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Cytotoxic effect of *Centaurea solstitialis L. (Asteraceae)* on cell cultures

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ABSTRACT

Aims: The aim of this study was to investigate the cytotoxic effect of *Centaurea solstitialis* (*C. solstitialis*) L. (*Asteraceae*) plant extract on A-549 lung cancer cells. Cytotoxicity is an important parameter in evaluating the lethal effect of plant extracts on cancer cells.

Methods: In this study, plant specimens were collected from the campus area of Kırıkkale University during flowering in May–June and identified in the ADO Herbarium. Dried and crumbled plant leaves were mixed with 70% ethyl alcohol to form a paste, and this mixture was kept in a magnetic stirrer for at least 48 hours. It was then kept in a fume hood for at least 48 hours to dry, and the dried material was labeled and stored in eppendorf tubes. Cytotoxicity was determined by the WST-1 assay and evaluated by examining the morphological characteristics of the cells.

Results: Different doses of *C. solstitialis* plant extract (200 ug/ml, 100 ug/ml, 0.05 ug/ml, 0.025 ug/ml, 0.025 ug/ml, and 0.0125 ug/ml) were tested, and the percentage of cell viability was calculated relative to the control group. The results revealed that dose 1 exhibited a cell viability of 26%, with dose 2 showing the highest cell viability at 38%, dose 3 at 74%, dose 4 at 80%, and dose 5 at 96%. The toxicity levels of *C. solstitialis* plant extract was determined, and apoptotic and necrotic effects were examined at the observed toxic doses. According to these results, it was determined that *C. solstitialis* alcohol extract at a dose of 200 mg/ml caused 21.5% apoptosis and 7.56% necrosis in A-549 cancer cells. At a 100 mg/ml dose, these rates were determined as 16.2% apoptosis and 5.39% necrosis, respectively. At lower doses, apoptosis rates of 50 mg/ml and 25 mg/ml *C. solstitialis* extract in A-549 cells were 9.94% and 5.1%, respectively, while necrosis rates were 4.12% and 3.25%, respectively. At the lowest dose of 12.5 mg/ml, the apoptosis rate was 3.02% and the necrosis rate was 2.16%.

Conclusions: The results showed that *C. solstitialis* showed a cytotoxic effect, and accordingly, the percentage of viability decreased and as the dose applied to the cell decreased, the percentage of viability increased, and the cytotoxic effect decreased. Apoptotic and necrotic effects observed at toxic doses suggest that programmed cell death mechanisms are induced. Further studies are needed to elucidate the underlying molecular mechanisms and compare the cytotoxic effects of *C. solstitialis* with those of other Centaurea species.

Keywords: Lung cancer, apoptosis, *Centaurea solstitialis*, cell viability, necrosis, cytotoxicity

INTRODUCTION

Asteraceae is one of the largest families of Angiosperms, and according to the latest classifications, it consists of 1535 genera and about 26,000 species gathered under 3 subfamilies and 17 tribes. When the limited studies conducted in Kırıkkale are examined, it can be said that there are approximately 30 genera belonging to the family.[1-3] One of a country's most important natural resources is its flora. For this reason, each country identifies the plants belonging to its flora and carries out studies on them, such as antimicrobial, antioxidant, cell culture, identification and evaluation of gene resources, and protection of plants in their natural environment.[4] *C. solstitialis*, used in this study, is a plant species belonging to the family *Asteraceae* (Daisy family) and is commonly known as Yellowthorn. This plant usually grows in steppe areas, fields, roadsides, and vacant lots.[5] Humans have utilized plants in the treatment of diseases since time immemorial. Before the modern pharmaceutical industry, many plants were used as natural medicines. Today, people still use plants or drugs derived from plants in the treatment of various diseases. The killing effect of plants on microorganisms and their important

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properties for human health have been investigated in various laboratories in Turkey and other countries since 1926. It is known that naturally occurring plant extracts and essential oils exhibit antifungal activity against bacteria and fungi, and antimicrobial activities are the basis for many applications, such as food preservation, pharmacy, alternative medicine, and natural therapy.[6] Potential for plant-derived medicines There are approximately 120 plant-derived medicines available worldwide, derived from only 95 different plant species. Today, only 5,000 species out of approximately 250,000 flowering plant species have been evaluated for their pharmaceutical potential.[7] The lethal effects of plants on microorganisms and their important properties for human health have been investigated in Turkey since 1926, as well as in various laboratories in other countries.[7] The role of plants in cancer treatment Today, many different methods, such as radiotherapy, surgical methods, hyperthermia, gene therapy, and chemotherapy, are used in cancer treatment. Chemotherapy is the most widely used treatment method and is often combined with other methods. Chemotherapy uses a variety of chemical agents as well as natural compounds of plant origin. Cytotoxicity refers to the toxic effect of a substance or agent on cells.[8] This toxic effect can disrupt the structural integrity of cells, affect their metabolic activities, or cause cell death.[9] Cytotoxicity is an important concept in many fields, such as drug discovery and development, toxicology, cancer research, and biomedical applications.[10] Cytotoxicity is a consequence of changes observed in the viability, proliferation, metabolic activities, and morphological characteristics of cells.[11] These changes may manifest as cell death (necrosis or apoptosis) or cell damage.[11] When assessing cytotoxicity, measurements of cell viability or cell membrane integrity are usually used.[11] Cytotoxicity tests are used to assess cytotoxicity and determine the effect of a substance or agent on cells.[12] These tests are usually performed in vitro cell culture systems and evaluate cytotoxicity by measuring different parameters. Commonly used methods in cytotoxicity tests include the MTT assay, the WST-1 assay, the XTT assay, LDH release, and the use of apoptosis markers.[10,11] Besides cytotoxicity tests, other parameters such as morphological changes of cells, DNA damage, and genetic alterations can also be evaluated to determine the effects of cytotoxic substances.[10] Such tests are used to gain a more comprehensive understanding of the effect of a substance or agent on cells.[12] Lung cancer is a high mortality cancer that is the most common cause of cancer-related deaths worldwide, accounting for approximately 1.6 million deaths each year.[13,14] Lung cancers are traditionally divided into two main histologic groups (small cell lung cancer [SCLC] and non-SCLC [NSCLC]) according to the natural history of the disease and treatment approaches. NSCLC accounts for approximately 85% of all lung cancers, and the most common subtypes are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.[15] In a study conducted in Turkey, lung cancer was observed in 90.5% of men and 9.5% of women.[16] According to the WHO 2014 report, 19.4% of cancer deaths are due to lung cancer.[17] The main aim of this research is to understand the effect of *C. solstitialis* plant extract on cancer tissues, and I think it will prioritize new treatment methods and existing treatments in the fight against cancer.

METHODS

Ethics

The study was conducted in a laboratory. No human or animal material was used, as this study did not require ethics approval. The approval of the institution was obtained. All procedures were carried out in accordance with the ethical rules and the principles.

Collection of Plant Specimens

C. solstitialis specimens were collected during the flowering period of May-June (2021–2022) in the campus area of Kırıkkale University. The collected plant specimens were identified in the ADO Herbarium. The plant specimens were washed with deionized water to remove dust and foreign debris and dried under room conditions in the shade. The leaves, which are the most commonly used part of the dried plants, were ground with a grinding device, and powders with a particle size between 0.50-1.00 mm were used to obtain plant extracts.

Preparation of Plant Extracts

Dried and crumbled plant leaves were mixed with 70% ethyl alcohol to form a paste. This mixture was kept in a magnetic stirrer for at least 48 hours. Then, the solution was kept in a fume hood for at least 48 hours and allowed to dry. The dried material was labeled and stored in eppendorf tubes for use.

Determination of Cytotoxicity

The WST-1 assay was used to determine cytotoxicity. Each of the cell lines to be used will be seeded in a separate 96-well plate with 5000 cells per well. The cells will be incubated for 24 hours. Then, the determined amounts of plant extracts will be prepared in ethanol, added to the cells, and incubated for 24, 48, and 72 hours. Only medium will be used as a positive control, and medium with H_2O_2 will

be used as a negative control. At the end of the incubation period, 15 µl of WST-1 solution will be added to each well and incubated at 37°C for 4 hours. Then, the absorbance intensity values of the 96-well plate will be measured at a wavelength of 440 nm in an ELISA plate reader to determine cell viability. Live cells will produce a yellow color, while dead cells will not show any color formation. Percent viability will be calculated based on the control group.[8]

Morphological Examination of Cells

The cells to be used in the study will be seeded with 10,000 cells per well of a 48-well plate. Then, the medium will be changed to apply plant extracts to the cells. After 24, 48, and 72 hours, the media will be removed and the cells will be fixed with 3% glutaraldehyde for 15 minutes, followed by fixation with 70% ethyl alcohol for 30 minutes. The cells will then be stained with Hematoxylin-eosin stain and examined under an inverted microscope to evaluate morphological changes such as cell membrane disruption and vacuoles. This evaluation will be done using the scoring method. Hematoxylin stains the nuclei blue (apoptosis appearance), while eosin stains the cytoplasm pink or red (necrosis appearance).[9]

Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). Statistical analysis of the data was performed with the IBM SPSS Statistics 22 package program (IBM SPSS Statistics®, Chicago, IL, USA). Mean comparisons were obtained by analysis of variance followed by a one-sample Wilcoxon signed rank test.

RESULTS

When the WST-1 test was used for *C. solstitialis* plant extract, live cells formed a yellow color, while no color formation was observed in dead cells. The percent viability was calculated based on the control group. The findings regarding the cytotoxic effect of *C. solstitialis* alcohol extract on A-549 cancer cells are presented in Table 1.

Table 1. Cytotoxic effect of *C. solstitialis* alcohol extract on A-549 cancer cells

Centaurea solstitialis	
Doses	**% Vitality**
200 µg/ml	26.2±0.003^{d}
100 µg/ml	38.3±0^{c}
50 µg/ml	74.4±0.014^{b}
25 µg/ml	80.5±0.033^{ab}
12,5 µg/ml	96±0.00^{a}
Control	100^{a}

*Different letters in the same column indicate differences between means. (p<0,05).

According to Table 1, it was observed that *C. solstitialis* alcohol extract at a 200 µg/ml dose provided 26.2% viability in A-549 cancer cells. At a 100 µg/ml dose, this rate was 38.3%. Lower doses of 50 µg/ml and 25 µg/ml of *C. solstitialis* extract provided 74.4% and 80.5% viability rates in A-549 cells, respectively. The lowest dose of 12.5 µg/ml indicated a viability rate of 96%. In the control group, it is noteworthy that the cells showed 100% viability. It shows that the cytotoxic effect of *C. solstitialis* plant extract on A-549 cancer cells increases in a dose-dependent manner. In other words, it is seen that the plant extract has a stronger cytotoxic effect on cancer cells with increasing doses.

Table 2. Apoptotic and necrotic index results of *C. solstitialis* alcohol extract

Centaurea solstitialis		
Doses	**% Apoptosis**	**% Necrosis**
200 mg/ml	21.5^{a}	7.56^{a}
100 mg/ml	16.2^{b}	5,39^{b}
50 mg/ml	9,94^{c}	4.12^{bc}
25 mg/ml	5.1^{d}	3,25^{c}
12.5 mg/ml	3.02^{e}	2,16^{cd}

*Different letters in the same column indicate the differences between the means. (p<0,05).

According to Table 2, it was determined that *C. solstitialis* alcohol extract at a 200 mg/ml dose caused 21.5% apoptosis and 7.56% necrosis in A-549 cancer cells. At a 100 mg/ml dose, these rates were determined as 16.2% apoptosis and 5.39% necrosis, respectively. At lower doses, apoptosis rates of 50 mg/ml and 25 mg/ml *C. solstitialis* extract in A-549 cells were 9.94% and 5.1%, respectively, while necrosis rates were 4.12% and 3.25%, respectively. At the lowest dose of 12.5 mg/ml, the apoptosis rate was 3.02% and the necrosis rate was 2.16%. Table 2 shows that *C. solstitialis* alcohol extract has apoptotic and necrotic effects on A-549 cancer cells. Apoptosis refers to controlled cell death, while necrosis refers to sudden death by cell damage. These results show that the apoptotic effect of the plant extract on cells is more predominant. In other words, the plant extract shows its antitumoral effect by inducing controlled cell death in cancer cells. These findings suggest that *C. solstitialis* stimulates apoptosis and necrosis processes in cancer cells and has a potential antitumoral effect. However, more comprehensive and mechanistic studies are needed. It is also important to investigate the effect of these effects on normal cells and possible side effects. In this way, *C. solstitialis* could contribute to the evaluation of its potential use as a natural compound in the treatment of cancer. In Figure 1, images of A-549 cells treated with 100 µg/ml *C. solstitialis* extracts are presented.

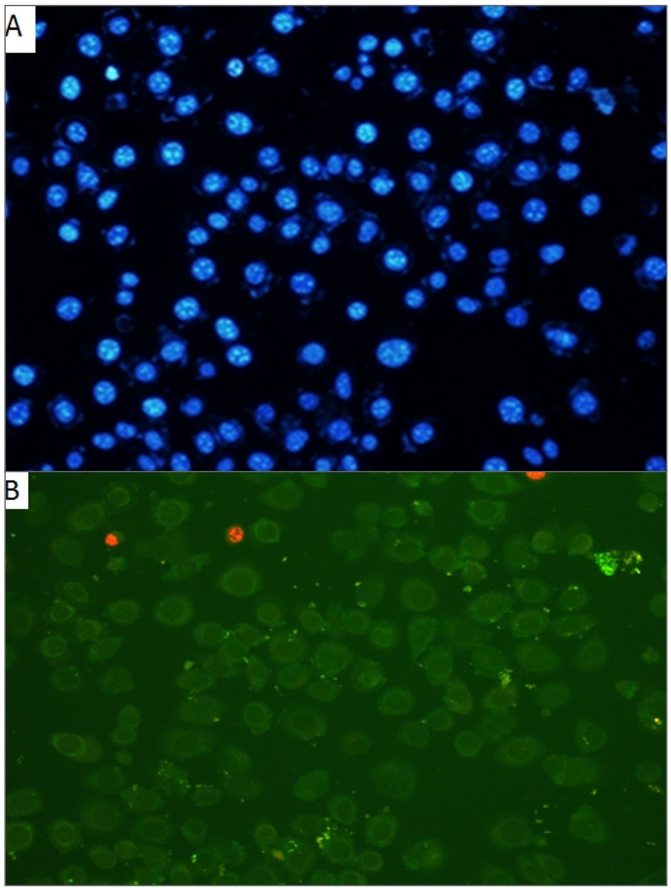

Figure 1. A-549 cells to which 100 ug/ml *C. solstitialis* extracts were applied

According to Figure 1, apoptotic cells are defined as having bright and fragmented nuclei, while necrotic cells are defined as having red nuclei. A: Apoptotic cells, B: Necrotic cells (Magnification of an inverted microscope image)

DISCUSSION

Various studies have revealed that *C. solstitialis* species may have various biological effects. In the study conducted by Akbar et al.[18] in 1995, they confirmed the hypothermic activity of repin isolated from *C. solstitialis* by using various pharmacological agents in rats and tried to explain its thermoregulatory effects and mechanisms of action. It was determined that repin induced dose-dependent and quite strong hypothermia in rats. Hypothermia caused by a 10 mg/kg dose of repin administration reached a maximum of 3 hours after injection, and the return to normal temperature was found to occur in more than 8 hours. Antimuscarinic agents such as atropine sulfate (10 mg/kg) and atropine methyl bromide (20 mg/kg), metergoline (non-selective serotonin receptor antagonist, 0.5 mg/kg), ketanserin (selective 5-HT2 receptor antagonist, 0.2 mg/kg), diphenhydramine (H1 receptor antagonist, 10 mg/kg), apomorphine (dopamine receptor antagonist, 0. 5 mg/kg) and propanolol (10 mg/kg), a non-selective β-adrenoreceptor antagonist and 5-HT1 receptor antagonist, 30 minutes before repin injection, did not directly antagonize the hypothermic effect of repin. In contrast, 2-4 hours after repin injection, hypothermia was partially but significantly reversed by atropine sulfate (20 mg/kg), metergoline, ketanserin, diphenhydramine, and apomorphine. According to the results obtained, it was stated that these late-onset effects may be due to secondary physiological mechanisms, and 20 mg/kg propanolol administration significantly increased the early and late-onset hypothermic effects of repin. The results of the study showed that cholinergic, serotonergic, histaminergic, and dopaminergic receptors were not involved in the hypothermic effects of repin.[18] In the study conducted by Yesilada et al.[19] in 1999, it was determined that the total extract prepared by mixing aqueous extracts (two extracts prepared at room temperature and with hot water) and methanol extract prepared from the aerial parts of *C. solstitialis* ssp. solstitialis provided 89.3% inhibition. In the results of the study, it was stated that the aqueous extract had a stronger antiulcerogenic effect compared to 100% inhibition of hot water extract prepared from 10 g of plant material, 80.1% inhibition of extract prepared from 5 g of plant material, and 81.6% inhibition of methanol extract prepared from 10 g of plant material.[19] In 2002, Arif determined the antibacterial effects of extracts and fractions of *C. solstitialis* and *C. depressa* species on Gram (+) (*Bacillus subtilis*) and Gram (-) (*Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) bacteria, and antifungal effects were determined by the microdilution method using *Candida tropicalis* strains.[20] The main extracts and fractions of both species showed an effect close to the control against *E. coli.* Chloroform fractions and ethanol extracts of the subsoil parts of the species were found to have a remarkable effect against Pseudomonas, and both species were found to have no antifungal effect.[20] In 2004, Yeşilada et al.[21] reported that fresh spiny flowers of *Centaurea solstitialis* ssp. (CSS) are used in the treatment of peptic ulcer in Turkey, and ethanol (80%) CSS extract exhibited a significant anti-ulcerogenic effect on an ethanol-induced ulcerogenesis model in rats. The ethanol extract was further fractionated by successive solvent extractions of n-hexane, chloroform, ethyl acetate, and n-butanol. All fractions showed significant anti-ulcerogenic activity, but the effect of the chloroform fraction was more pronounced with 99.5% ulcer inhibition. Bioassay-guided fractionation yielded sesquiterpene lactones as active components. The main components responsible for the activity of the chloroform fraction were identified as chlorojanerin and 13-acetyl solstitialin A, which were elucidated by HR-ESI and 1H, 13C, and 2D NMR spectroscopic techniques.[21] In 2009, Özçelik et al.[22] evaluated *Centaurea solstitialis* L. ssp. solstitialis

(*Asteraceae*) for antimicrobial and antiviral activities. Both standard and isolated strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans*, and *C. parapsilosis* were used for antimicrobial activity evaluation by the microdilution method. The antiviral activity of these three sesquiterpene lactones was determined using Vero cell lines of Herpes simplex type-1, a DNA virus, and Parainfluenza, an RNA virus. Ampicillin, ofloxacin, ketoconazole, fluconazole, acyclovir, and oseltamivir were used as reference drugs. 13-Acetyl solstitialin A was found to exhibit significant antibacterial activity against isolated E. faecalis strains at a concentration of 1 µg/ml, close to the effective concentrations of ampicillin. The same compound also showed significant activity against DNA viruses, which was as potent as the reference compound acyclovir at maximum and minimum concentrations of 16-<0.00006 µg/ml. This study is the first report proving that 13-acetyl solstitialin A has significant antiviral activity.[22] In 2016, Erenler et al.[23] reported that *Centaurea solstitialis* L. ssp. solstitialis (CSS) is used as a medicine for various diseases, and the root, stem, and flower parts of the plant were extracted separately with methanol for their isolation according to the bioassay guidance. The antiproliferative activities of each extract on C6 cells (rat brain tumor cells) and HeLa cells (human uterine carcinoma) were investigated in vitro. The methanol extract of the stem part of the plant exhibited the highest antiproliferative activity; therefore, isolation of active compounds for the stem part of the plant was carried out. The methanol extract of the stem part was boiled in water at 97°C for 2 h, followed by hexane and ethyl acetate extractions, respectively. Solstitialin A and 15-dechloro-15-hydroxychlorojanerin 2 were isolated from the ethyl acetate extract by column chromatography and identified by spectroscopic techniques. Solstitialin A 1 from CSS and 15-dechloro-15-hydroxychlorojanerin 2 were previously isolated from Saussurea lipschitz and Rhaponticum pulchrum. These two extracts exhibited very high antiproliferative activity on C6 and HeLa cells. The IC50 and IC75 values of extract 1 were 10.78 and 53.65 against C6 cells and 48.78 and 68.52 against HeLa cells, respectively. The IC50 and IC75 values of extract 2 were determined as 432.43 and 109.79 against C6 cells.[23] In 2019, Alper and Güneş evaluated the cytotoxicity of the crude ethanolic extract obtained from the flowering parts of *C. solstitialis* at seven different concentrations in A549, Daudi, HeLa, and Beas-2B cell lines to determine the IC50 value (µg/ml) causing 50% cell death.[24] According to the research findings, it was determined that the percentage of viable cells varied according to the cell lines used. It was reported that the viability of all cancer cells was significantly reduced by the extract in a concentration-dependent manner, as well as that the extract at concentrations of 15.6 and 31.2 µg/ml did not cause significant cytotoxicity in the normal BEAS-2B cell line, indicating that the extract has selectivity against cancer cells. The highest cytotoxicity was observed in HeLa cells with 63.18 µg/ml, while the IC50 values of A549 and Daudi cells were 252.5 µg/ml and 69.27 µg/ml, respectively. However, the extract exhibited a lower cytotoxic effect on normal BEAS-2B cells with an IC50 value of 75.25 µg/ml compared to the effects on HeLa and Daudi cancer cell lines. In other words, HeLa and Daudi cells were found to be the most sensitive cell lines in terms of cytotoxicity.[24] In 2021, Alper et al.[25] aimed to investigate the phenolic composition and antioxidant activity of *C. solstitialis* and Urospermum picroides and evaluate their possible cytotoxic effects. RP-HPLC analysis was used to elucidate the phenolic profiles of ethanolic extracts of the flowering parts of *C. solstitialis* and *U. picroides*. Both ethanolic extracts were evaluated for their antioxidant properties using DPPH, FRAP, phosphomolybdenum, and metal chelating assays. In addition, the effect of the extracts on cell viability was evaluated against MCF-7 and PC-3 cancer cells and the HEK293 cell line using the MTT assay. Caffeic acid was the most abundant phenolic compound in both extracts, and the amount of this compound was 24078.03 and 14329.59 µg/g in *C. solstitialis* and *U. picroides* extracts, respectively. Although the antioxidant activity of the extracts was similar, the *C. solstitialis* extract was found to have a higher potential for inhibition of cell viability compared to the *U. picroides* extract. The IC50 value of *C. solstitialis* on MCF cells was found to be 58.53 µg/ml. These findings suggest that *C. solstitialis* and *U. picroides* extracts can be considered as new and alternative natural antioxidant and anticancer sources.[25] In a study conducted in 2022 by Necip and Durgun, Mentha pulegium, *Lepidium draba*, and *Centaurea solstitialis* were traditionally used in different cultures for the treatment of various diseases. Total phenolic content analysis, chemical composition, and antioxidant activity of different solvent extracts, such as acetone, methanol, and n-hexane, obtained from the aboveground parts of *M. pulegium*, *L. draba*, and *C. solstitialis*, were investigated.[26] Total phenolic content was determined as gallic acid equivalent; the LC-MS/MS technique was used to determine the phenolic profiles of each extract; and the antioxidant activities of three extracts were determined by DPPH and ABTS methods. The highest total phenolic contents for acetone, n-hexane, and methanol extracts of *Centaurea solstitialis* were 99 507, 46 305, and 18 227 µg/mL, respectively. In the acetone extract of Mentha pulegium, the main component rosmarinic acid content was 128 195 µg/g extract, while this amount was determined as 780 383 µg/g extract in

the methanolic extract. The highest DPPH radical scavenging activity was found to be 77% and 79% in acetone and methanolic extracts of Mentha pulegium, respectively. ABTS radical scavenging activity was determined as 98% and 94% in acetone and methanolic extracts of Mentha pulegium, respectively. The antioxidant capacity of the extracts was found to be related to the total amount of phenolic substances.[26]

In 2023, Işık et al.[27] used nanoparticles obtained from the water extract of *C. solstitialis* leaves as green adsorbents for the removal of Reactive Red 180 (RR180) and Basic Red 18 (BR18) dyes in the Fenton reaction. Under optimum operating conditions, the nanoparticles showed high performance in the tested dye removal, with more than 98% elimination. Free radical capture, DNA nuclease, biofilm inhibition capacity, antimicrobial activity, microbial cell viability, and antimicrobial photodynamic therapy activities of iron oxide nanoparticles (FeO-NPs) obtained from water and methanol extracts of the plant were investigated. SEM-EDX, XRD, and Zeta potential were applied for the characterization of the prepared NPs and to explain their morphology, composition, and behavior in an aqueous solution, respectively. It was found that DPPH scavenging activities increased as the number of nanoparticles increased. The highest radical scavenging activity was obtained with FeO-NPs obtained from the water extract of the plant, with 97.41% at 200 mg/L. The new green synthesized FeO-NPs showed good DNA cleavage activity and good in vitro antimicrobial activity against human pathogens. As a result, both synthesized FeO-NPs showed 100% antimicrobial photodynamic therapy activity after LED irradiation. Water extract of FeO-NPs and methanol extract of FeO-NPs are also reported to have significant biofilm inhibition.[27]

When the literature findings were evaluated, hypothermic, antiulcerogenic, antibacterial, antiviral, antioxidant, and anticancer effects were found in studies with *C. solstitialis* extracts. The findings obtained from the study are similar to the literature findings and suggest that the effect of *C. solstitialis* extracts on A-549 cancer cells is due to the phenolic content that increases the antioxidant capacity of *C. solstitialis* extracts.

CONCLUSION

It was determined that alcohol extracts prepared from the leaves of *C. solstitialis* species showed an anti-carcinogenic effect that varied depending on the dose. Cytotoxicity and apoptosis-necrosis results show that *C. solstitialis* is a promising candidate for cancer treatment. It is thought that more comprehensive cancer studies on this plant species may be useful, and the phenolic compound that increases its antioxidant capacity should be further elucidated.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was conducted in a laboratory. No human or animal material was used, as this study did not require ethics approval. The approval of the institution was obtained.

Informed Consent: Because the study was designed as a laboratory study and did not involve human or animal material, no written informed consent form was obtained.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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