{-1}$ concentration ($82.54 \pm 0.285\%$). After 72 h of treatment, *E. rigida* methanol extract significantly reduced cell viability and proliferation due to the increase in concentration and showed cytotoxic effect compared to the control group, excluding 25, 50 and 100 $\mu\text{g mL}^{-1}$ ($p < 0.05$). DMSO, used as solvent control, had no significant cytotoxic effect at any treatment time. Farmorubicin, which was used as an anticancer agent, produced a very high cytotoxic effect on Hep3B cells during all treatment times and killed almost all of the cells.

Effect of *E. rigida* methanol extract on the proliferation and viability of HepG2 cells are also present in Table 3 and Fig. 5. 400 and 500 $\mu\text{g mL}^{-1}$ concentrations of *E. rigida* methanol extract reduced the viability of HepG2 cells at 24 h of treatment ($p < 0.05$), and all concentrations except for 25 and 50 $\mu\text{g mL}^{-1}$ at 48 h of treatment resulted in a significant decrease in cell viability. After 48 h of treatment with *E. rigida* methanol extract, the concentration that most reduced HepG2 cell viability and proliferation was 500 $\mu\text{g mL}^{-1}$ ($58.92 \pm 0.087\%$). The 72 h treatment of *E. rigida* methanol extract caused a concentration-dependently and significantly decrease in the

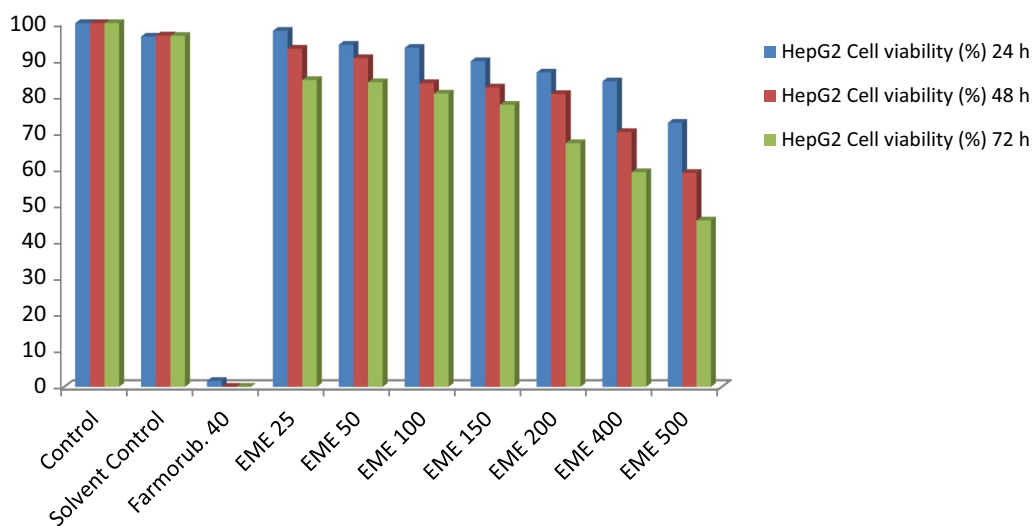


Fig. 5 The effect of *E. rigida* methanol extract on the viability of HepG2 cells (EME: *E. rigida* methanol extract)

viability of HepG2 cells in comparison with the control. HepG2 cell viability decreased to 45.91% at 500 $\mu\text{g mL}^{-1}$ concentration treatment (Table 3; Fig. 5). In HepG2 cells treated with *E. rigida* methanol extract decreased of the cell viability and proliferation was observed at most after 72 h of treatment, and this decrease was parallel to the increase in concentration. Farmorubicin killed almost all of the cells at all treatment time.

4 Discussion

Cancer is one of the most dangerous diseases with fast progression and high mortality around the world. Today, about 60–75% of anticancer agents are derived from natural sources [33–35]. The chemical constituents of the plants or crude extracts are known to be biologically active ingredients. Plant polyphenols have been highlighted as potential anticancer agents as well as being chemical inhibitors [36] because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage and/or transformation into cancer cells [36, 37]. In our literature review, we found antioxidant, anti-inflammatory and cell protective effects of *Arbutus* spp. but, we could not find any data regarding the cytotoxic effect of *Arbutus* species including *A. andrachne* stem (wood and bark). Furthermore, it has been shown that *Euphorbia* spp. is promising plants antitumor activity [38, 39]. Also, there are reports about antioxidant activities of different *Euphorbia* species [25, 26]. In this regard, the antioxidant activities of methanol extracts from *A. andrachne* and *E. rigida* were investigated using DPPH assay, phosphomolybdate assay and metal chelating assay. Also, cytotoxic effects of the

extracts on two human hepatocellular carcinoma (Hep3B and HepG2) cell lines were investigated using MTT assay.

When *A. andrachne* and *E. rigida* methanol extracts were compared in terms of antioxidant activities (DPPH free radical scavenging activity, total antioxidant capacity and metal chelating activity), *A. andrachne* methanol extract possess higher antioxidant activity. Differences between the antioxidant activities of the extracts may be attributed to different phytochemical contents of *A. andrachne* and *E. rigida* plants. The transition metal ion Fe^{2+} have the ability to maintain the formation of free radicals by gain or loss of electrons. Therefore, the decrease of reactive oxygen species genesis can be carried out by the chelation of metal ions with chelating agents. Considering the cellular damages of reactive oxygen species and their relation with various diseases, it is important that plant extracts possess metal chelating activity besides free radical scavenging activity [40, 41]. In our previous studies, we reported that diethyl ether, ethyl acetate and aqueous extracts from fruits of *Arbutus unedo* possess high total phenolic content and antioxidant activity [42]. Recently, it was suggested also, *Arbutus unedo* leaf aqueous extract has antioxidant activity, in vitro anti-inflammatory effect, and heat protective effect on the membrane integrity of human red blood cells against heat [43]. Furthermore, some studies reported vasorelaxant and antiaggregant effects of *Arbutus unedo* leaf extract on human platelets [44, 45] and antihemolytic and radical scavenging activities [46, 47]. Hmaidosh et al. [48] reported that *A. andrachne* flowers, leaves and barks contain high amounts of total phenolic compounds. They reported also that these parts of plant possess antioxidant activity (FRAP), which was similar

to the total phenolic contents. We reported also ethyl acetate, methanol and water extracts of *Euphorbia platyphyllos* possess high DPPH radical scavenging activity, previously [24]. Gapuz and Besagas suggested that leaf methanol extracts from three *Euphorbia* species (*E. milii*, *E. trigona*, and *E. antiquorum*) possess high total phenolic content and high DPPH radical scavenging activity [49]. They reported also that the extracts contain alkaloids, flavonoids, carbohydrates, saponin, and tannins.

In this study, it was investigated also whether *A. andrachne* and *E. rigida* methanol extracts possess cytotoxic effect on Hep3B and HepG2 cells. *A. andrachne* methanol extract showed no cytotoxic effect on Hep3B cells and increased cell viability and proliferation. Also, *A. andrachne* methanol extract showed no significant cytotoxic effect on HepG2 cells at 24 and 48 h treatments. Only 72 h treatment with 400 and 500 $\mu\text{g mL}^{-1}$ extract concentrations significantly reduced the viability and proliferation of HepG2 cells (Table 2; Fig. 3). This may be due to different responses of the cells to the components of the extract. When the results were examined, it was understood that methanol extract had no significant cytotoxic effect on both cells. Moreover, methanol extract appears to increase cell proliferation in Hep3B cells depending on time and concentration (Table 2; Fig. 2).

In previous studies conducted with cancer cells, it was found that extracts of *Arbutus* species don't possess cytotoxic and/or antiproliferative effect. Fortalezas et al. reported that hydroethanolic extract of *A. unedo* fruits caused no effect on viability of SK-N-MC human neuroblastoma cells [50]. Also, it was suggested that *A. andrachne* did not exhibit significant antiproliferative effect on breast, colorectal and skin cancer cells [4]. In addition, it was reported that arbutin, a main compound in *Arbutus* species, has not significant effect on cell viability, even at high concentrations on TCCSUP human bladder cancer cell line, HepG2 human hepatocellular carcinoma cells, and U937 human lymphoma cells [51–53]. In our study, very insignificant dose-dependent results were obtained for *Arbutus* extract. Because of the high antioxidant activity of the *A. andrachne* stem methanol extract, free radicals forming in the cell may be eradicated and cytotoxic effect is neutralized. In addition, it has been reported that the polyphenol compounds of plants to be anticancer agents [54–56].

Methanol extract of *E. rigida* exhibited statistically significant cytotoxic activity on HepG2 cells after 48 and 72 h treatment (Table 3; Fig. 5). However, the tried concentrations of *E. rigida* methanol extract were not as effective on Hep3B cells as on HepG2 cells (Table 3; Figs. 4 and 5). This result could be explained by different sensitivity of tumor cells to the chemical content of

the extract. Previous studies suggested that triterpenoids, alkaloids, diterpene esters, organic acids, acetophenone derivatives, and flavonoids are the main chemical ingredients of Euphorbiaceae plants [57, 58]. Therefore, the cytotoxic effect of *E. rigida* methanol extract on HepG2 cells may be considered to be due to the presence of these chemical ingredients. It was reported that *Euphorbia* plants possess a various biological activities such as anti-oxidative, antiproliferative, cytotoxic, anti-anaphylactic, antimicrobial, and anti-arthritic activities [59, 60]. Javidnia et al. [61] reported that *Euphorbia hebecarpa* extracts possess cytotoxic effect on K562 and U937 cells but, they had no effects on KB cells. Sadeghi-Aliabadi et al. [62] showed that ethyl acetate extracts of *E. macroclada* were cytotoxic against MDA-MB-468 cells. Metin and Bürün reported that the aqueous extract of *E. rigida* aerial parts possess negative impacts on mitosis and, cytotoxic and genotoxic effects on *Allium cepa* root meristematic cells at concentrations over 50 ppm [63].

5 Conclusions

Turkey has a great history of folk medicine, but this knowledge has not been documented extensively so far. In the recent years researchers have carried out many studies about traditional medicine in Turkey. Now we have important information, documents and great opportunities to study on medicinal plants. According to our findings it could be conclude that constituents extracted by methanol from *E. rigida* possess cytotoxic activity on HepG2 hepatocellular carcinoma cells and it required further investigations for the identification and purification of the active components. However, *A. andrachne* stem methanol extract possess cytotoxic activity neither on Hep3B nor on HepG2 cells. For this reason, different studies can be carried out on this plant as well as other potential effects other than anticancer effects in the future.

Abbreviations

AA: Ascorbic acid; AME: *A. andrachne* methanol extract; EME: *E. rigida* methanol extract.

Acknowledgements

Not applicable.

Authors' contributions

ÖSA, EŞY, and YG have designed the study and collected the data. ÖSA and TAÇ have performed Laboratory analysis and statistical analysis of the study. ÖSA has written manuscript; TAÇ, EŞY and YG have reviewed and edited manuscript. All authors have read and approved the final manuscript.

Funding

This study was partly supported by HMKU Scientific Research Project Fund (18.YL.010).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Biology, Faculty of Art and Science, Aydın Adnan Menderes University, 09010 Aydın, Turkey. ²Department of Biology, Faculty of Art and Science, Hatay Mustafa Kemal University, 31060 Hatay, Turkey.

Received: 23 June 2021 Accepted: 23 August 2021

Published online: 03 September 2021

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