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Biological activities of *Helleborus vesicarius*: Cytotoxicity, induction of apoptosis and antioxidant potential

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ABSTRACT: *Helleborus vesicarius*, an endemic species, grows in South Anatolia, the Amanos Mountains, Kahramanmaraş, and the Gaziantep region. Biological activity studies on *H. vesicarius* are restricted. Therefore, the study aims to investigate the antioxidant potential, DNA-protecting capacity, cytotoxic activity and apoptotic effect of *H. vesicarius*. In this study, the phenolic and flavanoid contents of *H. vesicarius* methanol extract were determined as 18.95 ± 0.65 mg/g (expressed as gallic acid equivalent) and 18.97 ± 0.17 mg/g (expressed as catechin equivalent), respectively. The extract displayed an inhibitory capacity against $FeCl_3$/ascorbic acid-induced phosphatidylcholine liposome oxidation (EC_{50} = 5.16 ± 0.12 mg/mL), eliminated stable 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^+$), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion (O_2^-) radicals (EC_{50} = 4.23 ± 0.05 mg/mL; 3.87 ± 0.24 mg/mL; 1.20 ± 0.06, respectively), and act as reducing agent as expressed by the Ferric reducing antioxidant power (FRAP) value (2.87 ± 0.04 mM/L Fe^{2+}) at 10 mg/mL. Furthermore, DNA-protecting activity was identified by DNA nicking assay. The extract dose-dependently protected pBR322 plasmid DNA against hydroxyl (OH) radical formed by the Fenton reagent. Also, cytotoxic activity was established on lung (A549), prostate (PC3), endometrial (ECC-1) cancer cells and primary dermal fibroblast (PCS) cells by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. The cytotoxic effect of the extract was more powerful on A549 (IC_{50}: 45.80 µg/mL) and PC3 (IC_{50}: 54.97 µg/mL) cell lines compared to that of the ECC-1 (IC_{50}: 367.62 µg/mL). The selectivity indexes for A549, PC3 and ECC-1 cell lines were 14.46, 12.09 and 1.81, respectively. Additionally, caspase-3 activity was also evaluated via the colorimetric method, the results revealed that the extract triggered apoptosis via active caspase-3 formation in the PC-3 cells. It was concluded that the extract may be used as a source of natural anticancer agents. Further investigations are needed to clarify the cytotoxic mechanism of the extract.

KEYWORDS: Anticancer; caspase-3 activity; DNA protecting activity; antioxidant; *Helleborus vesicarius*

1. INTRODUCTION

The increasing prevalence of cancer worldwide has highlighted the urgent need for effective treatment strategies. Among the promising strategies, compounds derived from natural sources offer significant potential. In this regard, plants serve as valuable sources of natural cytotoxic and antioxidant compounds [1, 2]. The beneficial effects of medicinal and edible endemic plants make them interesting for drug discovery [3]. Many herbal products may exhibit notable pharmacological activity that promises to be effective in the treatment of cancer [4].

The genus *Helleborus* L., belonging to the *Ranunculaceae* family, comprises approximately 20 species [5]. *Helleborus* species demonstrate a range of significant biological activities, including antimicrobial, antiinflammatory, antiparasitic, antihyperglycemic, antioxidant, and anticancer [6-8]. The application of this genus in traditional medicine due to their biological activities is particularly noteworthy. For instance, *Helleborus* extracts are used for their immunostimulatory effects in Romanian traditional medicine [9]. Additionally, an original drug, Boicil, developed and patented (US patent) in Romania from the extracts, has analgesic and antirheumatic effects [10]. Another *Helleborus* species, *Helleborus niger,* is used as an adjuvant

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agent in tumor therapy via subcutaneous injection in anthroposophical medicine in Germany. It is also used in the treatment of tumor-related conditions such as hydrocephalus, convulsions, and edema in hemopathy [9].

In Türkiye, the genus *Helleborus* L. is represented by two species: *Helleborus orientalis* Lam. and *Helleborus vesicarius* Aucher. *H. orientalis* is native to Greece and Türkiye. Its rhizomes have been used in Europe to treat heart failure and constipation. In Turkish folk medicine, they are commonly used to treat ailments such as rheumatism, toothache, and edema (for cattle) [11, 12]. *H. orientalis* has been reported to have antinociceptive and antiinflammatory properties [13], along with antioxidant properties [14]. Furthermore, compounds isolated from this plant have been found to display cytotoxic activity on cancer cells [12, 15, 16]. *H. vesicarius*, known by the vernacular names "patlak çiçeği" and "patlak otu", is an endemic species that grows in South Anatolia, the Amanos Mountains, Kahramanmaraş, and the Gaziantep region [17, 18, 19]. Definitive information on the traditional uses of *H. vesicarius* is lacking, and also, knowledge of its biological activities remains limited. To date, only two studies have documented its antimicrobial [20] and antioxidant [21] effects. In the antioxidant activity study of *H. vesicarius* [21], only total antioxidant and oxidant statuses have been investigated. More mechanistic antioxidant assays, such as Ferric reducing antioxidant power (FRAP) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH), have not been conducted. Expanding research into its biological properties is crucial for fully elucidating its therapeutic potential and understanding its broader medicinal applications. Given the limited existing literature, this study provides important new insights into its biological properties, highlighting its novelty and significance. Therefore, this study sought to further explore the antioxidant efficacy of *H. vesicarius* and, for the first time, to assess its DNA protecting capacity, cytotoxic activity, and apoptotic effects.

2. RESULTS AND DISCUSSION

Helleborus species are known to have antitumor, immunomodulatory, antioxidant, cytotoxic effects, and DNA protective activity due to their rich content of phytoconstituents [7, 22, 14]. In the present study, total phenolic (TPC) and flavonoid contents (TFC) of *H. vesicarius* methanol extract were determined as 18.95 ± 0.65 mg/g (expressed as gallic acid equivalent) and 18.97 ± 0.17 mg/g (expressed as catechin equivalent), respectively (Table 1).

There is no study to determine the TPC and TFC of *H. vesicarius* in the literature. However, numerous studies in the literature investigate the TPC and TFC of other *Helleborus* species, and these contents vary between different species and also within the same species. For instance, according to Čakar et al. (2011) [23], TPC in root and leaf extracts from *Helleborus odorus, Helleborus multifidus,* and *Helleborus hercegovinus* varied from 7.00 to 242.41 mg catechin equivalent (CAE)/g]. In another study, TPC of *Helleborus lucidus, Helleborus foetidus*, *H. niger*, *H. odurus* and *Helleborus purpurascens* root ethanol extract were 0.089 ± 0.054, 0.153 ± 0.012, 0.036 ± 0.023, 0.272 ± 0.073 and 0.039± 0.017 mg gallic acid equivalent (GAE)/g, respectively [24]. Additionally, TPC and TFC of *H. purpurascens* ethanolic extract were 39.18 ± 2.60 mg CAE/g and 19.32 ± 2.78 mg quercetin equivalent (QE)/g, respectively [25]. In a study conducted by Paun et al. (2014) [26], TPC and TFC of *H. purpurascens* extract were 463.1 ± 8.4 mg GAE/L and 29.3 ± 4.9 mg QE/L. Furthermore, TPC and TFC of different parts of *H. orientalis* varied [14]. Hence, our findings align well with the existing literature in this regard.

Table 1. Total extractable compounds, total phenolic and flavonoid contents in the extract.

	EC (g/g DW)	TPC (mg/g extract)	TPC (mg/g DW)	TPC/EC (%)	TFC (mg/g extract)	TFC (mg/g DW)
H. vesicarius methanol extract	0.343	18.95 ± 0.65	6.49 ± 0.23	1.8	18.97 ± 0.17	6.5 ± 0.05

Data were expressed as mean ± standard deviation (SD) (n=3). EC: Total extractable compounds, DW: Dry weight, TFC: Total flavonoid contents, TPC: Total phenolic content

The generation of reactive oxygen and nitrogen species (oxidants) results in the damage of macromolecules (protein, lipid, nucleic acid) when the balance with the antioxidant system is disrupted and oxidative stress is closely linked to various diseases such as cancer, Alzheimer's disease, atherosclerosis etc. [27]. Due to the importance of oxidative stress in the development of diseases, a large number of studies evaluated the antioxidant activity of different *Helleborus* species.

According to these studies, *H. purpurescens* is the most studied species for its antioxidant properties. *H. purpurescens* has a rich antioxidant content and, therefore, it shows high antioxidant activity [7, 24 , 28]. Apetrei et al. (2011) [29] carried out a study of *H. purpurescens* and stated that *H. purpurascens* reduces reactive oxygen species (ROS) levels, thereby decreasing ROS-induced damage. A study indicated that *H. orientalis* extract has a high antioxidant activity [14]. Similarly, *H. niger* extract has shown a high antioxidant activity [30]. Also, *H. odorus* Waldst. & Kit, *H. multifidus* Vis, *H. hercegovinus* Martinis, and *H. foetidus* L. extracts exerted antioxidant activity [23, 24]. Based on our literature survey, there is only one study reporting the antioxidant features of *H. vesicarius*. The study reported that the plant has high antioxidant potential with values 0.25, 5.55, and 13.78 for oxidative stress index, total antioxidant, and oxidant status, respectively [21].

In the present study, the extract was assessed for its antioxidant potential with ferric-reducing capacity, inhibitory activity of lipid peroxidation and radical scavenging activity against 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^+$), DPPH, and superoxide anion ($O_2^{\cdot-}$) radicals (Table 2). The extract (at 10 mg/mL) showed marked antioxidant activity of 87.12 ± 1.2 %, measured in the phospholipid model system. According to the half-maximal effective concentration (EC50) values, the lipid peroxidation (LPO) inhibitory (anti-LPO) activity of the extract with 5.16 ± 0.12 mg/mL was lower than that of rutin (0.79 ± 0.04 mg/mL) (Table 2). There is no study in the literature regarding the anti-LPO activity of *H. vesicarius*. In addition, there are few studies on the anti-LPO activity of *Helleborus* species. In a study conducted by Cakar et al. (2013) [31], the water and ethanol extracts of different parts of *H. odorus*, *H. multifidus*, and *H. hercegovinus* (at 2.5 mg/mL) showed anti-LPO activity in a wide range (4.41-81.44%). The highest anti-LPO activity was found in (81.44%) *H. multifidus* leaves water extract. In another study, the compounds isolated from *Helleborus cyclophyllus* ethyl acetate extract showed anti-LPO activity (EC_{50}: 15.7-97.3%) at a concentration of 100 µM [32].

The extract also showed the highest superoxide anion radical ($O_2^{\cdot-}$) scavenging activity by 92.27 ± 0.09 % at 5 mg/mL which is comparable to rutin (94.09 ± 0.12 %) at 1.25 mg/mL ($p>0.05$). Based on the EC_{50} values, $O_2^{\cdot-}$ scavenging activity of the extract with 1.20 ± 0.06 mg/mL was lower than that of rutin (0.59 ± 0.06 mg/mL)($p<0.05$)(Table 2). There is no data on the $O_2^{\cdot-}$ scavenging activity of *Helleborus* species in the literature.

The DPPH radical ($DPPH^{\cdot}$) scavenging activity of the extract (94.90 ± 2.6 %) at 10 mg/mL was similar ($p>0.05$) to that of rutin (93.50 ± 0.3 %) at 0.31 mg/mL. The results indicated that rutin having a lower EC_{50} of 0.150 ± 0.07 mg/mL, is a very potent $DPPH^{\cdot}$ scavenger, but the extract (3.87 ± 0.24 mg/mL) showed significantly lower ($p<0.05$) radical scavenging activity (Table 2). A previous study carried out on the $DPPH^{\cdot}$ scavenging activity of the leaf and root extracts from *H. odorus*, *H. multifidus,* and *H. hercegovinus* demonstrated that the leaf extracts had shown higher activity (EC_{50}: 0.19-0.92 mg/mL) than the root extracts (EC_{50}: 1.16 mg/mL for *H. odorus*; 3.51 mg/mL for *H. hercegovinus*) [23]. Studies displayed that *H. purpurascens* concentrated aqueous and hydro-alcoholic extracts showed high $DPPH^{\cdot}$ scavenging activity [7, 28]. On the other hand, some studies reported that *H. purpurascens* methanol/chloroform extract exhibited moderate $DPPH^{\cdot}$ scavenging activity (EC_{50}: 3.86 ± 0.16 mg/mL) and *H. purpurascens* root ethanol extracts had no $DPPH^{\cdot}$ scavenging activity [24, 33]. In a study conducted by Malik et al. (2017) [24], while no $DPPH^{\cdot}$ scavenging activity was observed in the root ethanol extracts of *H. lucidus, H. niger,* and *H. odorus*, $DPPH^{\cdot}$ scavenging activity of *H. foetidus* was 0.172 ± 0.013 trolox equivalent (TE). Also, the $DPPH^{\cdot}$ scavenging activity of different parts of *H. orientalis* methanol extract was EC_{50} (µL) values of 13–40 (µL/mL) [21]. Our results were similar to that of the root extract of *H. hercegovinus* [23] and the methanol/chloroform extract of *H. purpurascens* [33].

Moreover, in the present study, the $ABTS^+$ radical ($ABTS^{\cdot+}$) scavenging activity of the extract was 2.18 ± 0.005 mM/L Trolox equivalent at 10 mg/mL (Table 2). The EC_{50} values indicated that the extract has significantly lower $ABTS^{\cdot+}$ scavenging activity (4.23 ± 0.06 mg/mL) compared to that of the rutin with a value of 0.58 ± 0.02 mg/mL (Table 2)($p<0.05$). Our results, showing $ABTS^{\cdot+}$ scavenging activity for the extract, are supported by Trolox equivalent antioxidant capacity $(TEAC)^{ABTS}$ values for aqueous and hydroalcoholic extracts of *H. purpurascens* [25, 28].

Also, the FRAP of the extract was investigated according to the ability to reduce ferric (III) iron to ferro (II) iron. The extract showed similar antioxidant activity (2.87 ± 0.04 mM/L Fe^{2+}) at 10 mg/mL to that of the rutin (2.75 ± 0.04 mM/L Fe^{2+}) at 1.25 mg/mL ($p>0.05$). There is no study in the literature on the ferric-reducing power of *Helleborus* species.

Table 2. Antioxidant activities of the extract.

	EC_{50} (mg/mL)				$TEAC_{ABTS}$ [a] (mM TEAC/L)	FRAP [b] (mM Fe^{2+}/L)
	Anti-LPO	O_2^-	DPPH	$ABTS^+$		
H. vesicarius	5.16 ± 0.12	1.20 ± 0.06	3.87 ± 0.24	4.23 ± 0.05	2.18 ± 0.005*	2.87 ± 0.04*
Rutin	0.79 ± 0.04	0.59 ± 0.06	0.155 ± 0.08	0.55 ± 0.04	2.13 ± 0.004**	2.79 ± 0.03**

[a] expressed as mmol Trolox equivalent; [b] expressed as mmol Fe (II) ions
* was evaluated at 10 mg/mL; ** was evaluated at 1.25 mg/mL
Findings were expressed as mean ± standard deviation (SD) (n = 3).
ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-Diphenyl-1-picrylhydrazyl, EC50: half-maximal effective concentration, FRAP: Ferric reducing antioxidant power, LPO: Lipid peroxidation, TEAC: Trolox equivalent antioxidant capacity.

Considering the EC_{50} values, it was seen that the extract was a more efficient scavenger of $O2^{-\cdot}$ compared to the efficacy to protect liposomes from LPO and scavenge DPPH and ABTS+ radicals, but less active than rutin ($p<0.05$). Despite its mild reactivity, the $O2^{-\cdot}$ holds significance as it serves as the precursor to the hydroxyl radical, which is an aggressive radical and is formed through the Fenton/Haber-Weiss reaction within biological systems [34]. It is noteworthy that the extract demonstrates its most effective radical scavenging activity specifically against the $O2^{-\cdot}$.

DNA strand breaks arise in the cells from the attack by reactive oxygen species and other electrophilic molecules, and abnormal DNA repair mechanisms. These breaks in DNA strands are associated with diseases such as cancer, immune dysfunction, and neurodegeneration [35]. The DNA protective effect of the extract was assayed by "DNA nicking assay" which is based on the Fenton reaction, using pBR322 plasmid DNA. Our findings showed that *H. vesicarius* extract dose-dependently decreased the nicked DNA formation. These results are statistically significant ($p<0.005$). The extracts lead to maximum inhibition (100%) at 50µg/mL concentration. Even with the lowest dose, 1.5625 µg/mL, 80.6% inhibition was observed. So far, there is no knowledge in terms of the DNA protection capacity of *H. vesicarius* extract. Thus, this study reported the first time that *H. vesicarius* possessed DNA protection activity. According to the data, *H. vesicarius* has a quite high degree of DNA damage protection capacity (Figure 1). In the literature, only one study reported that *H. orientalis* extract has a significant DNA protective effect at doses of 2500 µg/mL and above [14].

Plants are an important source in discovering cancer drugs and have played a vital role in developing chemotherapeutic agents. While the mechanisms of action of plant-derived anticancer drugs are diverse, apoptotic cell death holds a significant place. Inducing cell death mechanisms through activation of apoptosis is an effective strategy in cancer treatment. Moreover, caspase family proteases play a crucial role in apoptosis, with caspase-3 acting as the primary executioner [36, 37]. A great deal of *Helleborus* species are currently being considered a promising cytotoxic plant. Regarding the potential antitumor effect of *H. vesicarius*, the cytotoxic activity of the extract was determined on A549 (lung), PC3 (prostate), and ECC-1 (endometrial) cancer cell lines and primary dermal fibroblast (PCS) cell line (Table 3). The findings showed that the extract possessed moderate cytotoxic activity on A549 (Half maximal inhibitory concentration (IC50): 45.80 µg/mL) and PC3 (IC_{50}: 54.97 µg/mL) cell lines but weak activity on ECC-1 (IC_{50}: 367.62 µg/mL) and PCS (IC_{50}: 664.90 µg/mL) cell lines (Table 3). The selectivity indexes of the extract for A549 and PC3 cell lines were higher than those for ECC-1. So, the extract was selective against the A549 and PC3 cell lines.

Furthermore, active caspase-3 activity was also evaluated in PC3 and A549 cell lines. The results indicated that the extract (21.62 µmol pNA/min/mL) triggers caspase-3 activity in the PC3 cell line ($p<0.05$) (Figure 2). However, there was no caspase-3 activation in the A549 cells treated with the extract. Hence, the extract has cytotoxic selectivity and its cytotoxic effect mechanism in PC3 is an activation of caspase-3. However, the cytotoxic effect mechanism of *H. vesicarius* may be different for various types of cancer.

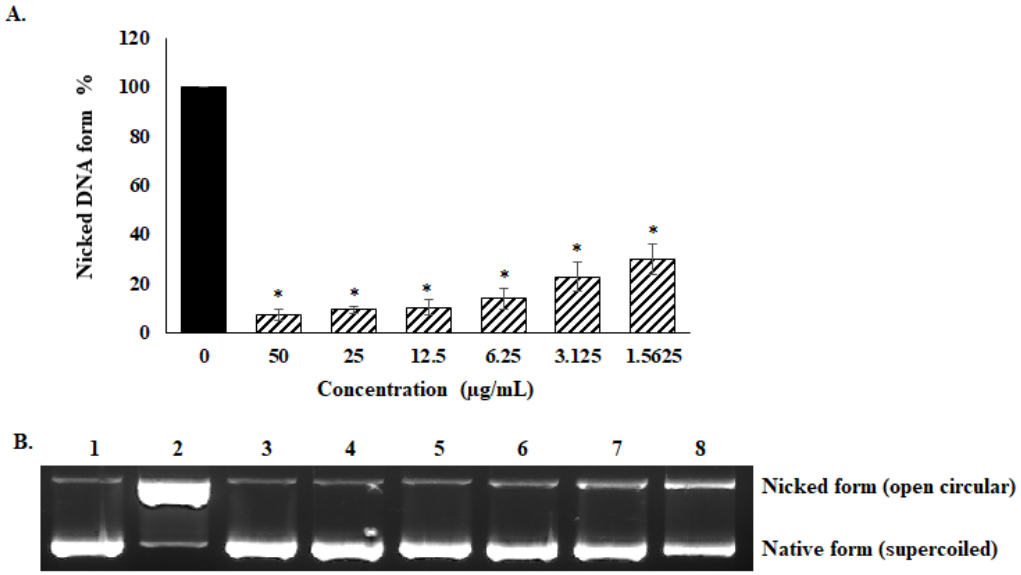

Figure 1. DNA protective effect of the extract against hydroxyl radicals.
*p<0.005
A. Density of nicked circular DNA form (Data were expressed as mean ± standard deviation).
B. The image of the DNAs on the gel.
Line 1. Control (ddH_2O and DNA)
Line 2. Negative control (DNA and Fenton reagent (FR))
Line 3. 50µg/mL the extract, DNA, and FR
Line 4. 25µg/mL the extract, DNA, and FR
Line 5. 12.5µg/mL the extract, DNA, and FR
Line 6. 6.25µg/mL the extract, DNA, and FR
Line 7. 3.125µg/mL the extract, DNA, and FR
Line 8. 1.5625µg/mL the extract, DNA, and FR

Table 3. Cytotoxic activity of the extract**.**

	IC_{50} (µg/mL)			
	A549	PC3	ECC-1	PCS
The extract	45.80 ± 1.26	54.97 ± 3.12	367.62 ± 13.54	664.90 ± 48.12
Selectivity Index (SI)	14.46	12.09	1.81	

Data were expressed as mean ± standard deviation (SD) (n = 3). IC_{50}: Half maximal inhibitory concentration

There are many studies in the literature investigating the cytotoxic and apoptotic effects of *Helleborus* species. In a study, *H. niger* extract was shown to have a cytotoxic effect and cell migration inhibitory effect on various cancer cell lines [38]. Similarly, another study indicated that the compounds isolated from *H. niger* exerted high cytotoxicity (IC_{50}: 0.0055-1.9 µM) against human cancer cells (HL-60, A549, and SBC-3) and some of the compounds caused activation of caspase-3/7 on HL-60 cells [39]. The studies stated that *H. niger* and *H. foetidus* inhibit cell proliferation in leukemia cells and this inhibition occurs as a result of induction of apoptosis through caspase-3 activation [40, 41]. The compounds isolated from *H. foetidus* showed potent cytotoxicity against HL-60 and A549 cells. One of the compounds displayed apoptotic effects such as caspase-3 activation and loss of mitochondrial membrane potential on HL-60 cells [42]. Furthermore, studies indicated that the compounds isolated from *Helleborus thibetanus* and *Helleborus lividus* were cytotoxic [43-45], and a novel cardiac glycoside isolated from *H. thibetanus* exhibited potent anticancer activity through induced apoptosis in cancer cells [46]. In the studies investigating the anticancer effects of *H. purpurascens*, the plant showed a strong antiproliferative effect on HeLa cells at a low dose [47] and the plant extract changed pro- and antiapoptotic protein expressions in favor of apoptosis by decreasing Bcl-2 and increasing Bad and Bax [7]. In like manner, the compounds isolated from *Helleborus caucasicus* showed potent cytotoxic effects on various cancer cell lines (Calu-1, HepG2, and Caco-2) and some of these effects have been associated with induction of apoptosis through decreased entry into S-phase, increased caspase-3 activity,

and decreased GRP78 protein expression [48]. These studies have concluded that the compounds responsible for the cytotoxic effects in *Helleborus* species are primarily steroidal in structure [39, 41, 42,45, 46, 48].

So far, there is no knowledge in terms of the cytotoxic and apoptotic effects of *H. vesicarius*. Thus, this study reported that *H. vesicarius* possessed cytotoxic and caspase-3 activating effects for the first time. Our results are consistent with the findings of previous studies regarding other *Helleborus* species.

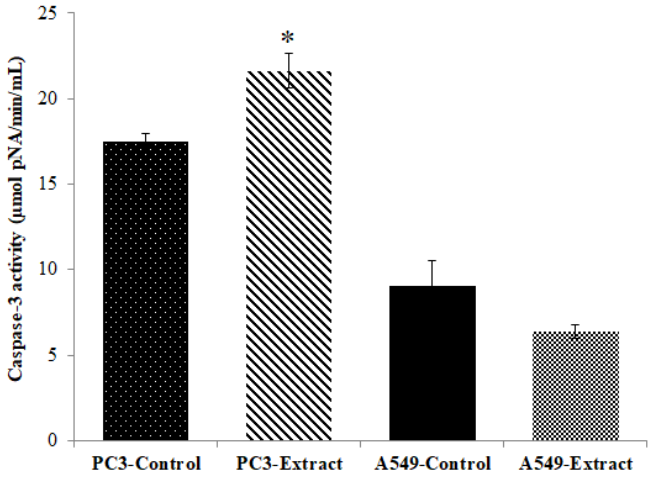

Figure 2. Caspase-3 activation on PC3 cells by the extract.
* $p<0.05$

3. CONCLUSION

In conclusion, *H. vesicarius* methanol extract exhibits moderate antioxidant and cytotoxic activities. The antioxidant effects were observed at higher concentrations than those required for cytotoxicity. Additionally, the extract demonstrated apoptotic activity in the PC3 cell line. These findings suggest that the plant may be a candidate for an anticancer agent instead of an antioxidant. Furthermore, it can be considered a natural antimutagenic source due to its DNA-protecting activity. However, further studies are required to elucidate the underlying mechanism of these effects and to identify the specific compounds in the plant responsible for these activities.

4. MATERIALS AND METHODS

4.1. Chemicals

Ferric chloride, thiobarbituric acid (TBA), trichloroacetate (TCA), and 2,4,6-tripyridyl-s-triazine (TPTZ) required for antioxidant activity were obtained from Merck (Darmstadt, Germany). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was purchased from Promega (Madison, Wisconsin, USA). The other chemicals and the caspase-3 assay kit (CASP3C) used in the present study were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Furthermore, fetal bovine serum (FBS) was purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany) and other cell culture requirements (medium, L-glutamine solution, 100 U/ml penicillin and 100 µg/ml streptomycin) were purchased from Wisent Bioproducts (St. Bruno, Canada).

4.2. Plant Material

The plant materials of *H. vesicarius* were collected from Kahramanmaraş, in April 2015. Identification and collection of the plant samples were performed by Prof.Dr. Sukran Kultur. Voucher specimens were stored in the Herbarium of the Faculty of Pharmacy, Istanbul University with the code (ISTE 116 557).

4.3. Preparation of the Methanol Extract

The dried aerial parts (160 g) of *H. vesicarius* were percolated with methanol in a percolator. The methanol extract was evaporated to dryness under reduced pressure and controlled temperature (40 to 50 ^{0}C) in a rotary evaporator. The extract (55 g) was kept at -20 ^{0}C for future experiments and dissolved in dimethyl sulfoxide before use.

4.4. Total Phenolic Compounds

Folin-Ciocalteu reagent was used to determine the quantity of total phenolic compounds in the extract [49]. Briefly, The Folin-Ciocalteu solution and the extract were mixed and incubated for 3 min. After that, 2% Na_2CO_3 solution was added and a further 2h incubation was performed. The absorbance was measured at 760 nm. The results were expressed as mg GAE/g dry weight (DW) of the plant material.

4.5. Total Flavonoids

The method of Sakanaka et al. (2005) [50] was used to quantify the flavonoids in the extract. The extract and $NaNO_2$ (5% w/v) were mixed and incubated for 6 min. Then, $AlCl_3$ (10% w/v) was added to the mixture and incubated for a further 5 min. The reaction was stopped by adding NaOH and the absorbance was recorded at 510 nm. The total flavonoid quantity was expressed as mg CE/g DW of the plant material.

4.6. The Inhibitory Effect on Lipid Peroxidation (LPO)

Thiobarbituric acid reactive substances (TBARS) were measured to evaluate the level of LPO [51]. Briefly, the extract was mixed with the lecithin solution (10 mM in phosphate buffer, pH 7.4) [52], $FeCl_3$, and ascorbic acid. Control (including all the contents except the test compounds) was prepared as well. After 1 h incubation at 37 ^{o}C, this mixture was added to TCA-TBA reagent containing 15% w/v TCA and 0.375% TBA in 0.25 N HCl and boiled for 10 min. The reaction mixture was left to cool down on the bench. Then, an equal volume of n-butanol was mixed with the solution and the mixture was centrifuged for 5 min at 3000 rpm and the formation of pink chromogen TBARS was recorded at 532 nm. Rutin was used as a standard. The percent inhibition of LPO was calculated based on the formula:

Inhibition effect of LPO (%)= [(1-Absorbance of sample/Absorbance of control)]x100

4.7. Total Antioxidant Capacity Assay

TEAC assay was performed to determine the total antioxidant capacity of the extract [53]. ABTS stock solution (7 mM) and potassium persulfate (2.45 mM) were mixed and kept in the dark at room temperature (RT) for 12-16 h to form $ABTS^{\cdot+}$. Then, the $ABTS^{\cdot+}$ solution was added to the extract, and any decrease in absorbance was recorded at 734 nm exactly at 6th min after the initial mixing. Rutin was used as a standard. The $ABTS^{\cdot+}$ scavenging capacity was determined based on the formula:

$ABTS^{\cdot+}$ scavenging activity (%)= [(1-Absorbance of sample/Absorbance of control)]x100
The results were expressed as mM Trolox equivalent.

4.8. DPPH Radical Scavenging Activity

The method of Brand-Williams et al. (1995) [54] was performed to determine the $DPPH^{\cdot}$ scavenging activity of the extract. Briefly, the extract was added to the methanolic solution of $DPPH^{\cdot}$ (60 µM). The mixture was incubated in the dark at RT for 30 min. The absorbance was determined at 517 nm. Rutin was used as a standard. The ability to scavenge $DPPH^{\cdot}$ was calculated based on the formula:

$DPPH^{\cdot}$ scavenging capacity (%)= [(1-Absorbance of sample/Absorbance of control)]x100

4.9. Superoxide Anion Radical Scavenging Activity

$O_2^{\cdot-}$ scavenging activity of the extract was determined using a modified nitroblue tetrazolium (NBT) reduction method [55]. NBT solution (150 µM, pH 7.4) and NADH solution (468 µM, pH 7.4) were added to the extract. The reaction was initiated by the addition of a 60 µM phenazine methosulfate solution. The mixture was incubated at 25 ^{o}C for 5 minutes and the absorbance was measured at 560 nm. Rutin was used as a standard. The $O_2^{\cdot-}$ scavenging capacity was determined based on the formula:

$O_2^{-\cdot}$ Scavenging activity (%)= [(1-Absorbance of sample/Absorbance of control)]x100

4.10. Ferric Reducing Antioxidant Power (FRAP) Assay

The procedure of Benzie and Strain (1996) [56] was used to assay the FRAP. Briefly, the extract and FRAP reagent (10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$, and 0.3 M acetate buffer (pH 3.6; ratio 1:1:10) were mixed, and the formation of a blue-colored Fe^{+2}-TPTZ complex was measured at 593 nm at 4th min. Rutin was used as a standard. The results were reported as mM Fe^{2+} equivalent.

4.11. DNA Nicking Assay

The DNA protecting capacity of the extract against hydroxyl radicals was estimated by the DNA nicking assay. The assay was accomplished using plasmid (pBR322) DNA and carried out based on the procedure previously described [57].

4.12. Cell Viability Assay (MTS Assay)

The effect of the extract on cell viability was executed on lung (A549, ATCC CCL-185), prostate (PC3, ATCC CRL-1435), and endometrial (ECC-1, ATCC CRL-2923) cancer cell lines and primary dermal fibroblast (PCS, ATCC PCS-201-012) cell line. Roswell Park Memorial Institute (RPMI 164), Dulbecco's Modified Eagle Medium (DMEM) and DMEM-F12 supplemented with 10% FBS, 1mM L-glutamine (for only ECC-1), 100 U/ml penicillin and 100 µg/ml streptomycin were used to maintained ECC-1, A549, PC3 and PCS cell lines, respectively. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2. To assess the cytotoxic activities of the extract, A549 ($2x10^4$), PC3 ($3x10^4$), ECC-1 ($5x10^4$) and PCS ($2x10^4$) were plated in 96-well plates and were exposed to different concentrations (1-0.0078 mg/mL) of the extract for 48 hours. Then, the MTS assay was performed as previously described [58]. The absorbance was read on a microplate reader (Biotek, Winooski, USA) at 490 nm. The inhibition of cell viability was expressed as an IC_{50} value.

4.13. Caspase-3 Activity Assay

The active caspase-3 activity was detected by a colorimetric kit (Sigma, CASP3C). Active caspase-3 breaks down the substrate (acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA)) into a p-nitroaniline moiety. The concentration of the p-nitroaniline was calculated by a calibration curve with absorbance at 405 nm using a microplate reader.

4.14. Statistical Analysis

An unpaired t-test and ANOVA variance analysis with the SPSS 21 (IBM SPSS, Türkiye) statistical computer package were performed to evaluate the results and expressed as means ± SD (n=3). The differences were considered statistically significant at $p<0.05$. The relationship between variables was evaluated using correlation analysis. IC_{50} values for the cytotoxic activity were calculated based on the inhibition % value of the extract. All the experiments were repeated 3 times.

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