

Investigation of antimicrobial and antioxidant activities of *Achillea setacea* Waldst. et Kit. extracts and extract loaded gel formulation

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ABSTRACT: Antibiotic resistance poses a significant problem in the treatment of infections. Plants, with their rich content, may contain compounds that can be used for this purpose. The dried flowers of *Achillea setacea* Waldst. et Kit. are prepared as an infusion and used internally for hemorrhoids, as an anti-diarrheal and wound healing agent, and also against abdominal pain. The antioxidant activities of different extracts and extract-loaded gel formulations of *A. setacea* were determined using DPPH, FRAP, and CUPRAC methods, and the total phenolic content was determined using FCR methods. The antimicrobial efficacy of the different extracts and the extract-loaded gel formulation was investigated using agar well diffusion and microdilution methods. The chloroform extract of *A. setacea* was selected for gel production due to its significant antioxidant activity (particularly iron(III) ion reducing activity), broad-spectrum antimicrobial activity, and efficacy against *Staphylococcus* species identified as primary causative agents of wound and skin infections. A pharmaceutical gel was prepared from the obtained chloroform extract using alginate, a natural polymer, characterized by FTIR, subjected to in vitro release testing, and its antimicrobial and antioxidant activities were evaluated. According to the findings, although the antimicrobial and antioxidant activity of the produced gel was lower than that observed at the extract level, it was determined that its antimicrobial and antioxidant efficacy was preserved. Based on these results, it is thought that *A. setacea*-loaded gel could potentially be used in wound treatment due to its antimicrobial and antioxidant activities after the completion of cytotoxic studies.

KEYWORDS: *Achillea setacea*; gel formulation; wound healing; antimicrobial effect; antioxidant activity.

1. INTRODUCTION

Antibiotics are used to treat infectious diseases caused by bacteria. In recent years, for various reasons, many infectious microorganisms have shown increasing resistance to antibiotics through different mechanisms. Today, due to the very limited or even non-existent antibiotic options available for treating resistant bacteria, difficulties are encountered in treating infections. The use of more toxic, broader-spectrum antibiotics in treating patients negatively affects the treatment process psychologically and physiologically, in addition to causing economic losses. For these reasons, there is an increase in studies on new compounds and methods that can prevent infections and have positive effects during treatment. In this regard, plants are of great importance. New antimicrobial compounds derived from plants can support the treatment of infectious diseases [1,2].

Some studies report that the therapeutic effects of plants stem from the synergistic effects of multiple compounds rather than a single active ingredient, thereby providing more effective treatment by countering the resistance of microorganisms that are difficult to kill with a single antibiotic. This has led researchers to investigate the inhibitory effects of natural antimicrobial agents derived from plant extracts [3,4].

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Wound healing is a complex process consisting of three phases that follow one another and overlap: inflammation, proliferation, and remodeling. Extracellular matrix components such as collagen, elastin, and hyaluronic acid, which support cells, provide flexibility, and ensure their continued viability, contribute to the wound healing process. Therefore, preventing the degradation of these compounds by inhibiting hyaluronidase, collagenase, and elastase may support wound healing [5,6].

Factors encountered in life, both internal and external, cause free radicals to form in the body. Free radicals are one of the fundamental causes of disease formation and the natural aging process in individuals. Antioxidant substances protect the body from the harmful effects of free radicals. Many natural products have antioxidant properties. The antioxidant effects of plant products are mainly due to phenolic compounds such as flavonoids, cinnamic acid derivatives, and coumarins. The main groups of polyphenols are flavonoids, phenolic acids, tannins, stilbenes (resveratrol), and lignans. These are known to protect substances found in foods that are easily oxidized from oxidation. For this reason, medicinal and aromatic plants, which have been used for many years as additives to enhance the aroma and taste of foods, are becoming increasingly important. As the amount of free radicals increases, aging accelerates, cell death occurs, followed by tissue death, and then damage leading to the destruction of brain vessels [7-12].

The genus *Achillea* L., consisting of 154 species (212 taxa in total) is a typical plant of Mediterranean climate regions, and its native range is the temperate and subtropical regions of the Northern Hemisphere [13,14]. In Türkiye, it is represented by 61 taxa, 33 of which are endemic [15]. *Achillea* has a mythological past. According to surviving information, Achilles, one of the heroes of the Iliad, distributed these plants to his soldiers after battles to stop their bleeding and heal their wounds. Since then, this genus has been known as *Achillea* after Achilles [16]. It is also one of the most popular hemostatic plants in folk medicine. In addition, it is used for gynecological pain, dyspeptic disorders, stomach aches, ulcers, gastritis, colds, fever and psychosomatic cramp pains. Yarrow, which is widely used as an herbal tea in many parts of the world, is also used in the form of ointments, herbal tinctures, or sitz baths [13]. Although the most common is 'yarrow', *Achillea* species are also known by different local names in our country. One of these is the *Achillea setacea* Waldst. et Kit. species, known by various local names in Türkiye such as Ayvabala, Ayvadana, Ayvadani, Ayvadene, Erkurtaran, Girtkesani ispi, Mayasıl otu, and Sancı otu [17]. Regarding the distribution of this species in our country, it is rare in Eastern Anatolia, while widespread in the rest of Türkiye. It grows in steppes, rocky slopes, meadows, and fallow fields at altitudes ranging from sea level to 2300 m [18,19]. *A. setacea* has various uses in Türkiye as a folk medicine for the treatment of digestive system disorders, gynecological diseases, skin diseases, and tuberculosis [20].

Species of the genus *Achillea* are regarded as some of the oldest medicinal plants and have been widely used in folk and traditional medicine in different countries for the management of wounds, bleeding, gastrointestinal disorders, headaches, inflammation, pain, and spasmodic diseases [21]. Numerous experimental and clinical studies have reported that species of the genus *Achillea* possess a broad spectrum of biological activities, including antimicrobial, anti-inflammatory, cytotoxic, antinociceptive, wound-healing, estrogenic, antispasmodic, as well as hepatoprotective and choleric properties [22-25]. There are relatively few scientific studies investigating *A. setacea*. These studies have reported that *A. setacea* contains essential oils, alkaloids, flavonoids, glycosides, tannins, resins, and organic acids, and that its essential oil exhibits antimicrobial activity against various microorganisms [23,26-28].

In this study, it was aimed to determine the antimicrobial and antioxidant activities of some extracts of *A. setacea* and to evaluate their potential for use in a gel formulation intended for wound healing.

2. RESULTS

2.1. Antioxidant activity results

2.1.1. Determination of DPPH Radical Scavenging Activity of *A. setacea* Extract and Extract-Loaded Gel Film

The free radical scavenging activity percentage inhibition values of the extracts from the plant were evaluated by comparison. The results obtained are shown in Table 1. When the extracts were compared with each other, the methanol extract showed higher activity. The chloroform-loaded gel was found to have a higher inhibition potential than the crude chloroform extract. When the results obtained were evaluated, all three extracts and extract-loaded gel showed lower activity than ascorbic acid (Table 1, 2).

Table 1. DPPH radical scavenging activities of extracts from plant

<i>A. setacea</i> extracts	DPPH (%inhibition)
Methanol	28.2060± 0.9348
Chloroform	4.9080± 0.2494
Petroleum Ether	3.6590± 0.3076
Ascorbic Acid	94.5305±0.9846

Table 2. DPPH Radical Scavenging Activities of Extract-Loaded Gel Films

	DPPH (%inhibition)
Chloroform Extract-loaded gel	20.7934± 0.9610
Extract free gel	9.5666± 1.7984
Ascorbic Acid	94.5305±0.9846

2.1.2. Determination of the Iron (III) Ion Reduction Antioxidant Power (FRAP) of *A. setacea* extract and extract-loaded gel

The FRAP method is quite important in evaluating the iron (III) ion reduction potentials of plant extracts. Iron reduction power is based on the reduction of Fe^{+3} to Fe^{+2} by the plant extract and the spectrophotometric analysis of this at 593 nm. In this method, high absorbance indicates high iron reduction potential.

When the FRAP values obtained in this study were compared with each other, it was found that the chloroform extract of *A. setacea* had the highest FRAP values. The chloroform extract-loaded gel was found to have lower FRAP values than the crude chloroform extract. In addition, it was observed that all extracts and extract loaded gel had lower radical scavenging activity than BHA (Table 3, 4).

Table 3. FRAP values of the extracts from plant

<i>A. setacea</i> extracts	FRAP (mM FeSO_4 /mg extract)
Methanol	1.3911± 0.0945
Chloroform	2.1138± 0.2197
Petroleum Ether	0.8938± 0.0697
BHA	5.81 ± 0.004

Table 4. FRAP values of extract-loaded gel

	FRAP (mM FeSO_4 /mg extract)
Chloroform Extract-loaded gel	0.0502± 0.0086
Extract free gel	0.0029± 0.0008
BHA	5.81 ± 0.004

2.1.3. Determination of Copper (II) Ion Reducing Antioxidant Capacity (CUPRAC) of *A. setacea* Extract and Extract-Loaded Gel Film

The antioxidant capacity of extracts and extract- loaded gel from the plant in reducing copper (II) ions was evaluated comparatively, and the results obtained are shown in Table 5. When the results obtained in this study are evaluated, the methanol and chloroform extracts showed the highest CUPRAC values. It was found that the gel loaded with chloroform extract had a lower copper (II) ion reducing antioxidant capacity than the crude chloroform extract. The findings showed that the extract-loaded gel and the extracts had lower CUPRAC values than the standard compound (Table 5, 6)

Table 5. CUPRAC values of the extracts from plant

<i>A. setacea</i> extracts	CUPRAC (mM Trolox equivalents/mg extract)
Methanol	1.4480± 0.0940
Chloroform	0.8124± 0.1079
Petroleum Ether	0.6237± 0.0387
Ascorbic Acid	5.6830±0.3372

Table 6. CUPRAC values of extract-loaded gel

	CUPRAC (mM Trolox equivalents/mg extract)
Chloroform Extract-loaded gel	0.0840± 0.0340
Extract free gel	-
Ascorbic Acid	5.6830±0.3372

3.1.4. Determination of Total Phenolic Content in *A. setacea* Extract and Extract-Loaded Gel

The total phenolic compounds contained in extracts from *A. setacea* were determined using the FCR method. The results obtained are given in Table 7 as mg gallic acid equivalent/mg extract. It was determined that the methanol extract of *A. setacea* contained the highest amount of phenolic compounds. The extract-loaded gel was found to contain phenolic compounds in amounts similar to those in the crude chloroform extract (Table 7, 8).

Table 7. The amount of phenolic compounds found in plant extracts

<i>A. setacea</i> extracts	TPC (mg GAE/mg extract)
Methanol	0.1090± 0.0093
Chloroform	0.0585± 0.0053
Petroleum Ether	0.0582± 0.0074

Table 8. The amount of phenolic compounds found in chloroform extract-loaded gels

	TPC (mg GAE/mg extract)
Extract-loaded gel	0.0407± 0.0060
Extract free gel	-

2.2. Antimicrobial Activity

2.2.1. Determination of the antimicrobial activity of *A. setacea* extracts and the gel by the agar well diffusion method

The results obtained from the determination of the antimicrobial activity of the extracts using the agar well diffusion method are presented in Table 9. It was determined that the petroleum ether and chloroform extracts exhibited antimicrobial activity, whereas the methanol extract showed no antimicrobial activity. Among the extracts, the chloroform extract was determined to have a broader antimicrobial spectrum (Table 9). The petroleum ether extract showed antimicrobial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*, while the chloroform extract additionally exhibited activity against *Acinetobacter Baumannii*

Table 9. Antimicrobial activity of *A. setacea* extracts evaluated by the agar well diffusion method

	ZD (mm)					
	PE	CE	ME	DMSO	M	AmB
<i>Pseudomonas aeruginosa</i> ATCC 27853	0	0	0	0	29.32 ±0.24	-
<i>Staphylococcus aureus</i> ATCC 29213	17.01 ±0.23	14.02 ±0.22	0	0	31.07 ±0.21	-
<i>Staphylococcus aureus</i> ATCC 43300	17.23 ±0.12	12.22 ±0.19	0	0	29.68 ±0.18	-
<i>Klebsiella pneumoniae</i> ATCC 4352	0	8.38 ±0.08	0	0	28.56 ±0.17	-
<i>Proteus mirabilis</i> ATCC 14153	0	0	0	0	29.39 ±0.18	-
<i>Salmonella typhimurium</i> ATCC 14028	0	0	0	0	31.01 ±0.24	-
<i>Staphylococcus epidermidis</i> ATCC 12228	9.96 ±0.09	12.01 ±0.21	0	0	42.39 ±0.20	-
<i>Acinetobacter baumannii</i> ATCC 19606	0	11.02 ±0.14	0	0	22.78 ±0.11	-
<i>Enterococcus faecalis</i> ATCC 29212	19.60 ±0.11	13.33 ±0.13	0	0	18.44 ±0.17	-
<i>Escherichia coli</i> ATCC 25922	0	0	0	0	27.81 ±0.27	-
<i>Candida albicans</i> ATCC 90028	0	0	0	0	-	21.14 ±0.09
<i>Candida tropicalis</i> kuen 1021	0	0	0	0	-	15.41 ±0.10
<i>Candida glabrata</i> ATCC 90030	0	0	0	0	-	21.37 ±0.16

ZD: Inhibition zone diameter, PE: Petroleum ether extract, CE: Chloroform extract, ME: Methanol extract, M: Meropenem, AmB: Amphotericin B, -: Not done

The data on the antimicrobial activity of the *A. setacea*-loaded gel film (AsG) are presented in Table 10. The data in Table 10 indicate that AsG retained its antimicrobial activity against the microorganisms for which the chloroform extract exhibited antimicrobial effects and remained effective against these microorganisms.

Table 10. Antimicrobial activity of *A. setacea*-loaded gel (AsG) evaluated by the agar well diffusion method

	ZD (mm)
	AsG
<i>S. aureus</i> ATCC 29213	9.60±0.11
<i>S. aureus</i> ATCC 43300	11.59±0.17
<i>K. pneumoniae</i> ATCC 4352	7.87±0.17
<i>S. epidermidis</i> ATCC 12228	11.18±0.18

<i>A.r baumannii</i> ATCC 19606	10.72±0.20
<i>E. faecalis</i> ATCC 29212	10.09±0.12

ZD: Inhibition zone diameter, AsG: *A. setacea*-loaded gel

2.2.2. Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC)

The MIC values of the extracts that exhibited antimicrobial activity in the agar well diffusion assay are presented in Table 11. The MIC values of the petroleum ether extract were determined to range between 0.20 and 0.78 mg/mL, while the MIC values of the chloroform extract ranged from 0.20 to 3.13 mg/mL.

Table 11. MIC and MBC values of the extracts exhibiting antimicrobial activity in the agar well diffusion assay

	MIC (mg/mL)/MBC (mg/mL)	
	PE	CE
<i>S. aureus</i> ATCC 29213	0.20/0.80	0.20/0.80
<i>S. aureus</i> ATCC 43300	0.20/0.80	0.20/0.40
<i>K. pneumoniae</i> ATCC 4352	-	3.13/6.20
<i>S. epidermidis</i> ATCC 12228	0.20/0.40	0.20/0.4
<i>A. baumannii</i> ATCC 19606	-	0.39/0.78
<i>E. faecalis</i> ATCC 29212	0.78/1.56	0.39/0.78

MIC: Minimal inhibitory concentration, MBC: Minimal bactericidal concentration, PE: Petroleum ether extract, CE: Chloroform extract, -: Not done

2.6.Characterization of Gel Formulations

Fourier-transform infrared (FTIR) spectroscopy was used to confirm the structure and possible interactions between the phytochemical content of the extract and the alginate matrix of the gel (Figure 1).

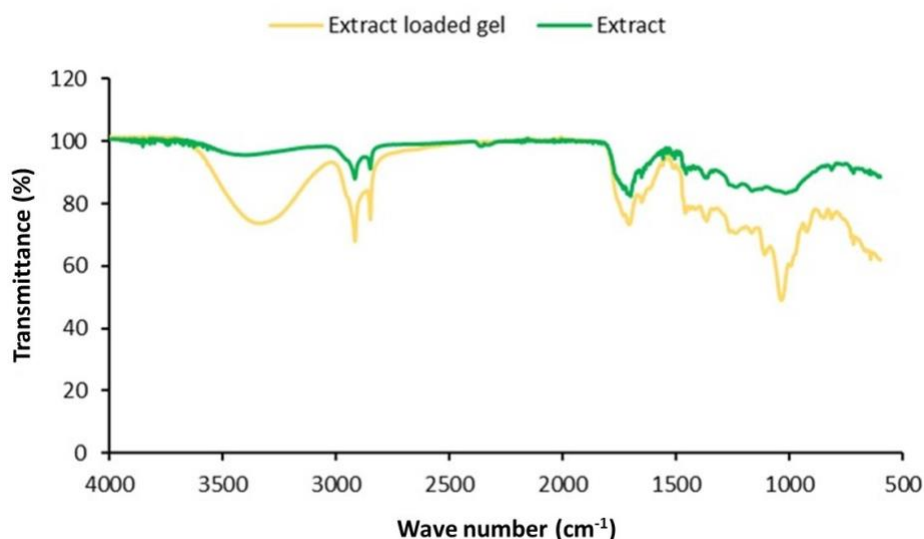


Figure 1 FTIR spectra of the extract and the extract loaded gel

It is important to observe the in vitro release profile of the extract loaded gel to show the application potential of the produced gel formulation in the drug delivery systems of the active phytochemicals. Figure 2 shows the in vitro release profile of the extract loaded gel formulation.

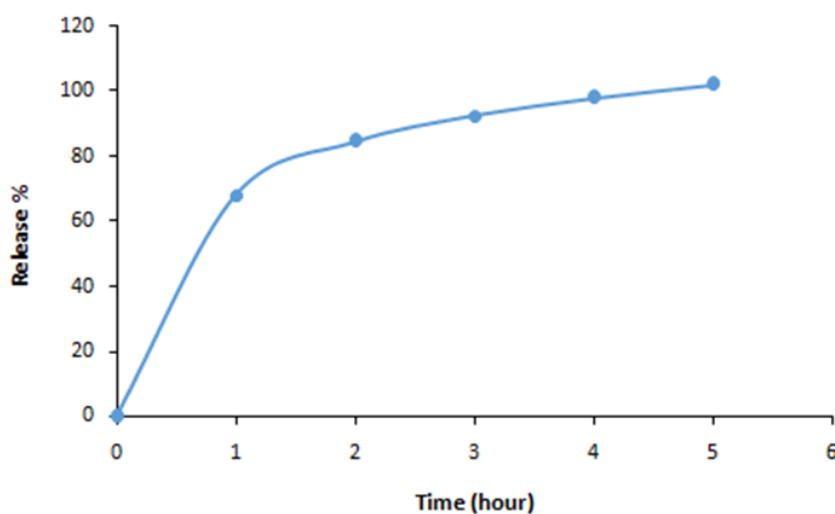


Figure 2. In vitro release profile of the gel

3. DISCUSSION

Wound care is currently considered a serious problem. Regardless of the cause, disruption of skin integrity renders wounds susceptible to infections. Moreover, wound healing is delayed in individuals with chronic diseases, particularly diabetes, as well as in immunocompromised patients. In addition, inadequate immune responses to infection and antimicrobial resistance may lead to severe outcomes, including death. Therefore, the development of new products that promote wound healing and possess antimicrobial activity is of great importance. Due to their rich composition, plants can be considered primary candidates for this purpose and can be utilized in various formulations [29-34].

Gel formulations are among the pharmaceutical dosage forms that can be used for this purpose. Gel formulations developed for wound care provide a moist wound environment due to their high water content, thereby supporting cell migration, epithelialization, and tissue regeneration. In plant-based gel formulations, extracts obtained from medicinal plants such as *Aloe vera* (L.) Burm.f., *Centella asiatica* (L.) Urb., *Achillea* species, and *Calendula officinalis* L. are frequently used. These components accelerate wound healing through their antioxidant, antimicrobial, and anti-inflammatory properties. Such gels are generally prepared using polymeric carriers including alginate, chitosan, gelatin, PVA, or carbopol, which may enable the controlled release of plant-derived active compounds. Plant-based gels may exhibit lower toxicity and better biocompatibility compared to synthetic agents. In addition, their ease of application to the wound surface, ability to reduce pain, and potential to decrease the frequency of application provide significant advantages in terms of usability and patient compliance. These characteristics render plant-based gel formulations promising biomaterials in modern wound care [29,30,34-40].

In the limited number of studies conducted on the antimicrobial activity of *Achillea* species, different results have been reported. In a study investigating the antimicrobial activity of methanol, water, and chloroform extracts of *Achillea schischkinii* Sosn. and *Achillea teretifolia* Willd. it was reported that the extracts of *A. teretifolia* possessed antimicrobial activity against *E. coli*, *S. aureus*, *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, *Enterobacter aerogenes*, and *Candida* sp., whereas the extracts of *A. schischkinii* did not exhibit antimicrobial activity against these microorganisms [24]. It has been reported that the n-hexane, ethyl acetate, and aqueous extracts of *A. setacea* and *Achillea coarctata* Poir. possess antimicrobial activity. Specifically, the hexane and ethyl acetate extracts of *A. setacea* were found to exhibit antimicrobial activity against *S. aureus*, *E. coli*, and *C. albicans*, whereas the n-hexane and ethyl acetate extracts of *A. coarctata* demonstrated antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*. In addition, the aqueous extract of *A. coarctata* was shown to have antimicrobial activity against *C. parapsilosis*, *C. tropicalis*, and *C.*

glabrata [25]. In our study, antimicrobial activity assays conducted with petroleum ether, chloroform, and methanol extracts of *A. setacea* against different bacterial strains revealed antimicrobial activity against the *S. aureus* strain (Table 9-11), consistent with the findings of [25]. However, in contrast to their results, no antimicrobial activity was detected against *E. coli* or *Candida* spp. strains. The discrepancies between the two studies may primarily be attributed to differences in extraction methods, the geographical origin and collection time of the plants, and the solvents used.

In recent decades, many studies have been conducted on the biological activities of species belonging to the genus *Achillea*, due to the biological activities of the secondary compounds they contain. The antioxidant activities of methanol extracts prepared from different parts (flowers, leaves, and roots) of the *A. setacea* plant were compared by Şabanoğlu and colleagues using DPPH and ABTS methods. Additionally, the amount of total phenolic compounds was determined using the FCR method. According to the findings, *A. setacea* leaf and root methanol extracts showed significant DPPH radical scavenging activity [41]. In contrast, our study investigated the antioxidant activity of different extracts from the aerial parts of the plant, and similarly, the methanol extract was found to have significant DPPH radical scavenging activity and contain total phenolic compounds.

Gel formulation prepared was characterized by using FTIR technique. In the FTIR spectra (Figure 1), the characteristic bands of the alginate matrix of the gel formulation are seen at approximately 3300, 2900, 1700 and 1400 cm^{-1} . Broad band which is seen between 3000 and 3500 cm^{-1} is corresponded to O-H stretching vibrations belongs to hydroxyl groups in the structure. The peaks at around 2900, 1700 and 1400 cm^{-1} were attributed to the C-H bonds, asymmetric and symmetric stretching of COO^- groups, respectively. Peak positions were not changed for the extract loaded gel, with the exception of a new peak formation at around 1050 cm^{-1} which is associated with C-O-C stretching vibrations in the spectra of the extract loaded gel [42-45].

In vitro release profile of the prepared gel was also evaluated and given in Figure 2 which shows a slow and controlled release where 100% of the extract was released gradually during the 5 hours. The slow and controlled release increases the duration of single-dose administration, significantly improving patient compliance with the treatment [46].

4. CONCLUSION

In our study, the chloroform extract of *A. setacea* was chosen for gel production due to its significant antioxidant activity (especially iron(III) ion reducing activity), broad-spectrum antimicrobial activity, and effectiveness against *Staphylococcus* species, which have been identified as the primary causative agents of wounds and skin infections. According to the findings, although the antimicrobial and antioxidant activity of the produced gel was lower than that observed at the extract level, it was found that the antimicrobial and antioxidant efficacy was preserved. Based on these results, it is thought that *A. setacea*-loaded gel could potentially be used in wound treatment due to its antimicrobial and antioxidant activities after the completion of cytotoxic studies.

5. MATERIALS AND METHODS

5.1. Procurement of Plant Material

A. setacea were collected from Çamlıyayla, Mersin on 27 June-2020 and identified by Dr. İsmail Şenkardeş. Dried specimens were cataloged in Marmara University Herbarium (No: 22448 MARE).

5.2. Extracts Preparation

The aerial parts of *A. setacea* were dried at room temperature and ground into powder. The aerial parts were sequentially extracted using the maceration method to obtain petroleum ether, chloroform, and methanol extracts. After maceration, the liquid portion was filtered through filter paper and collected. The solvents from the collected filtrates were evaporated using a rotary evaporator and then a lyophilizer to obtain crude extracts. The extracts obtained were stored at 4°C until the day the study began.

5.3. Antioxidant Activity Determination Method

5.3.1. DPPH radical scavenging activity assay

In this method, the free radical scavenging activity of the extracts is determined using 2,2-diphenyl-1-picrylhydrazyl(DPPH). About 10 μL of each extract was seeded onto microplates, then 240 μL of DPPH

solution at a concentration of 0.1 mM was added. The standard solution (ascorbic acid) and extract seedings were vortexed for 1 min and then kept in the dark at 25 °C for 30 min. Absorbances were measured at 517 nm with respect to the reference using a microplatereader. The results were calculated by expressing IC₅₀ values (mg/mL). % DPPH radical scavenging capacity = $[(A_0 - A_1) / A_0] \times 100$ A₀ : Absorbance of control solution, A₁ : Absorbance of plant extracts and standard solutions [43].

5.3.2. Ferric (III) reduction antioxidant power determination (FRAP)

The ferric ion reducing antioxidant potential (FRAP) assay was performed according to the method of Benzie and Strain (1996) [47]. The FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution (a solution of 10 mM TPTZ in 40 mM HCl) and 2.5 mL of 20 mM FeCl₃.6H₂O. It was incubated at 37°C for 30 min. 0.19 mL of the FRAP reagent was mixed with 0.01 mL of extract or standard, and the increase in absorbance at 593 nm at 4 minutes was measured against a blank prepared with distilled water instead of extract. Absorbance values of the extracts were read at 593 nm.

5.3.3 Copper (II) ion reduction antioxidant capacity method (CUPRAC)

The antioxidant capacity of extracts as a copper (II) ion reducer was determined according to the method developed by Apak and colleagues [48]. 60 µL of extracts, 60 µL of Cu (II)x2H₂O, 60 µL of neocuproine, and 60 µL of NH₄ Ac (1 M) were mixed on top, respectively. And finally, 10 µL of distilled water was added. The absorbance reference value of the mixture was measured at 450nm. CUPRAC values of the extracts were given as mM TroloxE/mg extract

5.3.4 Determination of Total Phenolic Compound Content

The amount of total phenolic compounds in the extracts was determined by the FCR method. 25 µL of extracts, 100 µL of Folin-Ciocalteu reagent (diluted 1/3 with distilled water) and 75 µL of 2% sodium carbonate solution were added to the plate. Following this, the mixture was left at room temperature for 2 hours and then the absorbance at 750 nm was measured by comparison with the reference. The total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE)/mg extract [44].

5.4. Antimicrobial activity

The antimicrobial activity of *A. setacea* extracts was first screened using the agar well diffusion method. For the extracts that exhibited antimicrobial activity in the agar well diffusion assay, the minimal inhibitory concentration (MIC) was subsequently determined.

5.4.1. Agar well diffusion assay method

For the determination of antimicrobial activity, the following reference strains were used: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Salmonella typhimurium* ATCC 140208, *Staphylococcus epidermidis* ATCC 12228, *Acinetobacter baumannii* ATCC 19606, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 90028, *Candida tropicalis* KUEN 1021, and *Candida glabrata* ATCC 90030. Bacterial strains were cultured on tryptic soy agar (TSA) at 37 °C for 18–24 h, while yeast strains were grown on Sabouraud dextrose agar (SDA) at 35 °C for 24–48 h. Microorganism suspensions were prepared from colonies grown on solid media in 0.85% physiological saline solution. Bacterial suspensions were adjusted to 0.5 McFarland standard turbidity, whereas yeast suspensions were adjusted to 1.0 McFarland standard. Using sterile swabs, the suspensions were spread onto Mueller–Hinton agar (MHA) for bacteria and SDA for yeasts. Wells with a diameter of 5 mm were aseptically punched into the agar using a sterile well borer, and 50 µL of each extract dissolved in its respective solvent was added to the wells. Dimethyl sulfoxide (DMSO) and physiological saline were used as negative controls, while meropenem (10 µg/well) for bacteria and amphotericin B (100 µg/well) for yeasts were used as positive controls. The plates were incubated at 37 °C for 18–24 h for bacteria and at 35 °C for 24–48 h for yeasts. After incubation, inhibition zones were measured in millimeters. All experiments were performed in triplicate, and the mean values were calculated. or the determination of the antimicrobial activity of the *A. setacea*-loaded gel, the gel was dissolved in DMSO, and its antimicrobial activity was evaluated using the method described above [49–52].

5.4.2. Determination of the minimal inhibitory concentration and minimal bactericidal concentration for bacteria

In accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines, bacterial suspensions were prepared from 24-h cultures and adjusted to 0.5 McFarland turbidity. The suspensions were then diluted to obtain a final inoculum concentration of 5×10^5 CFU/mL. Serial dilutions of the samples were prepared in sterile U-bottom 96-well microdilution plates by adding 100 μ L of Mueller-Hinton broth (MHB) to each well. Subsequently, 5 μ L of the bacterial suspension was added to the wells containing the extracts, and the plates were incubated at 37 °C for 24 h. After incubation, the lowest concentration of the extract showing no visible bacterial growth was defined as the minimal inhibitory concentration (MIC). For the determination of the minimal bactericidal concentration (MBC), 10 μ L aliquots from wells showing no visible growth in the MIC assay were subcultured onto tryptic soy agar (TSA) plates and incubated at 37 °C for 24 h. Following incubation, the lowest concentration showing no bacterial growth was recorded as the MBC. Mueller-Hinton broth (MHB), dimethyl sulfoxide (DMSO), and meropenem were used as controls. All experiments were performed in triplicate [51-53].

5.9 Preparation and characterization of the gel formulation

Chloroform extract loaded gel were produced by using 300 mg sodium alginate dissolved in 10 mL DI water, 300 mg extract dissolved in 10 mL DI water and 5 mL DMSO. They were mixed using a magnetic stirrer and 1 mL of glycerin were added to this mixture. Extract free gel was also produced using the same procedure. Fourier-transform infrared (FTIR) spectroscopy was used to analyze the chemical functionalities in the structure of the gel formulation. FTIR spectra of the extract loaded gel and the extract were obtained from 4000 to 500 cm^{-1} with an average resolution of 4 cm^{-1} (IRSpirit spectrometer, Shimadzu Corp, Kyoto, Japan). In vitro release of extract from the gel formulation was studied spectrophotometrically (Shimadzu 2100S) using a dialysis bag in PBS (phosphate-buffered saline, pH 7.4) medium. 5 mg of gel and 10 mL of PBS was used for the release experiments. Release experiments were conducted in a thermostatic shaking water bath at 37°C. Samples were taken at predetermined time intervals (at 1 h, 2 h, 3 h, 4 h and 5h) and the concentration of the samples were calculated by spectrophotometric method using the calibration curves prepared initially [54].

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