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Investigation of Antioxidant, Antimicrobial and *In Vitro* Cytotoxic Effects on PC-3 Cancer Lines of *Alchemilla holotricha* Juz. in Different Polarities Extracts

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Abstract: The extracts of *Alchemilla holotricha* Juz. with different polarities were obtained using hexane, dichloromethane, ethyl acetate, methanol, and water. The samples were further analyzed for their overall antioxidant activity, ability to scavenge DPPH free radicals, capacity to chelate metals, total reducing power, ability to scavenging hydrogen peroxide, and hydroxy radical scavenging activity. The ethyl acetate extract exhibited the highest concentration of total phenols (67.63± 0.88 mg GAE/g extract), whereas both the ethyl acetate and dichloromethane extracts demonstrated the highest levels of total flavonoids (14.82±1.02-14.82±0.03 mg CE/g extract). On the other hand, the hexane extract displayed the highest content of phenolic acids (0.72±0.21 mg SAE/g extract). The antimicrobial activity of each extract was assessed using the agar disk diffusion method. The *A. holotricha* strain's ethyl acetate fraction displayed the most potent antibacterial activity. The *Escherichia coli* exhibited the greatest antibacterial impact, measuring 17 mm, while the *Klebsiella pneumoniae* showed a somewhat lower effect of 15 mm. *Bacillus subtilis* had the highest level of resistance among the microorganisms tested, with a diameter of 8 mm. *A. holotricha* displayed the most potent antioxidant and antibacterial properties in the ethyl acetate extracts. The study showed that ethyl acetate, a solvent with moderate polarity, is more efficient at dissolving aromatic compounds in the above-ground portion of the plant compared to non-polar solvents like n-hexane and diethyl ether. Moreover, the water and methanol extracts exhibited cytotoxicity towards PC-3 cells at particular concentrations.

Alchemilla holotricha Juz.'un Farklı Polaritelerdeki Ekstraktlarında Antioksidan, Antimikrobiyal ve PC-3 Kanser Hattı Üzerinde *İn Vitro* Sitotoksik Etkilerinin Araştırılması

Anahtar Kelimeler
Alchemilla holotricha Juz. Antimikrobial, Antioksidan, Antisitoksit, Bitki ekstıraktları

Alchemilla holotricha'nın hekzan, diklorometan, etil asetat, metanol ve su ile farklı polariteli ekstreleri elde edildi. Daha sonra bu ekstrelerin toplam antioksidan aktivite, DPPH serbest radikal uzaklaştırma aktivitesi, metal şelatlama kapasitesi, toplam indirgeme gücü, hidrojen peroksit uzaklaştırma, hidroksi radikal uzaklaştırma aktivitesi tespit edilmiştir. Toplam fenol içeriği 67.63± 0.88 GAE/g ile en yüksek etil asetat, toplam flavonoid içeriği 14.82±1.02–14.82±0.03 mg CE/g ile en yüksek etil asetat ve diklorometan, fenolik asit içeriği 0.72±0.21 mgSAE/g ile en yüksek hekzan özütünde elde edilmiştir. Antimikrobiyal aktivitenin belirlenmesinde agar disk difüzyon yöntemi kullanıldı. *Alchemilla holotricha*'nın etil asetat özütü en yüksek antibakteriyel aktiviteyi göstermiştir. En yüksek antibakteriyel etkiyi sırasıyla 17 mm çap ile *Escherichia coli*, ardından 15

mm ile *Klebsiella pneumoniae* göstermiştir. *Bacillus subtilis* 8 mm çap ile en dirençli mikroorganizma olarak tespit edilmiştir. *Alchemilla holotricha* hem antioksidan hem de antimikrobiyal etkinliğini en iyi etil asetat ekstraktında göstermiştir. Çalışmada kısmen polar bir çözücü olan etil asetatın bitkinin toprak üstü kısmındaki aromatik maddeleri n-heksan ve dietil eter gibi polar olmayan çözücülere göre daha iyi çözdüğü görülmüştür. Ayrıca su ve metanol ekstireleri PC-3 hücreleri üzerinde belirli dozlarda toksik etki gösterdi.

1. INTRODUCTION

Current studies have revealed that; Plants have the ability to reduce and prevent many diseases, due to their high antioxidant activity and ability to eliminate free radicals. Free radicals, which are released as a result of some biochemical reactions in the body, cause damage to biopolymers such as lipids, proteins and DNA. Excessive production of these ROS (reactive oxygen species), RNS (reactive nitrogen species) and toxic agents, reactions such as DNA damage, carbonylation of cellular proteins or lipid peroxidation occur, resulting in many chronic and degenerative diseases, especially cancer. Antioxidants, which are molecules that reduce or slow down oxidation with their free radical scavenging effects, protect the body against diseases that can be caused by ROS (reactive oxygen species), RNS (reactive nitrogen species), and toxic agents. Exogenous antioxidant substances, especially the use of medicinal plants among the public, in which are a good source of antioxidants due to their secondary compound content such as phenolic and flavonoid, are used in many industrial areas [1]. In addition, the use of plant polyphenols, which are natural antioxidants, instead of carcinogenic synthetic antioxidants used in the food and pharmaceutical industry has increased [2]. For centuries, peoples have been using plants for the prevention and treatment of diseases and this is known as ethnobotany. In ethnobotany, *Alchemilla* L. is a plant believed to be healing. In Anatolia and the world geography, many species are mentioned in the category of medicinal plants in the ethnobotanical field [3]. In the ethnobotanical field, it is known that *Alchemilla* L. species improve wound healing by covering them on wounds. In many research articles, it has been shown that *Alchemilla* species have strong antibacterial effects and have antioxidant properties with high oxidant removal due to the presence of high phenolic and other bio compounds in their content. Anticancer properties have been demonstrated in the studies of this study group and in some other studies in recent years. Among the *Alchemilla* species, the antioxidant and antimicrobial properties of *A. alpina*, *A. cimilensis*, A. mollis, and *A. vulgaris* were studied [3-5]. In this study, the biological activities of different solvent extracts obtained from *Alchemilla holotricha* Juz, which have not been studied before, were evaluated. Our aim in this study was to determine the most effective of different extracts of *Alchemilla holotricha*. By identifying the most effective extract of this plant, we demonstrate its high biological activities are in the functional food concept that has developed in recent years and is expected to continue its development in the future. We think that adding plant-based compounds to foods instead of synthetic preservatives will be effective in the short term and in the long term, because with the rise of industrial society, the daily diet of people and the consumption of ready-made and packaged foods from outside are increasing together. The increasing intake of these agents in the daily diet can lead to adverse health effects. Since the use of additives in foods is important not only for these properties but also for taste, smell and sensory properties, the use of added plant-based compounds will be effective in the long term approach.

2. MATERIAL AND METHODS

The plant samples used in this study were obtained from Trabzon-Gümüşhane Passage, Turkey. The taxonomic identification of this plant was confirmed by Y. Menemen. This plant was kept in the herbarium of Kırıkkale University (Yusuf 1504).

2.1. Extraction Procedure

The powdered plant samples were extracted from the nonpolar solvent to the polar solvent. Starting from the hexane fraction, extraction was performed in dichloromethane, ethyl acetate, methanol, and finally in distilled water in a shaking mixer at 150 rpm for 24 hours, respectively. The obtained filtrate was extracted by evaporating the solvent at low speed in a rota evaporator. The extract, which was transferred to a vacuum desiccator filled with $CaCl_2$, was dried completely and protected from moisture. Extract in 5 different fractions was obtained to be used in the study [6].

2.2. Antioxidant Activity

The Folin Ciocaltaeu method was used to determine the total phenolic content. 0.5 mL of the plant extract was taken and 2.5 ml of Folin Ciocaltaeu reagent was added into it. Then, 7.5 mL of sodium carbonate solution was added into the test tube and kept at 25°C for 2 hours. The total amount of phenol was calculated in UV Spectrophotometer at 760 nm, as equivalent to gallic acid [7].

2.2.2. Determination of total flavonoid content

To calculate the total flavonoid content; we added 1.25 mL distilled water to 250 µL plant extract. To this mixture 75 µL of 5% $NaNO_2$ solution was added. 6 minutes later, 500 µL NaOH was added, followed by 275 µL distilled water and mixed gently. Total flavonoid content was calculated as mg/kg by reading at 415 nm according to the catechin standard calibration chart. [8].

2.2.3. Determination of total phenolic acid content

2 ml HCl (0.5 M) was added into 1 ml plant extract. 2 mL reagent prepared by dissolving 10 g $NaNO_2$ and 10 g Na_2MoO_4 in 100 mL water. Then 2mL NaOH (8.5%) was added into that mixture. Then 2mL of NaOH (8.5%) was added. The final volume was made up to 10 mL. Total phenolic acid content was calculated as sinapic acid equivalent (mg/g) by reading at 505 nm [9].

2.2.4. Determination of total lycopene and B-carotene content

The method developed by Barros et al. was used to determine the total content of β-carotene and lycopene. For this, 100 mg of the plant extract was weighed and mixed with 10 ml of acetone-hexane (4:6). The mixture was filtered through filter paper. Beta-carotene and lycopene levels were calculated from absorbance values read at 453, 505 and 663 nm wavelengths. Use the formulas below to calculate beta-carotene and lycopene levels. [10].

$$\beta\text{-carotene (mg/100 ml)} = 0{,}216A_{663} - 0{,}304A_{505} + 0{,}452A_{453} \quad [1]$$

$$\text{Lycopene (mg/100 ml)} = -0{,}0458A_{663} + 0{,}372A_{505} - 0{,}0806A_{453} \quad [2]$$

2.2.5. Determination of DPPH radical scavenging

DPPH removal activity of the plant extracts was measured by the violet/purple decolorization abilities of the 2,2-diphenyl-1-picrylhydrazil radical. To determine the activity, 0.3 mL of plant extract was added to 2.7 mL of 0.1 mM DPPH. Afterwards, the solution was mixed and kept in the dark area for 1 hour. The DPPH radical scavenging effect of plant extracts was determined by measuring absorption at 517 nm. This radical scavenging activity was calculated using the following formula [11].

$$\%\ \text{Inhibition} = (A_{Control} - A_{Sample}/A_{Control}) \times 100 \quad [3]$$

2.2.6. Assessment of the reductive force property

To determine the reducing power properties of plant extracts, 2.5 mL of sodium phosphate buffer and 2.5 mL of potassium ferrocyanide (1%) were added to 2.5 mL of plant samples. The resulting solution was incubated at 50°C for about 20 minutes. To this mixture was added 2.5 mL of 10% prepared trichloroacetic acid and centrifuged at 1000 rpm. The supernatant was taken and 5 mL of deionized water and 1 mL of ferric chloride (0.1%) were added to it and read at 700 nm against BHA and α-tocopherol standards. [12].

2.2.7. H_2O_2 (Hydrogen Peroxide) removal activity

0.6 mL (40 mM) H_2O_2 in phosphate buffer was added to 0.4 mL of plant extract solution at different concentrations prepared in water. The final mixture was quickly read at 230 nm against the blank. The removal activity of the plant extract was calculated using the following formula [12].

$$H_2O_2\ \text{Removal Activity} = (A_{Control} - A_{Sample}/A_{Control}) \times 100 \quad [4]$$

2.2.8. Assessment of the $OH^{\cdot}$ removal activity

For hydroxyl radical scavenging activity, 60 µL $FeCl_2$, 90 µL 1,10-phenanthroline, 2.4 mL phosphate buffer, 150 µL H_2O_2 were added to the plant extracts at different concentrations, respectively. Samples incubated at 25 °C for 5 minutes were read at 560 nm. The radical scavenging activity was carried out according to the following equation [13].

$$\text{Hydroxyl Radical Removal} = (A_1 - A_2/A_0) \times 100 \quad [5]$$

Absorbance reading without plant extract was given as "A_0", absorbance reading in the presence of plant extract was given as "A_1" absorbance reading without 1,10-phenanthroline was given as "A_2".

2.2.9. Iron chelating capacity

The plant extracts were tested for their ferrous metal chelating activity. For this, 0.5 mL samples were taken from different fractions of the plant extract and mixed with 1.6 mL distilled water and 0.05 mL $FeCl_2$ (2 mM). Then 0.1 mL of Ferrozine (5 mM) was added to the mixture. Measure the absorbance of the Fe-Ferrozine complex of the mixture at 562 nm. The iron chelate activity was calculated using the following equation [14].

$$\text{Iron chelating capacity} = (A_{control} - A_{sample}/A_{control}) \times 100 \quad [6]$$

2.2.10. Evaluation of the total antioxidant capacity

For the antioxidant activities of the samples, 0.4 mL of the sample dissolved in methanol was taken, mixed with 4 mL of 0.6 M sulfuric acid containing 4 mM ammonium molybdate and 28 mM sodium phosphate, and the final mixture was vortexed and kept at 95 ^{0}C for 90 minutes. The antioxidant activity was read at 695 nm [15]. (y= 0.001x + 0.062, R2= 0.946).

2.3. Determination of Antimicrobial Activity

For the measurement of antimicrobial activity of extracts, the plant extracts were prepared with dimethylsulfoxide at 0.1 g/mL. The resulting solution was filtered with 0.22 µm Nylon membrane filters. 50 µl of each extract was absorbed into the blank discs. 200 µL of test organisms adjusted according to Mc Farland were taken and spread in a petri dish containing the medium. Then, these discs

were transferred to the petri dish under sterile conditions with the help of a forceps. Streptomycin was used as a reference antimicrobial agent and discs impregnated with dimethylsulfoxide were used as a negative control. Petri dishes were kept in the refrigerator at 4°C for 1 hour in order to spread the active ingredients in the extract absorbed into the discs to the medium and to keep the microorganism growth at the lowest level. At the end of the period, it was kept in an oven at 37°C for 24 hours in order to incubate for the development of microorganisms. For incubation, it was kept in an oven at 37°C for 24 hours. The antimicrobial activity of effective compounds in plant extracts was determined by measuring the zones formed around the discs. [16]. *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli (*ATCC 25922), *Klebsiella pneumoniae (EMCS), Candida albicans, Saccharomyces cerevisiae* obtained from Refik Saydam Hygiene Center were used for antimicrobial activity.

2.4. Antiproliferative Potential of Methanolic and Aqueous Extracts

WST-1 cell viability test was performed using Human prostate cancer cells (PC-3) to determine the efficacy of plant extracts. Human prostate cancer cells (PC-3) incubated and grown in cell growth medium containing 10% FBS and 1% penicillin-streptomycin, and then seeded in 96-well cell plates. It is adjusted to have approximately $5x10^3$ cells in each well. The cells in the 96-well plates were incubated for 24 hours for penetration. Then, water and methanolic extracts of the plant extracts were added to the cells in the wells at different doses and left for incubation. After 24 hours, 5 µL of 4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate (WST1) per well containing PC-3 added and incubated for 4 hours. The absorbance of each well at 450 nm was taken at the reference wavelength of 630 nm and read in ELISA.2.5. Statistical Analysis.

Student's t-test was used for analysis and measurements were made in three replicates.

3. RESULTS AND DISCUSSION

Since the antioxidant components in the content of plants remove the free radicals in the environment and thus prevent the oxidative stress that triggers many diseases, studies in this field are becoming more and more popular with each passing day. Numerous medicinal plants and their components, such as purified flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins, and isocatechin, contain antioxidant compounds that show beneficial therapeutic potential [17]. Therefore, in this study, the antioxidant effect of the aerial parts of *Alchemilla holotricha* was determined. In the study, it was observed that ethyl acetate, which is a partially polar solvent, dissolves aromatic substances in the above-ground part of the plant better than nonpolar solvents such as n-hexane and diethyl ether [18].

3.1. Antioxidant Assay Results

3.1.1. Total antioxidant capacity

The total antioxidant capacity results of the extracts are given in Table 1. The lowest antioxidant capacity was observed in the hexane fraction with 24.15 mg AAE/g plant, and the highest value was observed in the ethyl acetate fraction with 359.64 mg AAE/g. In a study conducted with *Alchemilla persica*, the total antioxidant capacity of ethyl acetate extract was found to be 292.18 mg AAE/g [3]. Extract yields of *Alchemilla holotricha* were also examined. The highest extract yield was obtained with 0.93% in ethyl acetate. In the study, the highest antioxidant activities were mostly obtained in this fraction.

3.1.2. Total phenolic compounds

Total phenolic content was plotted using the Gallic acid calibration curve in the spectrophotometer at 760 nm. ($y=0.002+0.052$, $R^2=0.920$). The ethyl acetate fraction has the highest total phenolic content as a solvent with relatively moderate polarity. Hexane, a nonpolar solvent, and its extract were observed to have the lowest phenolic content (Table 1). Similarly, in our study with *Alchemilla cimilensis*, the highest values of total phenolic content were obtained in the same solvent, namely ethyl acetate solvent [19].

3.1.3. Total flavonoid content and Total Phenolic Acid content (TPA)

One of the basic compounds in plants that eliminate tissue damage caused by radicals and prevents cells from being damaged is active compounds such as phenolics [20]. Flavonoids are low molecular weight phenolic compounds responsible for the antioxidant potential of plants. The total flavonoid amounts of the plant extracts were read in the spectrophotometer at 415 nm, and calculations were made in milligrams according to the (+)-catechin standard calibration curve ($y=0.163x-0.012$, $R^2=0.977$). The total flavonoid content in the examined plant samples ranged from 9.85 to 14.82. As with the phenolic content, the flavonoid concentration was also high in the ethyl acetate fraction (Table 1). Sözmen U.E. et al. [21] evaluated the total phenol and flavonoid contents of the *Alchemilla mollis* plant and reported that the phenolic compounds of the water extract were higher than the alcohol (methanol) extracts. Total flavonoid substance amounts were higher in ethyl acetate and dichloromethane extract. It was observed that hexane and water extracts demonstrated the lowest total flavonoid content. The fact that *A. holotricha* plant exhibits high antioxidants in the ethyl acetate fraction can be interpreted as the high content of polyphenols and flavonoids in this fraction. The flavonoid profile revealed in the study makes the plant medicinally important. In another study examining the flavonoid content of dichloromethane, ethyl acetate, methanol, and water extracts of *A. cimilensis*, it was reported that the ethy lacetate solvent medium gave higher results [19].

The TPA (total phenolic acid) content of the plant extracts was determined at 505 nm according to the sinapic acid standard calibration curve. (y=6960x-501.2, R^2=0.962). The total amount of phenolic acid substance was the same in all studied fractions. While there are many studies on total phenolic acid content by chromatographic methods, studies with spectrometric measurement are limited. The TPA content was obtained in the hexane fraction in the spectrometric study with *A. persica* [3].

3.1.4. Evaluation of Total β-Carotene and Lycopene Content

Carotenoids are secondary plant pigments and consist of an aliphatic chain with methyl groups attached and a conjugated double bond system that gives the carotenoids their color. Carotenoids show maximum absorbance at wavelengths of about 430-480 nm, and the color is the result of this conjugated double bond system. Since carotenoids are lipophilic compounds, they are more soluble in apolar organic solvents than alcohol [22]. In this study, the best results were obtained in dichloromethane solvent for β-carotene and methanol for lycopene. Results were calculated in mg carotenoid and lycopene per g extract (Table 1Total lycopene content in various solvent extracts for *A. holotricha* could be ordered as methanol > water= dichloromethane > hexane > ethyl acetate respectively. Total β-carotene content in various solvent extracts for *A. holotricha* could be ordered as extracts of dichloromethane >ethyl acetate > methanol > hexane > water respectively (Table 1). The biological importance of β-carotene is that it is a lipid antioxidant and neutralizes free radicals, especially singlet oxygen. Linoleic acid-free radical binds to highly unsaturated β-carotene models. In addition, the presence of carotenoids in the medium not only reduces the free radical concentration but also reduces Fe^{3+} to Fe^{2+} [23]. Lycopene has greater radical scavenging activity as an antioxidant in in-vitro systems than β-carotene [24]. Lycopene, one of the most powerful antioxidants, has a singlet oxygen holding capacity 2 times that of β-carotene and 10 times that of α-tocopherol [25].

Table 1. Effect of solvent type on total antioxidant, total phenolic, total flavonoid, total phenolic acid, total β-carotene and total lycopene content of extracts from *A. holotricha*

Antioxidant Activity	H	D	E	M	W
Total antioxidant capacity (TAC), (mg g^{-1})	24.15	135.96	359.64	197.84	234.06
Total phenolic content (mg GAE g^{-1})	8.05	28.32	67.63	45.98	48.16
Total flavonoid content (mg CE g^{-1})	10.46	14.82	14.82	11.29	9.85
Total phenolic acid content (mg SAE g^{-1})	0.72	0.72	0.72	0.72	0.72
Total β- carotene content (mg g^{-1})	1.04	1.68	1.62	1.47	0.92
Total lycopene content (mg g^{-1})	0.81	0.82	0.76	0.94	0.82

(H: hexane D: dichloromethane, E: ethyl acetate, M: methanol, W: distilled water)

3.1.5. DPPH Radical scavenging capacity (%)

The plant extracts showed close results with alpha-tocopherol, BHT and BHA studied as standard in the medium of water, ethyl acetate, and methanol. DPPH results are given in Table 2 as a percentage. An increase in the percentage of DPPH radical removal was observed depending on the concentration increase of the plant extracts. In one study, it was found that the DPPH radical scavenging effect of *Alchemilla alpina* methanol extract increased in the range of 45.4% -94.4% depending on the increasing concentrations [26]. Percent inhibition of DPPH radical scavenging effect of *Alchemilla vulgaris* extract has been reported as 71.8±4.1% [27]. The DPPH radical scavenging effect of *Alchemilla ellenbergiana* Rothm, in which methanol and hexane extracts were used, was found to be 243.1 µg mL^{-1} in the methanol extract. Low DPPH radical scavenging activity was also detected in hexane extract (7.1 µg mL^{-1}) [28]. Similarly, in our study, the radical scavenging effect of methanol extract (87%) was significantly higher than hexane (47 %). In another study, it was observed that the radical scavenging efficiency of water and methanol extracts was low in the *Alchemilla. mollis* species of this *Alchemilla* genus. This significant difference in the DPPH effectiveness of these two types in the same solvents may be attached to environmental impacts [21]. The variability of results depends on plant ecological conditions, harvest time, plant species, concentration studied and solvent used [5]. It is emphasized that there is a parallel relationship between phenolic content and antioxidant capacity, especially with radical scavenging activities. In our study, this parallel relationship between phenolic content and DPPH was observed especially in the ethyl acetate extract [29]. Since the DPPH method is technically simple, it is a widely used method for measuring the antioxidant activity of plant extracts. However, DPPH does not react to all antioxidants at the same rate, and the reaction rate is very slow for some antioxidants. For this reason, it is not an adequate method for the determination of antioxidant activity, and it is recommended to be supported by other methods.

3.1.6. Reductive force property

The reducing force of *Alchemilla holotricha* and synthetic antioxidants (BHA, BHT and α-tocopherol) are given in Table 2. As seen in Table 2, methanol, water and ethyl acetate showed a higher reduction than synthetic antioxidants. In the reducing power analysis, the presence of antioxidants in the plant extract reduces the Fe^{3+} / ferricyanide complex to the Fe^{2+} form. Fe^{3+} reduction is an indicator of electron-donating activity of phenolic antioxidants and is strongly associated with other antioxidant properties. In addition, it was observed that the reducing force activity increased in parallel with the increase in concentration at the studied concentrations. Therefore, the reductive force property of *Alchemilla holotricha* ethyl acetate extracts can generally be associated with phenolic hydroxyl groups. Because, in the same way, it was found in this solvent the most in phenolic groups. In a study, it was observed that the

reducing properties of *Alchemilla vulgaris* extracts, which were tested in ethanol, methanol, and ethyl acetate, were the most in ethyl acetate solvent [30].

3.1.7. Hydrogen peroxide removal activity assay

Percentage H_2O_2 removal activity for each sample were calculated and displayed in Table 2.

In the study, there was a high removal of hydrogen peroxide in the hexane fraction at all concentrations studied. In other fractions of the studied plant, the scavenging effect increased from 0.1 mg. The hydrogen peroxide removal activity in the hexane fraction can be attributed to the saturation of the fatty acids in this fraction [3]. The highest removal was observed at the highest concentration of 0.2 mg/mL in all fractions studied. The hydrogen peroxide concentration in the environment is formed according to phenolic compounds and flavonoids. Since the phenolic compounds in the plant extract are good electron donors, they can accelerate the conversion of H_2O_2 to H_2O [31]. Being good electron donors also shows reduced power [32].

3.1.8. Hydroxyl radical removal property

The hydroxyl radical is a highly reactive compound that attacks most organic molecules, and they are highly oxidizing by nature. The OH radical scavenging activity of the studied plant extracts and standards (BHA, BHT, α-tocopherol) is given in Table 2. The concentration ranges of the extracts prepared for the hydroxyl radical removal method is 0.05-0.2 mg/mL. The highest % inhibition was observed in the fraction of dichloromethane (11%) and ethyl acetate (13 %) at 0.2 mg/mL. It was observed that the OH radical scavenging effect also increased depending on the concentration increase of the plant extracts. Kaya reported that the OH scavenging activity of *Alchemilla persica* was the best in dichloromethane, and this was due to the low polarity of dichloromethane, which resulted from functional groups binding free groups better [3].

3.1.9. Metal chelating activity

Metal chelating activity was evaluated according to the competition of plant extracts with ferrozine to bind Fe^{+2} ions in solution. The metal chelation potential of the plant extracts used in the study is given in Table 2. Metal chelating activity of the hexane, dichloromethane and ethyl acetate extract were found to be high for *Alchemilla holotrchia* solvent extracts, while methanol and water extracts showed very little activity. On the other hand, in the present study, *Alchemilla. holotrchia* solvent extracts showed stronger metal chelating activity than synthetic antioxidants. This proves that the plant extract is a peroxidation inhibitor. Boroja et al. [33] using $ABTS^{\cdot+}$ and $^{\cdot}OH$ assays showed that the methanol extract of *A. vulgaris* has a greater antioxidant potential than the synthetic antioxidant. However, they reported that the methanol extract of *Alchemilla vulgaris* L. did not have Fe^{2+} reducing properties [33]. In our study where different solvent extracts were used, the lowest iron chelating activity was observed in methanol.

Table 2. Antioxidant activity of extracts from *Alchemilla holotricha* in different solvents

DPPH scavenging capacity (%)	0.05 mg mL^{-1}	0.1 mg mL^{-1}	0.15 mg mL^{-1}	0.2 mg mL^{-1}
Hexzane	43.83±2.11	44.51±4.44	46.57±3.12	47.63±6.23
Dichlorometan	45.98±6.12	46.66±.4.16	47.42±1.23	53.6±7.26
Ethyl acetate	85.50±3.21	86±6.21	86.57±3.12	88.14±4.12
Methanol	85.57±5.13	86.66±.3.12	86.69±6.23	87.17±.5.23
Water	86.93±0.23	87.27±.3.31	87.48±3.21	87.62±.8.21
α-tocopherol	83.19±1.26	85.44±.3.23	85.52±.4.03	85.72±6.36
BHA	83.81±3.32	85.18±.5.36	85.33±5.06	85.66±4.44
BHT	75.91±6.19	79.42±.6.12	82.22±.3.96	83.58±4.00

Reducing power activity	0.05 mg mL^{-1}	0.1 mg mL^{-1}	0.15 mg mL^{-1}	0.2 mg mL^{-1}
Hexane	0.294±0.01	0.303±0.03	0.304±0.23	0.481±0.06
Dichlorometan	0.355±0.03	0.381±0.04	0.452±.0.26	0.554±0.05
Ethyl acetate	2.194±0.23	2.891±0.32	3.158±0.12	3.333±0.24
Methanol	1.132±0.11	1.746±0.15	2.485±0.26	3.011±0.12
Water	1.439±.0.12	2.340±0.42	2.758±0.46	2.775±0.23
α-tocopherol	0.375±0.05	0.622±0.46	0.944± 0.05	0.994± 0.11
BHA	0.788±0.07	1.433±0.59	2.068± 1.12	2.441± 0.28
BHT	0.247±0.12	0.265±0.71	0.355± 0.08	0.385± 0.02

%OH scavenging activity	0.05 mg mL^{-1}	0.1 mg mL^{-1}	0.15 mg mL^{-1}	0.2 mg mL^{-1}
Hexane	0.25±0.02	0.44±0.09	1.04±0.02	2.13±0.01
Dichloromethane	0.41±0.23	1.95±0.05	9.74±0.25	11.95±0.32
Ethyl acetate	2.96±0.36	3.34±0.02	12.48±0.51	13.29±0.12
Methanol	1.21±0.21	1.99±0.12	3.24±0.96	3.57±0.03
Water	1.36±0.06	1.81±0.08	2.38±0.74	2.62±0.12
α-tocopherol	0.86±0.05	1.32 ± 0.96	1.41 ± 0.58	2.50 ± 0.21
BHA	0.43±0.09	0.54 ± 1.16	0.65 ± 0.32	0.86 ± 0.45
BHT	0.15±0.11	0.16 ± 0.87	0.27 ± 0.74	0.33 ± 1.56

Metal chelating activity (%)	0.05 mg mL^{-1}	0.1 mg mL^{-1}	0.15 mg mL^{-1}	0.2 mg mL^{-1}
Hexane	60.62±2.02	64.54±0.21	69.28±0.32	73.15±0.21
Dichlorometane	57.88±2.14	66.87±0.12	71.47±0.12	96.46±1.23
Ethyl acetate	45.46±0.36	59.50±2.14	61.93±0.21	86.49±1.56
Methanol	44.43±0.13	44.94±1.15	45.43±0.15	45.78±3.25
Water	41.24±0.21	42.15±1.11	47.59±0.18	51.23±0.89
BHT	5.74± 2.03	13.17 ± 0.0	16.37 ± 1.4	36.57± 2.08
α-tocopherol	33.28±2.14	33.35 ± 0.0	34.56 ± 2.0	36.91± 1.78
BHA	33.58±0.21	34.32± 1.15	38.14± 1.61	43.67± 0.65
Trolox	3.84±0.52	34.95± 1.18	36.78± 0.74	36.94± 0.85

Hydrogen peroxide scavenging activity (%)	0.05 mg mL^{-1}	0.1 mg mL^{-1}	0.15 mg mL^{-1}	0.2 mg mL^{-1}
Hexane	52.24±0.12	54.76±1.23	58.44±0.89	59.92±1.25
Dichlorometane	46.40±0.56	47.67±0.13	47.69±4.25	53.12±4.12
Ethyl acetate	28.16±0.03	34.05±2.10	42.07±4.51	50.23±6.25
Methanol	41.03±0.13	42.61±1.36	52.37±3.23	53.21±4.65
Water	42.84±0.54	46.98±3.23	57.73±4.63	57.75±4.43

3.2. Antimicrobial Activity

Antimicrobial activity findings for *Alchemilla holotricha* strain are displayed in Table 3. The antibacterial activities of the extracts *A. holotricha* were tested against bacteria, and one yeast strain. As evident from Table 3, the hexane extracts from *A. holotricha* weak activity against bacteria. However, the ethyl acetate and methanol extract of *A. holotricha* was the most active showing a strong inhibitory effect against all tested Gram-positive bacteria, Gram-negative bacteria and yeast strain. On the other hand, the water and dichloromethane extracts showed low antimicrobial activity similar to that of *A. holotricha* extracts.

Studies on the antimicrobial activities of different strains of this *Alchemilla* are available in the literature, and different rates of antimicrobial activity were observed in the same bacterial strains [26, 28, 34, 35, 36]. In the study with *Alchemilla ellenbergiana*, it showed an equal inhibition zone of 11 mm in ethanol and ethyl acetate extracts against *K. pneumoniaea*, a gram-negative bacterium, while an 18 mm zone of inhibition against *C. albicans* in the ethanol extract, and *S. aureus* (MRSA) in ethyl acetate and methanol extracts of 13 mm and 12 mm, respectively. [28]. In another study, increasing concentrations of *Alchemilla glabra* Neygenf formed an inhibition zone of 12-22 mm in gram negative bacteria *E. coli*, 22-17 mm in *P. vulgaris*, and 13-15 mm in *K. pneumoniae*. It was determined that *S. aureus* showed antimicrobial activity at different rates with a 12-22 mm inhibition zone. In the same study, it formed a 14-16 mm inhibition zone against *C. albicans* at different concentrations [36-37].

Table 3. *In vitro* antimicrobial activities of *A. holotricha* different solvents

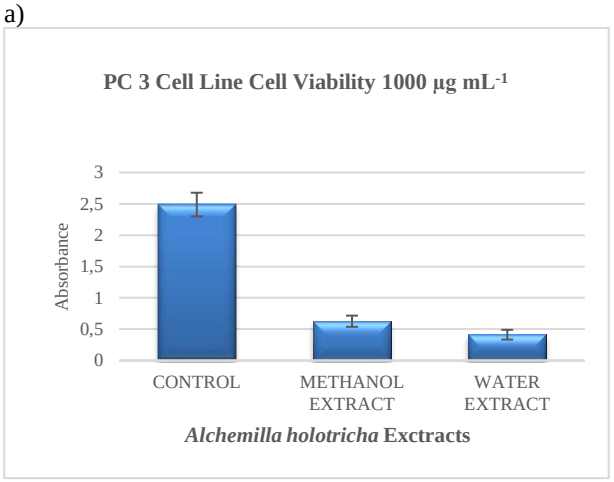
Microorganism	*Alchemilla holotricha* Extracts						
	H	D	*E*	*M*	*W*	*S.*	*DMSO*
	Inhibition zone (mm)						
B. subtilis	-	7±0,1	8±0,2	8±0,1	7±0,2	25±0,7	-
S. aureus	8±0,1	10±0,3	11±0,1	12±0,4	7±0,3	40±0,8	-
E. coli	7±0,1	12±0,4	17±0,3	10±0,4	8±0,5	40±1	-
K. pneumoniae	-	9±0,5	15±0,2	10±0,2	8±0,2	26±0,3	-
C. albicans	-	8±0,3	13±0,6	9±0,3	8±0,3	16±0,5	-
S. cerevisiae	-	-	8±0,1	11±0,2	7±0,2	23±0,4	-

***** Hexane (H), Dichloromethane (D), Ethyl acetate (E), Methanol (M), Water (W), Dimethylsulfoxide (DMSO), Streptomycin (S)

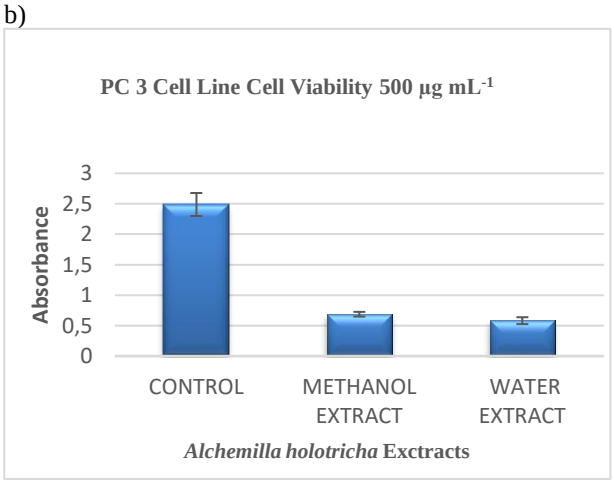
3.3. Antiproliferative Potential of Methanolic and Aqueous Extracts

PC-3 cell lines were used to evaluate the effect of the extracts on tumor cell growth. To determine the proliferation effects of methanol and water extracts of *Alchemilla holotricha* in the PC-3 cell line, applications at different concentrations between 125-1000 µg mL^{-1} were performed. water and methanol extracts of *A. holotrchia* decreased PC-3 cell proliferation significantly at concentrations from 125 to 1000 µg mL^{-1} when compared to control cells ($p < 0.001$). (Fig. 1a, b, c and d). All extracts showed cytotoxic effect on PC-3 cells. A study was conducted using L929 and MDA-MB 231 cells to determine the cytotoxic effects of extracts obtained from *A. mollis* roots. In this study, no toxic effects were observed in the concentration range of 62.5 and 3.25 µg mL^{-1} of different extracts, while it was reported that the ethyl acetate extract at a concentration of 250 µg mL^{-1} had a higher toxic effect than the other extracts. [38-39]. In the study conducted to determine the cytotoxic effect of methanol and water extracts of *A. persica* on PC-3 cell line, methanol extract showed a higher antiproliferative effect when compared to the other two concentrations at 250 µg mL^{-1} [3]. However, in this study, the cytotoxic activity of the methanol extract of *A. holotricha* on the cell PC-3 line was higher than the water.

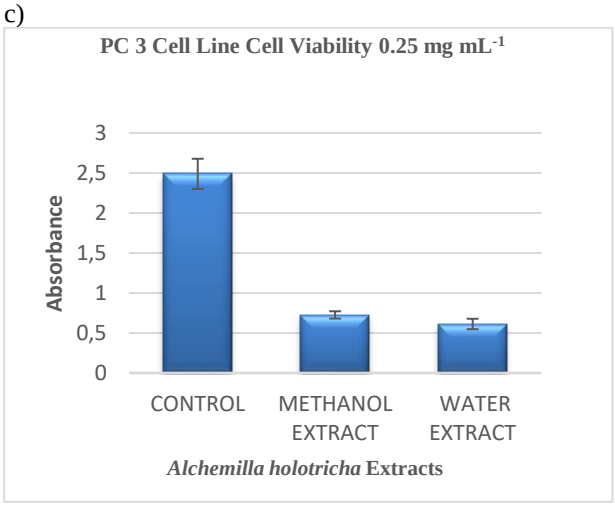
a)



b)



c)



d)

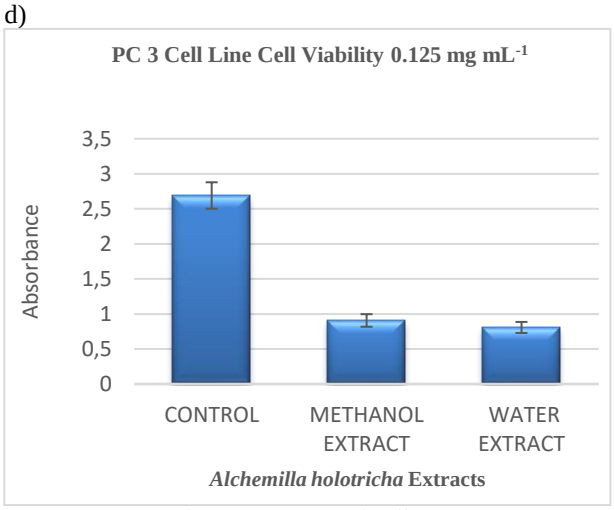


Figure 1. Antiproliferative potential of different concentrations of methanol and aqueous extracts of *A. holotricha*; a) 1000 µg mL^{-1}, b) 500 µg mL^{-1}c) 250 µg mL^{-1} d) 125 µg mL^{-1}

4. CONCLUSION

Natural antioxidants such as polyphenols, flavonoids, and phenolic compounds found in various parts of plants are responsible for preventing the harmful effects of stress by scavenging free radicals. As a result, due to its antioxidant content, *Alchemilla holotricha* is a natural source of antioxidants. The gram-negative bacteria were mainly affected by the ethyl acetate solvent. It also showed high antimicrobial activity in *C. albicans*, a pathogenic yeast. The water and methanol extract of *Alchemilla holotricha* showed significant cytotoxicity on the PC-3 cell line. In the light of the data obtained from this study, when antioxidant antimicrobial and anticancer were evaluated, it was determined that although the activities of the lion's claw (*Alchemilla holotricha*) plant in different solvents vary, the activity levels, in general, were high.

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