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RESEARCH ARTICLE

Characteristics of the biological activities of the piperidine complex: an anticancer and antioxidant investigation

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ABSTRACT

Aim: To determine the anticancer and antioxidant activity levels of the synthesized heterocyclic molecule named 1-benzyl-1-(2- methyl-3-oxo-3-(p-tolyl)propyl) piperidin-1-ium chloride.

Methods: The molecule 1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl) propyl)piperidin-1-ium chloride was synthesized solvent-free via microwave synthesis. Piperidine purification involved dichloromethane extraction with 2 M HCl, followed by 5% NaHCO3 and precipitation with n-hexane. Anticancer activity on A549 lung cancer cells was assessed using the MTT assay. Antioxidant activity was evaluated by DPPH and CUPRAC methods at five concentrations (250-15.6 µM), with ascorbic acid as a control.

Results: The heterocyclic molecule dissolved in PBS was tested for anticancer activity on A549 cells at concentrations ranging from 6.25 to 100 µM. Cytotoxicity was highest at 66.90% for 100 µM and decreased to 5.57% at 6.25 µM, with an IC50 of 32.43 µM. In DPPH assays, the absorbance for AscA varied from 1.263±0.057 to 0.675±0.093, while the piperidine molecule ranged from 1.339±0.044 to 1.072±0.120. In CUPRAC assays, AscA absorbance was 0.227±0.052 and 1.768±0.176, and for the piperidine molecule, it was 0.132±0.042 and 0.142±0.031.

Conclusion: Piperidine is considered a saturated heterocyclic ring and possesses a wide range of biological activities. In this study, it was observed that the synthesized piperidine molecule showed limited DPPH radical scavenging activity. It also showed a high level of cytotoxic effect on A549 cancer cells and could be an important molecule for anticancer studies.

Keywords: antioxidant activity, cancer, cytotoxicity, drug synthesis

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INTRODUCTION

Oxidants are reactive molecules that are formed during biochemical processes in the body and can damage cells. Free radicals are highly reactive oxidants that originate from endogenous or exogenous factors, are usually formed naturally during oxygen metabolism, and can damage cells when they exceed a certain level. Excessive production of these molecules or inadequate antioxidant defense mechanisms can lead to oxidative stress and cellular damage. Oxidative stress plays a role in the development of various chronic diseases such as aging, cancer, cardiovascular diseases, and neurodegenerative disorders by damaging cellular components such as DNA, proteins, and lipids (1,2).

Long-term oxidant exposure as a result of antioxidant deficiency has been associated with cell damage and many diseases (3). For this purpose, providing antioxidant support from exogenous sources is thought to prevent the occurrence of these undesirable conditions. Research continues to synthesize and discover new antioxidant molecules with different effects in order to prevent the occurrence of these diseases or to stop their progression (4).

Cancer, in which the antioxidant/oxidant imbalance plays important roles, is a group of diseases caused by the uncontrolled and abnormal growth and proliferation of cells. Normal cells are subject to a specific process of growth and division, which ends with programmed cell death (apoptosis) in the event of cell aging and damage. However, in cancer cells, these regulatory mechanisms are disrupted, causing the cells to proliferate uncontrollably (5).

Cancer can develop in almost any tissue in the body, can cause tumor formation and spread to other organs through metastasis (6). Both in the stages of cancer development and during treatment, depending on the tissues and systems it affects, it is accompanied by significant health problems. It is an important health problem worldwide and threatens the lives of many people. The most common treatment modalities are surgery, radiotherapy, chemotherapy, and immunotherapy. However, since the most effective treatment method may vary depending on the type of cancer and the individual, one or more of these treatment methods can be applied together or at different times (7). The nature of cancer and its ability to metastasize complicate the treatment process and necessitate the development of new treatment methods (6).

Lung cancer is globally prevalent and one of the deadliest cancers due to its significant impact on vital systems and the poor prognosis associated with the disease. This type of cancer is often diagnosed late because it does not show symptoms in the early stages, which reduces the chances for effective treatment (8).

Innovative approaches to cancer treatment aim to prevent the growth and metastasis of cancer cells (9,10). The complex nature of lung cancer and its potential for resistance to treatment necessitates the development of new and effective treatment strategies (11). In this context, the anticancer potential of piperidine and its derivatives emerges as a promising area of research. Piperidine and its derivatives have the potential to stop the cell cycle and induce apoptosis in cancer cells with their potent antioxidant and anticancer properties (12). Therefore, studies on the biological activities of piperidine complexes may contribute to the development of new and effective strategies in cancer treatment.

Piperidine is a saturated heterocyclic ring that is considered an important compound due to its various roles in biological activities. It is noted for its strong antioxidant properties and diverse biological activities. These activities include anti-microbial, anti-inflammatory, anti-viral, anti-malarial, general anesthetic, anti-depressant, anti-oxidant, anti-epileptic, anti-tumor, anti-convulsant and anti-hyperlipidemic activities (13). The planar structure of piperidine allows the addition of substituent groups at different positions of the ring, which can enhance its biological activities. For example, piperine (PubChem CID: 638024, Figure 1b), a piperidine derivative, is an alkaloid derived from the plant Piper nigrum L. (black pepper) that shows potent antioxidant activity due to its ability to inhibit or quench free radicals. Piperine shows antioxidant, antiplatelet, anti-inflammatory, antihypertensive, hepatoprotective, antithyroid effects (14).

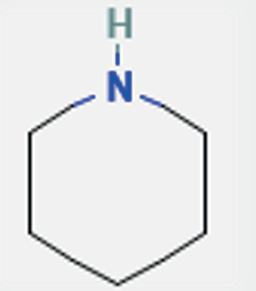
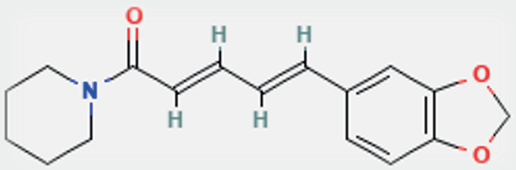
a) Piperidine	b) Piperine
	
PubChem CID: 8082 **Molecular Weight**: 85.15 g/mol Hexahydropyridine Cyclopentimine Azacyclohexane	**PubChem CID**: 638024 **Molecular Weight**: 285,34 g/mol

Figure 1. Some chemical properties of piperidine and piperine compounds

Piperidine (PubChem CID: 8082, Cyclopentimine, Hexahydropyridine) is a heterocyclic compound consisting of a six-membered ring containing five methylene groups (-CH_2-) and one amine group (-NH-) (Figure 1a). This compound can be found in barley (*Hordeum vulgare L., Poaceae*) and black pepper (*Piper nigrum L., Piperaceae*) and provides the characteristic flavor of black pepper and plays an important role in the pharmaceutical industry (12,15). Piperine, a piperidine derivative, has therapeutic potentials against cancers such as breast cancer, ovarian cancer, stomach cancer, glioma cancer, lung cancer, oral squamous, chronic pancreatitis, prostate cancer, rectal cancer, cervical cancer and leukemia (12). Research has shown that piperidine nitroxides, such as TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy radical), a piperidine derivative, exhibit potent antioxidant effects in various ways (16). The planar structure of the heterocyclic core of piperidine allows different groups to be attached at different positions of the ring (13). Due to the skeletal structure of piperidine, it can also be investigated as an important anticancer agent by acting on important receptors or with different derivatives to be created (17). These properties make it possible to study a wide range of biological activities of piperidine derivatives (13).

Piperidine and its derivatives contribute to various biological processes that induce cell apoptosis and inhibit the growth of cancer cells. Activation of cell apoptosis by contributing to anticancer biological processes such as activation of mitochondrial cytochrome C, release of Bax-protein from mitochondria and down-regulation of Bcl-2 protein (12). Its wide range of biological activities makes piperidine an important compound in pharmaceutical research and drug development. The aim of this study was to determine the anticancer and antioxidant activity levels of the synthesized heterocyclic molecule with the chemical structure of 1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl) propyl)piperidin-1-ium chloride (Figure 2).

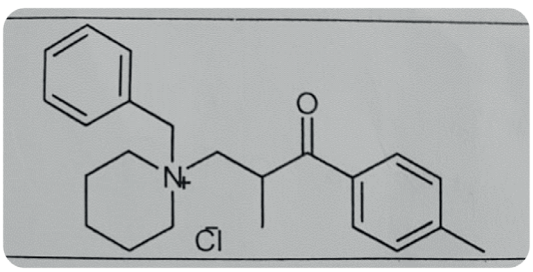


Figure 2. 1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl) propyl)piperidin-1-ium chloride

MATERIALS AND METHODS

Piperidine Derivative Synthesis

In the first step of the study, **1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl)propyl)piperidine-1-ium chloride** molecule was synthesized in a solvent-free medium using the microwave synthesis method (Figure 2). In the purification of piperidine, the amide dissolved in dichloromethane was first extracted with 2 M HCl followed by 5% sodium bicarbonate ($NaHCO_3$) and precipitated with n-hexane.

Cytotoxic Effect of Piperidine on A549 Cell Line

The lung cancer cell line (A549) was used for anticancer activities. The cytotoxic effect of the test compounds on the cells was determined by the MTT method, an enzymatic method widely used in the determination of cytotoxicity. This method is based on the ability of the MTT compound to cleave the tetrazolium ring. The compound (MTT) is absorbed into living cells and the reaction is catalyzed by mitochondrial succinate dehydrogenase and reduced to blue-violet, water-insoluble formazan (Figure 3) (18-20).

After thawing the stock A549 (Lung Cancer) cell line in a cryotube in a sterile water bath at 37 0C, studies were continued under sterile conditions in a laminar flow cabinet. Cells were homogenized by gentle pipetting. Dulbecco's Modified Eagle Media (DMEM) medium containing 20% (50 ml) Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA), 1% (5 ml) Penicillin/ Streptomycin (Sigma-Aldrich, USA) and 2.2 g/L

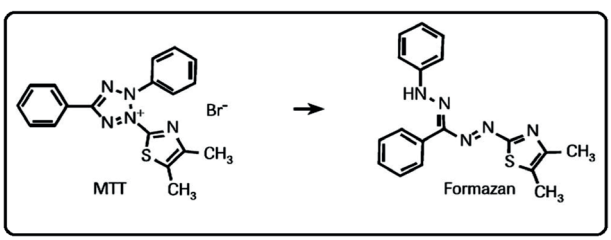


Figure 3. MTT-Formazan conversion via mitochondrial enzymes in living cells (20)

Sodium bicarbonate for A549 cell line was used. A549 cells were transferred to 25 cm2 sterile cell growth dishes (flasks) containing culture medium. The flask was placed in an incubator containing 5% CO2 at 37 oC. The flasks were checked daily under an inverted microscope, the medium was changed, and once the cells became confluent, they were passaged and the cells were multiplied and transferred to larger flasks. In 75 cm^2 culture flasks, the cells were cultured in DMEM medium prepared by adding 10% FBS, penicillin and streptomycin at 37°C in an incubator with 5% CO_2. Passaging was performed when the cells to be examined under the inverted microscope were confluent (when they reached 80-85% density). Tyrpan blue dye and cell suspension were mixed in a one-to-one ratio and cell counting was performed on a Thoma slide and the number of cells was determined as follows:

Equation 1:

$$Live\ cell\ count/mL = Mean\ live\ cell\ count\ x\ Dilution\ coefficient\ x\ 10^4$$

The respective serial dilutions of the piperidine complex were dissolved in 1% PBS (1ml) to a final concentration of 100, 50, 25, 12.5, and 6.25 uM and passed through a 0.22 µm filter (Millipore, USA). These samples were then added to 96-well plates in which cells were seeded and incubated for 24 hours.

For cytotoxicity assay, MTT solution was prepared in sterile phosphate buffer at a concentration of 0.5 mg/mL, and applied to each well and incubated for 3 hours. After incubation, the contents of each well were discarded and 100 µL dimethylsulfoxide (DMSO) was added to dissolve formazone crystals. The 96-well plate was covered with aluminum foil and mixed for 10 minutes. The optical densities (OD) of the cells in the wells were measured in an ELISA microplate reader (Thermo MultiskanGo, USA) at 570 nm wavelength (19). The absorbance values of the control group were averaged and this value was considered as 100% viable cells and cytotoxicity was determined by proportioning each well to which different concentrations of piperidine were added.

Determination of Antioxidant Activity of Piperidine

Assays used to evaluate antioxidant compounds can be classified into those associated with lipid peroxidation (thiobarbituric acid assay [TBA] and others) and those associated with electron or radical scavenging [2,2-diphenyl-1-picrylhydrazyl (DPPH) ABTS, FRAP, FTC and aldehyde/carboxylic acid assay] (13). DPPH and CUPRAC activities were evaluated to determine the antioxidant effects of piperidine.

Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

The DPPH antioxidant activity determination method developed by Blois (21) was modified and used. A 1 mM solution of DPPH- radical was used as a free radical. Different concentrations were prepared by dissolving the synthesized piperidine derivative molecule in water with final concentrations of 250, 125, 62.5, 31.25, and 15.63 µg/mL. Water was used as a blind and L-ascorbic acid (Merck, Germany) at the same concentrations was used as a positive control. 150 µL of DPPH- solution and 50 µL of sample were added to each well of the 96-well plate, incubated for 30 minutes at room temperature and in the dark, and the absorbance was measured at 517 nm. Three replicates were run for each different concentration and the data were calculated by the formula given below and presented as mean and standard deviation.

Equation 2:

$$DPPH\ Inhibition\% = \left(\frac{A_{Control} - A_{Sample}}{A_{Control}}\right) x100$$

$A_{Control}$: *Control Absorbance*

A_{Sample}: *Sample or Ascorbic acid Absorbance*

Determination of CUPRAC (Cupric Reducing Antioxidant Capacity)

CUPRAC is an analysis method used to determine the antioxidant capacity of a molecule. In this method, antioxidants react with copper(II)-neocuproin complex (Cu(II)-Nc) to form copper(I)-neocuproin complex (Cu(I)-Nc), which is determined by color change. This method is widely used because it is simple, rapid and sensitive and is useful in assessing the activity of various antioxidant compounds.

In order to determine the antioxidant activity, 0.01 M CuCl2 solution, 7.5x10-3 M ethanolic neocuprin solution and 1 M ammonium acetate buffer were prepared. 75 µL of each of these solutions were added to 96 wells. 75 µL of five different concentrations of piperidine or ascorbic acid solution were added, with a final concentration of 250 - 15.63 µg/ml. After a half-hour incubation in the dark, the absorbance at 450 nm was recorded. Increasing absorbance of the reaction mixture indicates increasing copper ion (Cu2+) reducing capacity. Three replicates were run for each concentration and absorbance values are presented as mean and standard deviation.

Statistical analysis

Statistical analysis results of the MTT study were obtained with GraphPad Prism 8 program. The data of the study were analyzed on a computer using SPSS 22 software. Two-way ANOVA test was performed to determine the differences between the groups studied in the MTT method. The significance level in different groups was determined according to $p<0.05$. The graphs and IC_{50} (sample concentration providing 50% inhibition) values of DPPH and MTT results were obtained using GraphPad Prism 8 program and A_{50} (sample concentration halving the maximum absorbance) values of CUPRAC results were obtained using the Microsoft Excel program.

RESULTS

Cytotoxic Effect of Piperidine on A549 Cell Line

The cytotoxic activity of the synthesized heterocyclic molecule in A549 cell line was determined by MTT method. At the highest concentration of 100 µM, 66.90% of A549 cells, 60.81% at 50 µM, 46.66% at 25 µM, 30.77% at 12.5 µM and 5.57% at 6.25 µM showed cytotoxic effect. According to these findings, IC_{50} value was obtained as 32.43 µM (Table 1 and Figure 4).

Table 1. Cytotoxicity ratio versus increasing piperidine concentration

	Concentration of Piperidine Complex (µM)				
NT	**6.25**	**12.5**	**25**	**50**	**100**
100	97.9	55.3	45.4	39	32.2
100	100	62.3	56.2	41.4	36.0
100	100	73	59.6	41.1	34.4
100	87.3	73.5	54.2	37.6	33.2
100	90.4	76.1	53.1	37.7	31.1
100	91	75.2	51.5	38.4	31.7
Mean±SD	94.4±5.05	69.2±7.72	53.3±4.37	39.2±1.52	33.1±1.68
	p<0.05	p<0.05	p<0.05	p<0.001	p<0.001

SD: Standard Deviation; NT: Non-Treatment.

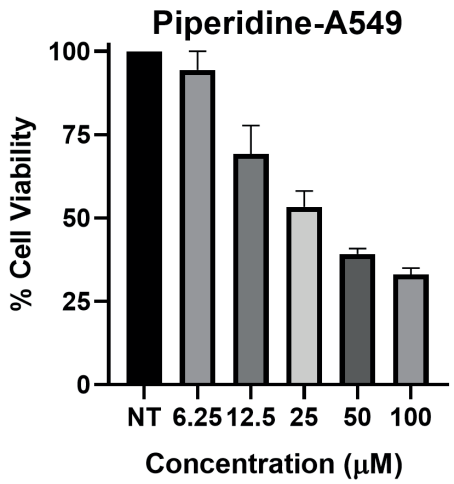


Figure 4. Plot of cytotoxicity rates against increasing piperidine concentration

(NT: Non-Treatment)

Antioxidant Activity of Piperidine

DPPH Radical Scavenging Activity

DPPH inhibition levels of ascorbic acid were determined as 58.2±0.8%, 52.8±0.6%, 48.0±0.4%, 35.1±0.8% and 17.6±1.1% at different concentrations between 250-15.63 µg/mL, respectively. For the piperidine complex synthesized under the same conditions, these values were 23.4±1.9%, 19.3±4.4%, 11.5±5.8%, 6.5±4.5% and 3.5±2.4%, respectively. Based on these findings, the IC_{50} value of ascorbic acid was calculated as 106.85 µg/mL, whereas the IC_{50} value was not calculated since the piperidine complex did not show 50% or more inhibition at the indicated concentrations and can be expressed as IC_{50} > 250 µg/mL (Table 2 and Figure 5).

CUPRAC Identification

The antioxidant activity of the synthesized piperidine complex was also evaluated by the CUPRAC method. During this evaluation, absorbance values for different concentrations of ascorbic acid between 250-15.63 µg/mL were read as 1.818±0.074, 1.244±0.012, 0.762±0.011, 0.578±0.014 and 0.227±0.037, respectively. Absorbance values for the piperidine complex synthesized under the same conditions were 0.142±0.025, 0.140±0.026, 0.142±0.023, 0.134±0.043 and 0.132±0.034, respectively. While the A_{50} value for ascorbic acid was calculated as 60.67 µg/mL, this value was not calculated since no significant activity was observed for the synthesized piperidine concentration (Table 3 and Figure 6).

DISCUSSION

Piperidine is a saturated heterocyclic secondary amine associated with various biological activities such as antimicrobial, anti-inflammatory, antiviral, antimalarial, general anesthetic, antidepressant, antioxidant, antiepileptic, antitumor, anticonvulsant and antihyperlipidemic activities (13).

Table 2. DPPH Inhibition Ratio Against Increasing Piperidine and Ascorbic Acid Concentration

Concentration (µg/mL)	Ascorbic A. (Mean±SD)	Piperidine (Mean±SD)
250	58.2±0.8	23.4±1.9
125	52.8±0.6	19.3±4.4
62.5	48.0±0.4	11.5±5.8
31.25	35.1±0.8	6.5±4.5
15.63	17.6±1.1	3.5±2.4
IC_{50}	106.85 µg/mL	> 250 µg/mL

Table 3. CUPRAC activity against increasing Piperidine and Ascorbic Acid concentration

Concentration (µg/mL)	Ascorbic A. (Mean±SD)	Piperidine (Mean±SD)
250	1.818±0.074	0.142±0.025
125	1.244±0.012	0.140±0.026
62.5	0.762±0.011	0.142±0.023
31.25	0.578±0.014	0.134±0.043
15.63	0.227±0.037	0.132±0.034
A_{50}	**60.67 µg/mL**	

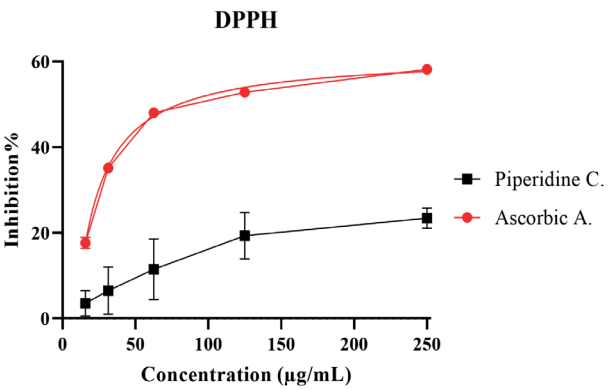


Figure 5. Plot of DPPH inhibition rates against increasing concentration of Piperidine complex and Ascorbic Acid

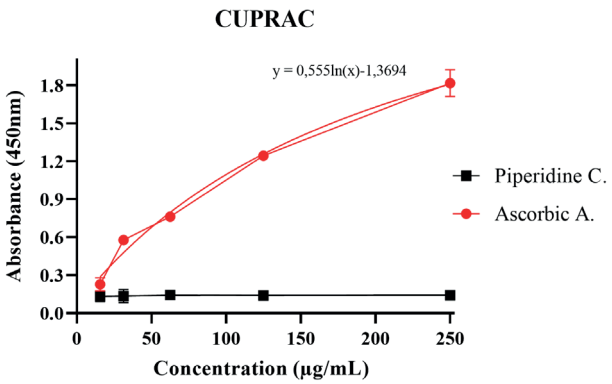


Figure 6. Plot of CUPRAC Activity against increasing Piperidine and Ascorbic Acid concentration

When the anticancer activities of the synthesized piperidine complexes were examined; Benaka et al. (22) synthesized new molecules containing more than ten piperidine groups. The antiproliferative activities of these compounds against HT-29 (colon carcinoma), HeLa (cervix-cervix carcinoma), MCF-7 (breast carcinoma) and HepG2 (hepatocellular carcinoma) cell lines at 100 µM concentration for 24 hours were evaluated by MTT assay. For these cell lines, 45.11-78.55%, 39.22-78.50%, 38.68-73.75% and 39.11-68.74% cell viability was maintained, respectively. In this study, the average survival for A549 cell line was 33.1±1.68% under the same conditions. It is understood that the molecules synthesized by Benaka et al. (22) showed a more cytotoxic effect on A549 cancer cell line. However, variables such as the structure of the molecules and the morphological structure of the treated cells should not be ignored.

Bezerra et al. (23) determined the cytotoxicity effects of piperine (Figure 1b), a piperidine derivative obtained from black pepper seeds and commercially available, on CEM and HL-60 (human leukemia), B16 (mouse melanoma) and HCT-8 (human colon) cell lines at increasing concentration by MTT method. They determined that these cell lines were cytotoxic with IC_{50} values of >87.6 µM, >87.6 µM, 69.9 µM and 66.0 µM, respectively.

Vinaya et al. (24) synthesized 9 different piperidine derivatives (4-[3-(piperidin-4-yl)propyl]piperidine derivative) at increasing concentrations and different incubation times and examined their MTT and some other cytotoxic effects on K562 and Reh (human leukemia cells) cell lines and concluded that they showed anti-leukemic effect with IC_{50} values of 2-125 µM. According to the investigations carried out in

this study and the studies presented in the literature, different piperidine derivatives can show anticancer effects at quite different levels according to the side groups that they confer.

Lahmidi et al. (25) with their study on determining the DPPH radical scavenging activity of three newly synthesized piperidine derivatives; they concluded that quercetin, which they used as a control, did not show significant activity, with IC_{50} values between 8.2±0.2 and 19.5±0.5 mM, compared to its IC_{50} values of 0.012±0.003 mM.

Prashanth et al. (26) determined the antioxidant activities of 10 newly synthesized piperidines and ascorbic acid by the same method. According to the results of this study, ascorbic acid showed an IC_{50} of 12.6±0.43 µg/mL, while piperidine derivatives showed IC_{50} values of 8.3±0.02 and 36.9±0.17 µg/mL. According to these data, some piperidine derivatives may show even better DPPH activity than ascorbic acid.

Karaman et al. (27) evaluated the DPPH activity of 19 different piperidine compounds and α-tocopherol as a control: IC_{50} value for α-tocopherol was calculated as 12.26±0.07 µM, while the IC_{50} value for piperidine compounds was calculated between 19.99±1.03 and 96.71±0.28. Thus, it was observed that none of these synthesized molecules showed DPPH activity as much as α-tocopherol.

In this study, a significant IC_{50} value (>250 µg/mL) could not be calculated since 50% inhibition of DPPH activity was not observed by the synthesized piperidine complex compound. Compared to ascorbic acid used as control, 58.2±0.8% inhibition was observed at the maximum ascorbic acid concentration (250 µg/mL), while 23.4±1.9% inhibition was observed at the same piperidine concentration.

Karaman et al. (27) evaluated the CUPRAC activity of 19 different piperidine compounds and α-tocopherol as a control: They calculated the A_{50} value for α-tocopherol as 40.48±1.87 µM, while the piperidine compounds were calculated between 4.16±0.04 and 92.13±0 µM. Thus, some of these synthesized molecules showed better CUPRAC activity than α-tocopherol. In this study, the compound showing the best CUPRAC activity compared to α-tocopherol (4.16±0.04 µM) did not show the same level of DPPH activity (74.83±0.46 µM, α-Tocopherol= 12.26±0.07 µM). Karaman et al. (27) synthesized 17 piperidine derivatives and α-tocopherol as control in DPPH and CUPRAC activity study; IC_{50} value between 31.35±1.07- 92.00±0.49 µM for DPPH activity of 17 piperidine derivatives and 17.72±0.03-87.82±0.01 µM A_{50} value for CUPRAC activity were calculated. For the DPPH and CUPRAC activities of α-Tocopherol, the data given in the other study are presented. In this study, ascorbic acid, which is used as a positive control for antioxidant studies, showed activity as expected with the CUPRAC method, and its A50 value was calculated as 60.67 µg/mL. While the synthesized piperidine compound exhibited some DPPH activity compared to ascorbic acid, no CUPRAC activity was observed.

CONCLUSION

Piperine and piperidine heterocyclic molecules have gained interest in the pharmaceutical industry as anticancer agents. Piperidine is considered as a saturated heterocyclic ring and is a structure with a wide range of biological activities. In this study, the synthesized piperidine molecule was also observed to exhibit limited DPPH radical scavenging activity. In addition, it showed a high level of cytotoxic effect in A549 cancer cells and it was concluded that it could be an important molecule for anticancer studies.

The cytotoxicity effects of this newly synthesized compound, which exhibits anticancer properties, can be investigated in healthy and other cancerous cell lines and its more comprehensive effects can be investigated with further in vivo studies. More comprehensive data can be obtained by investigating its antioxidant effects with other antioxidant methods other than CUPRAC and DPPH.

Ethical approval

Since the A549 cell line used in this study is a commercially available cell line, ethical approval was not required. Therefore, no ethical committee approval was obtained for this study.

Author contribution

Concept: İS, SK; Design: İS, SK; Data Collection or Processing: İS, MA, SK; Analysis or Interpretation: İS, SK; Literature Search: İS, SK; Writing: IS, MA, SK. All authors reviewed the results and approved the final version of the article.

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Conflict of interest

The authors declare that there is no conflict of interest.

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