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Cytotoxic and apoptotic effects of Prunus spinosa fruit extract on HT-29 colon cancer line

Fulya Dilek Gökalp[a*], Egzona Qipa[a]

[a] *Trakya University, Science Faculty, Biology Department, 22030, Edirne, Türkiye*

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ABSTRACT

Colon cancer holds the position of the third most common type of cancer and stands as the third leading cause of cancer-related deaths for both men and women. Modern strategies in cancer prevention center around the use of natural compounds, which demonstrate a range of effects, including preventive, inhibitory, and latency-inducing impacts on the progression of cancer. In the present study, aqueous extracts derived from the fruits of *Prunus spinosa* L. (blackthorn, Rosaceae) are employed to assess their cytotoxic potential against the HT-29 colon cancer cell line. The fruit extract is administered to the HT29 cell line in different concentrations over 24 and 48-hours to evaluate the induction of apoptosis. The MTT cell viability test is employed to quantify the cytotoxic effect, indicating the extent of the impact. Additionally, the EB/AO (ethidium bromide/acridine orange) dual staining method is utilized to gather supplementary information regarding the cytotoxic effects. Observations after 24 hours of exposure showed no significant cytotoxic effect; however, 48-hour exposure revealed IC_{20}, IC_{50}, and IC_{80} values of 1.27, 173.7, and > 1000 µg/ml, respectively, as determined by MTT analysis. Correspondingly, values of 5.06, 123.8, and > 1000 µg/ml were recorded by the EB/AO dual staining method. Our results show that *P. spinosa* fruit water extract has an inhibitory effect on the HT-29 cell viability by exerting cytotoxic and apoptotic effects in a concentration-dependent and time-dependent manner. Toxicity studies have shown that MTT and EB/AO support each other and achieve similar results. Further extensive research into the metabolic and functional effects of *P. spinosa* could illuminate its potential and increase its economic importance in the field of anticancer treatments as a natural drug.

1. Introduction

For centuries, plants and natural products have been employed in treating diverse ailments, leading to the utilization of medicinal compounds derived from a multitude of plants (Badal et al., 2024; Chunarkar-Patil et al., 2024; Gunnar & Bohlin, 1999). Presently, numerous plant-derived compounds have been isolated, displaying activity against various cancer cell types (Nirmala et al., 2011). According to the findings from these studies, an increased intake of fruits and vegetables plays a crucial role in promoting healthy nutrition and preventing chronic diseases like heart attacks, cancer, diabetes, and neurodegenerative conditions (Liu, 2013).

Fruits and vegetables, abundant in polyphenols such as flavonoids, phenolic acids, anthocyanins, and tannins, as well as other compounds like sugars, essential oils, carotenoids, vitamins, and minerals, are recognized for their health benefits. Polyphenolic compounds, categorized as secondary metabolites in plants, exhibit potent antioxidant properties (Scalbert et al., 2005). These compounds function as antioxidants by chelating or inhibiting metal ions during the initiation of free radical formation, thereby suppressing radical species. Additionally, polyphenols act as radical scavengers by donating hydrogen molecules (Aviram et al., 2005; Halliwell, 1996).

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Ivana Kmetič: University of Zagreb, Zagreb, Croatia
Sevim Feyza Erdoğmuş: Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye

* Corresponding author(s):
E-mail address:
fulyadilek@trakya.edu.tr (F. D. Gökalp)
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Polyphenols have been documented to possess both in vitro and in vivo antioxidant, anticancer, antimutagenic, anti-inflammatory, and anti-neurodegenerative properties (del Rio et al., 2013; Nile & Park, 2014). Interest in plant phenolic extracts has increased due to their potential antioxidant activity and extensive pharmacological properties, including anticancer activity (Carocho & Ferreira, 2013). Anthocyanins, a subgroup of flavonoids primarily found in fruits, contribute to the red, blue, and purple hues in many plants, particularly in grapefruits (Mazza, 1993).

Along with the beneficial effects of anthocyanins against cancer and cardiovascular disorders; antioxidant, anti-inflammatory, and antiproliferative activity is reported to affect cellular signaling pathways, cause cell cycle arrest, and activate apoptotic redox-sensitive transcription factors (Calabriso et al., 2016; Slatnar et al., 2012; Zhang et al., 2008).

The blackthorn fruit is a rich source of compounds with antioxidant and antibacterial characteristics, including flavonoids, anthocyanins, phenolic acids, vitamins, minerals, and organic acids (Natić et al., 2019; Ürkek et al., 2019). *Prunus spinosa* L., also known as blackthorn and belonging to the Rosaceae family, is grown in the north, west, and south of Türkiye and central Anatolia up to 1350 m high, grows in and at the edge of forests, the high variety between 1-3 m, perennial, shrub plant. The fruits of the *P. spinosa* grown in Türkiye are consumed in various ways for treatment among the population. It is reported that the flowers of blackthorn are used for respiratory diseases, diarrhea, stomach cramps, intestinal diseases, edema, and kidney and bladder diseases (Fraternale et al., 2009). *P. spinosa* fruits grown in our region are the best source of phenolic compounds containing flavonoid, coumarin, phenolic acid, and A-type proanthocyanins (Orhan et al., 2007), which are used as astrejan, diuretic, and purgative (Guimarães et al., 2013; Pinacho et al., 2015).

Extracts of different parts of *P. spinosa* obtained using ethanol, water, methanol, and ethyl acetate were tested on various cancer cell lines, and their antiproliferative and cytotoxic effects were studied (Condello & Meschini, 2021; Dedić et al., 2023; Guimarães et al., 2014; Meschini et al., 2017; Murati et al., 2016; Pinacho et al., 2015). It was demonstrated that ethanol extract of P. spinosa fruits on PC-3 and DU145 (Dedić et al., 2023) and methanol-water extract on MCF-7, NCI-H40, HCT-15, HeLa, and HepG2 cancer cell cultures (Guimarães et al., 2014) induced antiproliferative and cytotoxic activity. Proanthocyanidins obtained from *P. spinosa* extract were tested in GLC and COLO320 cell lines, and the cytotoxic effect of proanthocyanidins on the cancer cell line was obtained (Kolodziej et al., 1995). Phenolic compounds containing anthocyanin obtained from *P. spinosa*, *Rosa micranta*, and *R. canina* have antioxidant and antitumor effects on NCL-H460 cell lines (Guimarães et al., 2014). Furthermore, ethanol and water extracts of *P. spinosa* fruits showed antitumor effects on the colorectal cancer cell line (HT-29) (Popović et al., 2020).

In many studies with plant extracts, the MTT viability test is used to investigate the cell proliferation and cytotoxic effects of extracts or secondary metabolites on cancer cell lines. The MTT assay is a colorimetric experiment to assess cell metabolic activity. This method is based on a mitochondrial enzyme that breaks down MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium salt by using it as a substrate. Mitochondrial dehydrogenase enzymes found in the mitochondria of living cells break down MTT into formazan crystals. When the crystals were dissolved with DMSO or acid isopropanol, the purple-colored solution formed and was measured using a spectrophotometer at 560 nm and absorbance at 620 nm (Mosmann, 1983).

Ethidium bromide/acridine orange (EB/AO) cell viability test is employed in toxicology studies. AO stains both live and dead cells, while EB exclusively stains dead cells due to membrane structure disintegration. AO causes DNA to appear orange but is very weakly bound to the RNA. Although AO stains dead cells as green, EB suppresses the green color and reflects an orange-red color that can be observed by fluorescence microscopy. Cells entering necrosis stain the same as dead cells but do not have an apoptotic morphology. Early apoptotic cells are observed as green. Green cells with normal morphology observed in the cell population are alive, and red-orange cells with normal morphology are necrotic cells (Kasibhatla et al., 2006).

The water extract of *P. spinosa* fruit is known to possess a high phenolic and flavonoid content, albeit not as much as its branches. Additionally, the highest amount of anthocyanin is detected in the water extract of *P. spinosa* fruit (Pinacho et al., 2015). Studies have demonstrated that *P. spinosa* extracts were non-toxic to non-tumor cell lines (Gerardi et al., 2016; Guimarães et al., 2014; Meschini et al., 2017). This study aims to assess the cytotoxic and apoptotic effect of water extracts from *P. spinosa* fruits on HT-29 colon cancer cell lines using the MTT viability test and EB/AO staining methods.

2. Materials and methods

2.1. Extraction and isolation

P. spinosa fruit extraction and isolation were performed according to Stanković et al. (2019) with minor modifications. *P. spinosa* fruits were collected from Edirne, Türkiye. Plant material was identified by Prof. Dr. Çiler Kartal and the voucher specimen (No. 4315) was deposited at the herbarium of Trakya University, Biology Department (Republic of Türkiye Trakya University, EDTU Herbarium). *P. spinosa* fruits were extracted, homogenized and centrifuged at 9000 rpm for 2 minutes. The extract was diluted with water (1:1 ratio), infused with a magnetic stirrer for 30 minutes, and filtered through a 0.45µm filter. The filtrates were frozen and lyophilized in a vacuum freeze dryer (Virtis SP Scientific). Extracts were stored at –18 °C until tested (Stanković et al., 2019).

2.2. Chemicals used

Reagents and chemicals used in the study were Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), trypsin (Gibco, USA), MTT, phosphate buffered saline (PBS) salts, penicillin-streptomycin, AO, EB (Sigma Aldrich, USA), and Annexin V-FITC apoptosis detection kit (Biovision, USA).

2.3. Cell culture and application

HT-29 cells were cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin and were incubated in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C. HT-29 cells (5×10^3 cells/well) were grown in 96 well plates. After 24 hours, the cells showed a growth increase of 80%, and the medium was replaced with a new medium. The new medium contained herb extracts at different concentrations. No extract was added in the control group (Dewi et al., 2022). To determine IC_{20}, IC_{50}, and IC_{80} values, cells were exposed to different concentrations of the water extract of *P. spinosa* for 24 and 48 hours (43.95, 87.89, 175, 351.56, 703.13, 1406.2, 2812.5, 5625, 11250, 22500 and 45000 µg/ml). IC_{20}, IC_{50},

and IC_{80} concentrations obtained by MTT test were applied to cell cultures, and the apoptotic effects were assessed with the EB/AO method.

2.4. Determining apoptosis with the EB/AO dual staining method

EB/AO dual staining method based on the separation of viable and dead cells, was used to detect apoptosis. Viable cells were stained with EB/AO to determine the number of apoptotic cells. With fluorescence microscopy, AO enables the visualization of normal and apoptotic nuclei of living cells as green. EB allows the normal and apoptotic nuclei of dead cells to appear red (Kasibhatla et al., 2006; Petit et al., 1993). The experiment was repeated 3 times, 500 cells were counted for each concentration in each repetition.

2.5. Preparation of EB/AO dual staining solution

EB/AO staining solution used for counting live and dead cells consisted of a 100x stock solution prepared by mixing 50 mg of EB, 15 mg of AO with 1 ml of 98% ethanol, and 49 ml of distilled water and frozen in 1 ml aliquots. 1x working solution was prepared by mixing stock solution and PBS in a 1:1 ratio. The cell suspension was prepared at 1–5 x 10^6 cells/ml in a cell culture medium. Cell suspension and staining solution were mixed at a 1:1 ratio to observe dead and live cells by using fluorescence microscopy at 460 nm and 510 nm wavelengths (Parks et al., 1979). The phases of the cells in the apoptosis process were described by Gasiorowski et al. (2001) and at least 500 cells were counted. Apoptosis was determined by the deformation of the colon cancer cells. The percentage of apoptotic cells was calculated as the apoptotic index.

2.6. MTT assay

Antiproliferative effects were determined in the concentration range of 1–45000 µg/ml and dose-response curves and IC_{50} values were determined by Probit analysis. In this study, plant extracts with different concentrations (1–45000 µg/ml) were applied to evaluate their impact on cell viability on the HT-29 colon cancer cell line during 24 and 48-hour incubation periods. The experiment was repeated three times for each concentration, and average absorbance values were determined. IC_{50} values were then calculated for both the extract and negative control.

HT-29 cells were plated in 96-well plates as 5 x 10^3 per 100 µl suspension. Plates were incubated in an incubator for 24 hours to ensure adherence of colon cancer cells and duplication of cell number. Then, the medium in the wells was discarded and replaced with varying concentrations of plant extract in culture medium (43.95, 87.89, 175, 351.56, 703.13, 1406.2, 2812.5, 5625, 11250, 22500, and 45000 µg/ml). Only wells without cells containing the assay medium were evaluated as blind. The plates were incubated for 24 and 48 hours. Subsequently, 10 µl of MTT solution was introduced into each well, and the plates were incubated in the incubator for 4 hours. Following this, 100 µl of the solvent solution was added to each well, and the plates were incubated overnight. It is measured using a spectrophotometer at 560 nm and absorbance at 620 nm. Cytotoxicity levels were determined using the formula:

$$Cytotoxicity\ level = 1 - \frac{Absorbance\ of\ the\ test}{Absorbance\ of\ the\ control}\ x\ 100$$

The concentration of cytotoxic effect at 50% relative to the control was regarded as the cytotoxic dose (Ghasemi et al., 2023).

2.7. Statistical analysis

The results of the MTT and EB/AO assays were analyzed using the Probit analysis program (SPSS 17). Pearson correlation test was used to determine the relationship between concentration and cell death (statistical tests were performed at the confidence interval of $p < 0.05$).

3. Results and discussion

P. spinosa fruit extract caused the death of HT-29 cancer cells at different concentrations after 24 and 48-hour treatments. The impact of various concentrations on cell death following 24 and 48 hours of treatment, as assessed by the MTT test, is presented in Figure 1. The cell death ratio did not reach 50% after 24 hours of treatment. However, IC_{20}, IC_{50}, and IC_{80} values were determined after 48 hours of treatment, showing an increase in cell death rate inhibition up to 80%. Following 48 hours of treatment, the IC_{20}, IC_{50}, and IC_{80} values were found to be 1.27 µg/ml, 173.7 µg/ml, and > 1000 µg/ml respectively (Table 1). The MTT assay showed that the number of viable cells decreased after 24 and 48 hours of treatment compared with the control group. The apoptotic effect was observed at a maximum concentration of the extract after 48 hours.

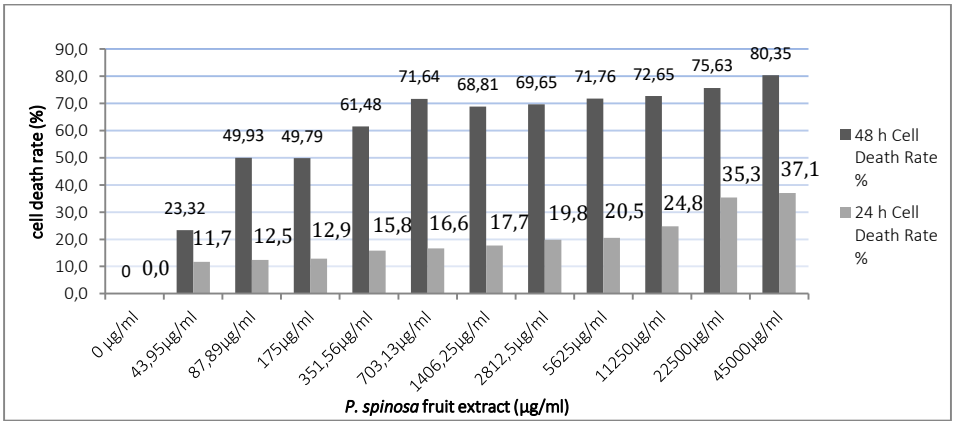

Figure 1. HT-29 colon cancer cell line proliferation inhibition after 24 and 48-hour *P. spinosa* fruit extract treatment measured by using MTT assay.

The cell death rate increased as the concentration increased.

Table 1. IC_{20}, IC_{50}, and IC_{80} inhibition concentration values after 24 and 48-hour treatment on HT-29 cell using MTT and Probit analyses

Treatment period	IC_{20}	IC_{50}	IC_{80}
24 h	> 1000 µg/ml	> 1000 µg/ml	> 1000 µg/ml
48 h	1.27 µg/ml	173.7 µg/ml	> 1000 µg/ml

The apoptotic effect was assessed through cell counting utilizing the EB/AO test method, a technique employed to distinguish between live and dead cells. Cell viability was analyzed after treating the cells with the herb extract at concentrations of 44 µg/ml, 88 µg/ml, and 45000 µg/ml for 24 and 48-hour time points. Morphological evaluations of cells were conducted, revealing the presence of viable, necrotic, and apoptotic cells. The morphological alterations of the cells are depicted in Figure 2. Table 2 provides the percentages of viable, necrotic, and dead cells after treatment with three different concentrations of *P. spinosa* extract on the HT-29 cell line over 24 and 48 hours. The results of the 24 and 48-hour treatments indicate that *P. spinosa* extract induces cell death, with the extent of cell death showing a significant increase with both concentration and duration ($p < 0.01$, $p < 0.001$). Notably, the results exhibit a substantial rise in cell death with escalating concentrations, as illustrated in Figure 3. The IC_{20}, IC_{50}, and IC_{80} values obtained from the EB/AO double coloring test, analyzed using Probit analysis software for both 24 and 48-hour treatment periods, are presented in Table 3. According to the Probit analysis, the IC_{50} value is 159.3 for the 24-hour treatment and 123.8 µg/ml for the 48-hour treatment.

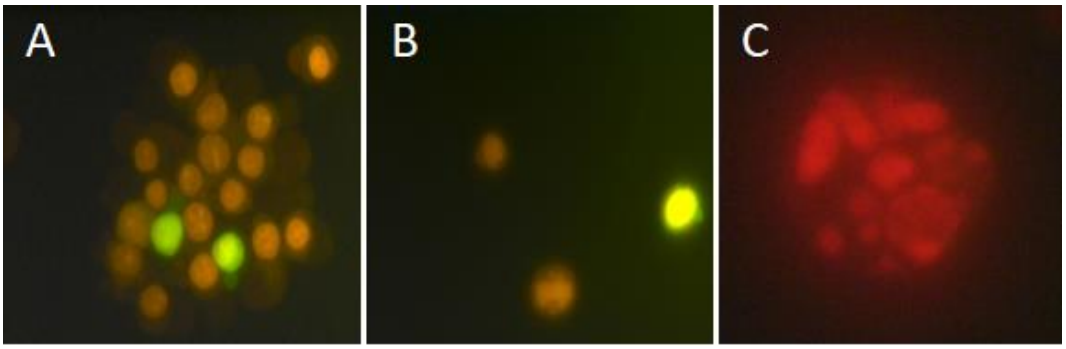

Figure 2. Fluorescence images of HT-29 cells after treatment with *P. spinosa* fruit water extract using the EB/AO dual staining method
(A) green cells are live and orange cells are necrotic (40x). (B) Orange cells were identified as apoptotic due to changes in morphology (40x). (C) apoptotic cell (100x).

Table 2. Percentage of viable, necrotic, and apoptotic HT-29 cells observed after three different concentrations of *P. spinosa* extract treatment after 24 and 48 hours, by EB/AO staining

Concentration (µg/ml)	Viable normal cells (%)	Necrotic cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Total cell viability (%)	Total apoptosis (%)
24 h						
Control	86.78 ± 6.05	7.7 ± 6.4	0 ± 0	5.4 ± 1.2	94.5 ± 6.2	5.44
44	58.63 ± 7.83[b]	0 ± 0	36.63 ± 5.04[b]	4.7 ± 2.7	58.6 ± 7.8[b]	41.38[b]
88	53.79 ± 5.51[b]	0 ± 0	42.97 ± 6.61[b]	3.2 ± 1.1	53.7 ± 5.5[b]	46.2[b]
45000	17.37 ± 0.26[b]	0 ± 0	81.62 ± 1.34[b]	1.01 ± 1.0	17.3 ± 0.2[b]	82.63[b]
48 h						
Control	75.74 ± 7.67	21.9 ± 8.9	0 ± 0	2.3 ± 1.3	97.6 ± 7.5	2.32
44	54.52 ± 5.64[a]	0 ± 0	42.45 ± 5.74[b]	3.0 ± 0.1	54.5 ± 5.6[a]	45.48[a]
88	52.34 ± 2.2[a]	0 ± 0	42.76 ± 2.27[b]	4.9 ± 0.6	52.3 ± 2.2[a]	47.66[a]
45000	5.51 ± 0.17[b]	0 ± 0	0 ± 0	94.4 ± 0.2[b]	5.5 ± 0.17[b]	94.49[b]

[a]= $p < 0.01$, [b]= $p < 0.001$

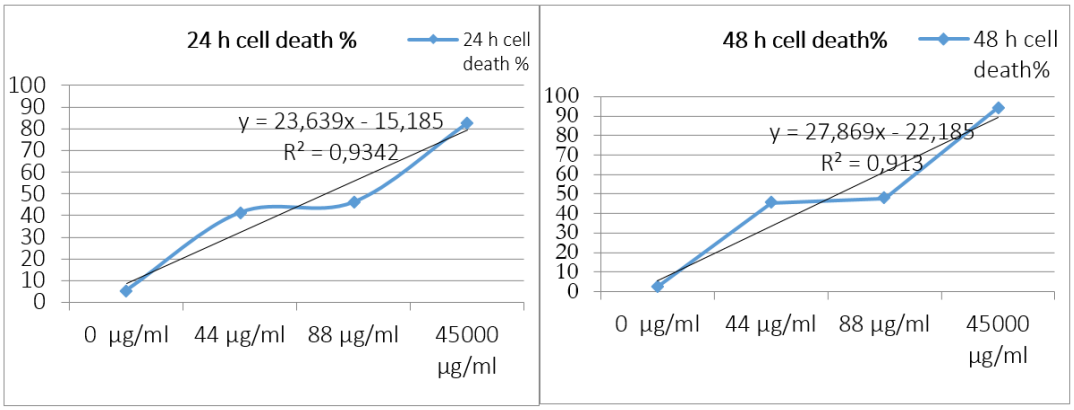

Figure 3. Apoptosis rates obtained after 24 and 48-hour treatment of *P. spinosa* fruit extract on HT-29 cells using the EB/AO dual staining method

Cell viability was detected on the HT-29 cancer cell line after exposure to different concentrations of *P. spinosa* water extract using the EB/AO dual staining method (Table 2).

The MTT viability test revealed a decrease in absorbance values after 24 and 48 hours compared to the control group. Notably, the IC_{50} value was determined to be 173.7 µg/ml following 48 hours of treatment. Similarly, employing the EB/AO method demonstrated

the maximum apoptotic effect at 48 hours, revealing an IC_{50} value of 123.8 µg/ml.

In recent years, to demonstrate the high cytotoxic effect of *P. spinosa*, extracts of different parts of the plant prepared in different solvents have been tested on cancer cell lines. *P. spinosa* fruit ethanol extract was tested on HepG2, Hepa1-6 (Murati et al., 2016), PC-3, and DU145 cells (Dedić et al., 2023), and methanol extract on MCF-7, NCI-H40, HCT-15, HeLa, and HepG2 cells (Guimarães et al., 2014), stem, leaves, fruits of *P. spinosa* with different solvents (Pinacho et al., 2015), and flowers (Karakas et al., 2019; Murati et al., 2019) on cancer cell lines. The types and quantities of phenolic compounds found in plant extracts differ, as well as their antiproliferative and cytotoxic effects on the cell lines, and the IC_{50} values detected may differ depending on the cell line used, the duration of the extract, the solvent, and the plant part. In our study, the effect on the cell viability of *P. spinosa* fruit water extract was tested by using MTT and EB/AO staining methods on the HT29 cell line. According to the probit analysis of *P. spinosa* fruit water extract, the MTT-viability test absorbance values were found to be decreased compared to control after 24 hours and 48 hours of treatment, and the IC_{50} value of 48th hour was found to be 173.7 µg/ml. Similarly, according to the EB/AO method, the apoptotic effect was detected at 48-hour treatment, and the IC_{50} value was determined as 123.8 µg/ml.

Table 3. IC_{20}, IC_{50}, and IC_{80} values obtained after 24 and 48-hour treatment by EB/AO double staining, according to Probit analysis

Incubation time	IC_{20}	IC_{50}	IC_{80}
24 h EB/AO	1.018 µg/ml	159.3 µg/ml	24908 µg/ml
48 h EB/AO	5.06 µg/ml	123.8 µg/ml	3031 µg/ml

Different solvents and different plant parts were used in other studies with *P. spinosa*. According to the part of the plant and the type of solvent; the variety, amount, antiproliferative and cytotoxic activities of the obtained phenolic compounds differ. Pinacho et al. (2015) reported that phenolic, flavonoid, and anthocyanin amounts obtained from *P. spinosa* stem, leaves, and fruits by extraction with different solvents (dichloromethane, ethyl acetate, ethanol, and water) differ in containing phenolic, flavonoid and anthocyanin amounts relating to the herb parts. The antiproliferative activities of water and ethanol extracts from different parts of plants exhibit variations (Nabende, 2015). The methanol extract from the leaves of *P. africana* was found to have higher antiproliferative activity on the mouse breast cancer cell line (4T1) and mouse colon cancer cell line (CT-26) compared to its water extract. However, body shell water extract had a higher antiproliferative effect on a colon cancer line than a breast cancer line (Nabende, 2015). Murati et al. (2016) found that alcohol extract of *P. spinosa* showed antiproliferative and prooxidant activity in mouse (Hepa1-6) and human (HepG2) liver cancer cell lines. 72nd hour IC_{50} values of *P. spinosa* ethanol extract in HepG2 and Hepa1-6 cell lines were determined as 68.2 µg/ml and 85.41 µg/ml, respectively (Murati et al., 2016). The reason behind these values being lower than those in our study might be the extended duration (72 hours), the utilization of ethanol extract, or the specific assay applied. In our investigation, the IC_{50} values of the water extract and the 48-hour results were evaluated and were found to be higher than the 72nd hour IC_{50} values.

The growth inhibition (48 hour GI_{50}) values of the non-anthocyanin phenolic compound enriched extract (methanol:water 80:20) of *P. spinosa* fruits on MCF-7 (breast carcinoma), NCI-H40 (non-small lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) were determined as 270.65, 154.25, 220.44, 193.62, 169.56 µg/ml, respectively (Guimarães et al., 2014). These values were nearly identical to the IC_{50} values obtained in the results of the present study, where the IC_{50} values were 173.7 µg/ml and 123.8 µg/ml for MTT and EB/AO assays, respectively. This correlation could be attributed to the exclusive presence of phenolic acids and the highest levels of flavones and flavonols for *P. spinosa* phenolic extract. The researchers also confirmed that the samples were non-toxic on non-tumor liver primary culture (Guimarães et al., 2014). Dedić et al. (2023) also tested *P. spinosa* fruit extract on PC-3 and DU145 malignant prostate cell lines, indicating that the extract had dose-dependent antiproliferative activity on malignant cells. The fruit extracts exhibited 72-hour IC_{50} values ranging from 637 µg/ml to 950 µg/ml for PC-3 and from 385 µg/ml to > 1000 µg/ml for DU145 cells (Dedić et al., 2023). To compare our results, the aqueous extract of *P. spinosa* fruit IC_{50} values were lower (173.7 µg/ml and 123.8 µg/ml) indeed in the short-term (48 hours) treatments. Similar to the study of Dedić et al. (2023), a dose-dependent antiproliferative effect was also observed in the present study.

The 24-hour cell growth inhibition of HT-29 cells was found as IC_{50} 4790 µg-dw/ml after exposure to methanol/water extract of *P. spinosa* (BL1 genotype). IC_{50} values of the other genotypes ranged between 5880 and 28480 µg-dw/ml (Popović et al., 2020). In our study, 24-hour IC_{50} was found at over 1000 µg/ml, consistent with the study. Popović et al. (2020) also found a positive correlation between HT-29 cell toxicity and the 3-pCoQA hydroxycinnamic acid component of the extract. Hydroxycinnamic acids are the largest class of phenolic compounds, scavenge free radicals, and are associated with reduced cancer risk (Herrmann & Nagel, 1989; Huang et al., 1986; Manach et al., 2004; Spencer et al., 2008). It is also suggested that these compounds might modulate the activity of specific enzymes and inhibit cell proliferation (Manach et al., 2004). The anticancerogenic effects of hydroxycinnamic acids were studied on several cancer cell lines (Janicke et al., 2011; Kurata et al., 2007; Puangpraphant et al., 2011). It was shown that diCQA fractions inhibited the proliferation of RKO and HT-29 human colon cancer cells by inducing apoptosis rather than arresting the cell cycle. Apoptosis occurs via induction of Bax and Bcl-2 protein expression and diCQA induced the cleavage of procaspase-3 to active caspase-3 (Puangpraphant et al., 2011), which might explain the apoptotic activity in the present study. Similar results were obtained in our study; *P. spinosa* fruit water extract showed antiproliferative effects on the HT-29 colon cancer cell line according to the MTT test, and it was found to induce apoptosis according to the EB/AO test. IC_{50} values were obtained after 24 and 48 hours of treatment, as examined according to the EB/AO dual staining method; after 48-hour treatment, late apoptotic cells were observed more than at 24 hours. Remarkably, there was a significant decrease in the number of cells at high concentrations. It was found that the antiproliferative effect increased and showed a statistically significant difference compared to the control cells.

4. Conclusions

Our findings reveal that the water extract from *P. spinosa* fruit exerts an inhibitory impact on cell viability of the HT-29 cell line, demonstrating cytotoxic and apoptotic effects in a concentration- and time-dependent manner. Leveraging more advanced extraction methods to isolate metabolites from fruit extracts and conducting further investigations to elucidate the activities of these metabolites have the potential to enhance the economic value of *P. spinosa* and position it as a natural remedy in the anticancer pharmaceutical industry. This study highlights the cytotoxic impact of *P. spinosa* fruit water extract on the HT29 colon cancer cell line and its capacity to

impede cell viability. In toxicity-focused examinations, it has been observed that MTT and EB/AO methods complement each other, yielding comparable outcomes.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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CRediT authorship contribution statement

Fulya Dilek Gökalp: Investigation, Writing - original draft, Visualization, Supervision
Egzona Qipa: Investigation, Resources, Methodology

ORCID Numbers of the Authors

F. D.Gökalp: 0000-0001-8219-6657
E. Qipa: 0000-0002-1828-4123

Supplementary File

None.

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