



Antibiofilm Potential and Chemical Characterization of Crude Methanolic Extracts from *Astragalus gummifer*

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Abstract

Astragalus species are widely recognized for their potent bioactive properties; however, the antibiofilm potential of *Astragalus gummifer* remains unexplored. In the present study, we assessed the antimicrobial and antibiofilm activity of *A. gummifer* metabolic extracts against referenced bacterial and *Candida* strains. Plant material was collected from the Palandöken Mountain region in Erzurum, Turkey. The crude extract was obtained by methanol extraction and concentrated at 62 °C, followed by dissolution in distilled water for microdilution and crystal violet assays. In addition to these assays, untargeted metabolite profiling was performed on the methanolic extract. Fourier Transform Infrared Spectroscopy (FT-IR) and Quadrupole Time-of-Flight Liquid Chromatography–Mass Spectrometry (Q-TOF-LC/MS) analyses were employed for compound identification. A total of nine known secondary metabolites were successfully identified in the crude extract of *A. gummifer*.

Keywords: *Astragalus gummifer*, Biofilm, Qtof lcms, Kristal violet

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Introduction

Bacterial biofilms are complex microbial communities encased in a self-produced extracellular polymeric substance (EPS) that adhere to surfaces. Unlike planktonic (free-floating) bacteria, biofilm-forming bacteria exhibit enhanced resistance to antibiotics, disinfectants, and the host immune system, making them a major concern in medical, industrial, and environmental settings [1], [2], [3]. Biofilms are responsible for a wide range of persistent infections, such as chronic wounds, dental plaque, catheter-associated infections, and implant-related complications [4]. Additionally, they contribute to industrial biofouling and water contamination, leading to significant economic and health challenges [5]. Understanding biofilm formation and developing effective strategies to inhibit or disrupt these structures is crucial for improving infection control and industrial hygiene [6], [7], [8]. Plants produce many metabolites important for human health [8], [9], [10]. Some of these plants are the *Astragalus* species that have gained importance in recent years. The flora of Turkey includes more than 440 species of the *Astragalus* genus, 211 of which are known to be endemic [11]. *Astragalus* species are used in various ways. Because of their high nutritional properties, some *astragalus* species are used as animal feed. *Astragalus* species that are not eaten by animals play an important role in preventing soil erosion. In addition, some *astragalus* species are uprooted and used as fuel, while others are preferred as ornamental plants due to their aesthetic appearance and beautiful flowers [12]. *Astragalus* species attract attention not only with their industrial use but also with their immune system modulatory, antioxidant, anti-inflammatory, hepatoprotective and neuroprotective properties. Studies show that this plant supports the immune system, delays cellular aging and may positively affect cardiovascular health [13]. Many of the *Astragalus* species can also be consumed by humans due to their antiviral and liver

protective properties. The *Astragalus gummifer*, which grows in the mountains of Eastern Anatolia, especially Turkey and Iran, in high altitude and arid regions is a remarkable medical plant. It is a perennial plant belonging to the Fabaceae (legume) family, distributed across the arid and semi-arid regions of Turkey. When the chemical composition of *A. gummifer* is examined, it is known that there are many secondary metabolites such as saponins, flavonoids, polysaccharides, phenolic compounds and alkaloids. However, studies investigating the effects of *Astragalus* species on biofilm formation are quite limited, and this indicates that more research is needed to evaluate the antibiofilm potential of *A. gummifer* [9], [14], [15]. In the light of this information, the antibiofilm activity of *A. gummifer* extract against pathogenic microorganism was shown for the first time in the present study. Moreover, chemical structure was investigated.

Material and methods

Chemicals

All chemicals used in the study, such as ethanol, crystal violet dye, phosphate buffered saline (PBS), Mueller-Hinton Agar (MHA), broth (MHB) and potato dextrose agar (PDA) were of analytical grade and were purchased commercially. Extraction of *Astragalus gummifer* Fresh *Astragalus gummifer* was collected from Palandöken Mount, Erzurum city in Turkey. The plant (leaves and petiole) was air dried. Then, 20 g of plant parts were added to 99% methanol for 72 hours for maceration. It was then filtered through whatman paper. The methanolic extract obtained was evaporated with the help of a rotary evaporator (Scilogex, RE100 Pro) at 62°C. The remaining extract was dissolved in 1 mL of sterile dH₂O.

Bacterial and Yeast Culture Conditions

Four bacterial strains (*Pseudomonas aeruginosa* PAO1 and ATCC 27853 *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and

Enterococcus faecalis (ATCC 29212)) and four yeast strains (*Candida albicans* ATCC 90028, *C. tropicalis* KUEN 1025, *C. parapsilosis* ATCC 22019 and *C. dubliniensis* CBS 7987) were used to test antimicrobial and antibiofilm activity assay. Bacteria and yeast strains were grown on MHA and PDA medium, respectively.

Microdilution Assay

The microdilution assay was conducted using modified EUCAST protocol [16]. In brief, an overnight culture was grown in medium at 37°C with continuous shaking at 150 rpm. After incubation, the final inoculum was adjusted to an optical density of 0.08–0.1 at 600 nm. In a 96-well plate, 100 µL of the extract at increasing concentrations (0.5–512 mg/L) was combined with 100 µL of the microbial culture, bringing the total volume to 200 µL. Medium served as the negative control. The plates were then incubated under static conditions at 37°C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no microbial growth was observed. The results were determined by taking the averages of three repeated experiments.

Crystal Violet Assay

The antibiofilm activity was assessed using a modified crystal violet (CV) assay [17]. Initially, microbial cultures were prepared in a 96-well plate following a similar procedure to the microdilution test. After 48 hours of incubation, non-adherent cells were discarded, and the wells were rinsed with sterile water. Subsequently, 0.1% crystal violet dye was added to each well and allowed to stain for 20 minutes. Excess dye was then removed, and the wells were washed thoroughly with tap water. Finally, 30% acetic acid was added to dissolve the bound dye, and absorbance was measured at 590 nm using a spectrophotometer

(Thermo, Multiscan). All measurements were performed in triplicate, and the results are expressed as the mean ± standard deviation.

Fourier-Transform Infrared Reflectance Spectroscopy (FT-IR)

FT-IR Attenuated total reflectance (ATR) spectroscopy (Bruker VERTEX 70V) was used to characterise the crude extract. The spectra were calculated in the frequency range of 500–4000 cm⁻¹ [18].

Quadrupole Time-of-Flight Liquid Chromatography Mass Spectrometry (Q-TOF-LC/MS)

The crude extract was characterised using Q-TOF-LC/MS system (Agilent Technologies, CA, USA). The gradient system with the mobile phase consisted of solvent A: ultra-pure water/%1 formic Acid and solvent B: formic Acid/acetonitrile. The column temperature was set to 35 °C. The electrospray ionization (ESI) mode was selected using a fragmentation voltage of 90. The gradient profile used in the Q-TOF-LC/MS was taken in Table 1. Compounds of the unknown peaks were investigated using devices library, PubChem and Mass bank [19]

Table 1: The gradient profile used in the Q-TOF-LC/MS separation of crude extract of *Astragalus gummifer*.

Time (min)	Flow Rate (mL/min)	%A	%B
0-1	0,4	90	10
1-15	0,4	80	20
15-18	0,4	100	0
18-24	0,4	80	20

Result

In this study, the antimicrobial and antibiofilm effects of the methanolic extract of the *A. gummifer* on bacterial and fungal culture was examined. Interestingly, in our study, the methanolic extract of *A. gummifer* was not found to have any lethal or growth inhibitory properties against either bacteria or yeast. Therefore, in the microdilution test we performed, no results were obtained up to the value of 512 mg/L. Despite this, crystal violet staining revealed significant antibiofilm activity against *S. aureus*, *E. coli*, *C. albicans*, and *C. parapsilosis*. At a concentration of 8 µg/mL, the extract inhibited biofilm formation by 66% in *S. aureus* and 70% in *E. coli*. Similarly, at 64 µg/mL, biofilm inhibition rates reached 83% for *C. albicans* and 86% for *C.*

parapsilosis. These results are illustrated in Figure 1. Notably, no antibiofilm activity was detected against two clinical strains of *P. aeruginosa*, or against *C. dubliniensis* and *C. tropicalis*.

FTIR-ATR spectra provide information about the chemical characteristic functional groups of crude extract [20]. The absorbance wavelength graph of the FTIR result is presented in figure 2. The peak at 1074-1112/cm was ascribed to C-N stretching amine. The peak at 1116,49/cm was attributed to C=C stretching. The peak at 1651/cm was attributed C=N stretching imine/oxime or C=O stretching conjugated ketone or alkenes. The peak at 2947/cm was ascribed to CH₃ asymmetry stretching. The peak at 3338/cm was attributed intermolecular bonded alcohol O-H stretching.

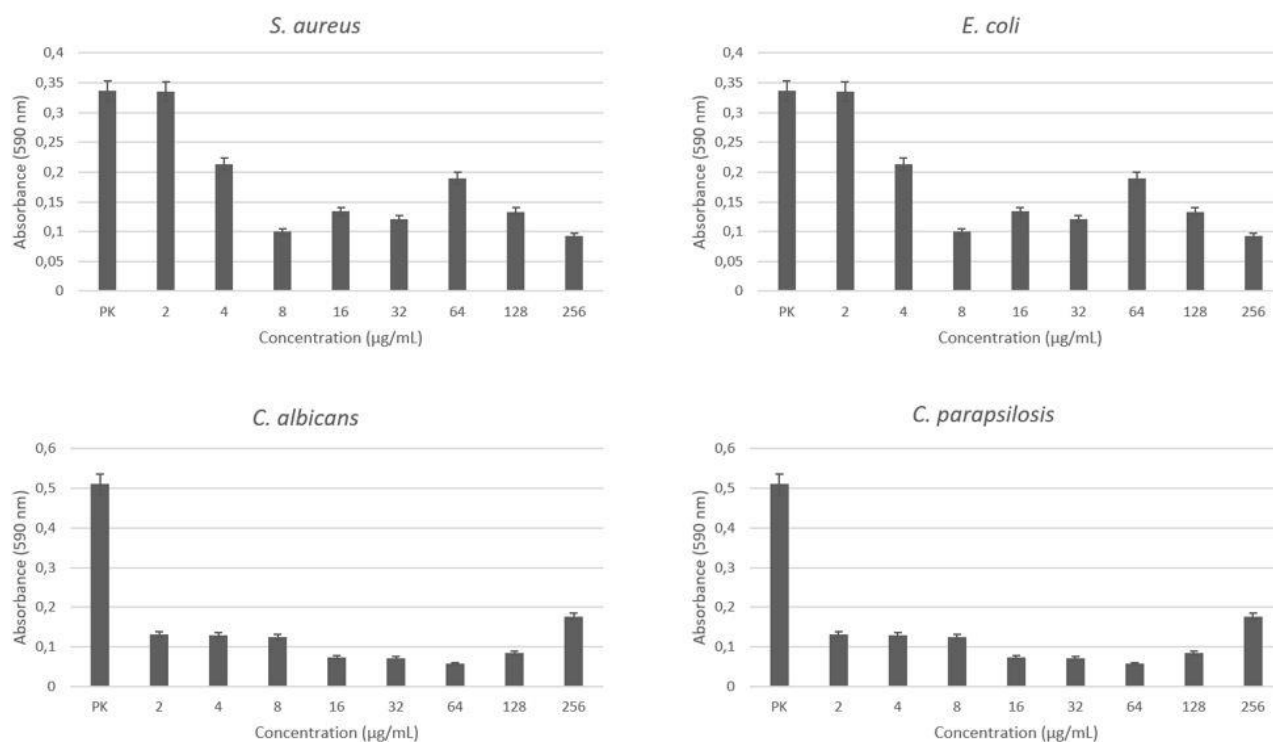


Figure 1. Antibiofilm activity of methanolic extract of *A. gummifer* against *S. aureus*, *E. coli*, *C. albicans* and *C. parapsilosis*. Results obtained from the average of three replicates. Standard error was taken in the results. PK is the group that contains only cells, without astragalus extract.

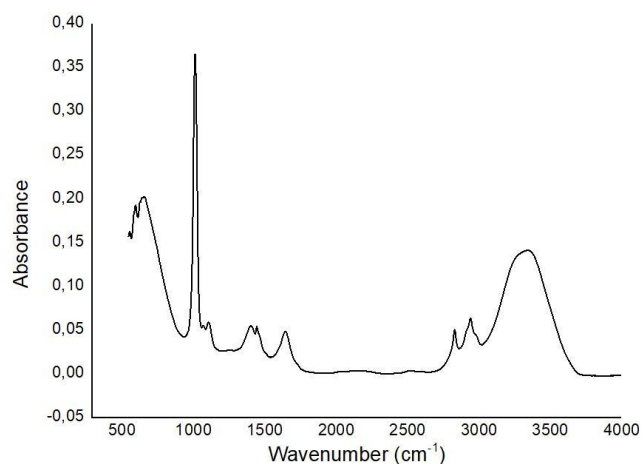


Figure 2. FTIR analysis of methanolic extract of *A. gummifer*

Q-TOF-LC/MS analyses were carried out to determine the molecular weight and possible structure of the compounds in the crude extract [19], [21] As an

outcome of the analyses, the most abundant peak was 871.57232 m/z. The library search revealed that this peak resembles Pheophytin a. Pheophytin a is a derivative of chlorophyll. It is found in plants, algae, and cyanobacteria and is formed when the magnesium (Mg) ion in chlorophyll a is replaced by hydrogen (H) ions. The other most abundant peak was m/z. 607.28967 m/z. The library search revealed that this peak resembles Euphornin. It is an alkaloid of plant origin. According to the library screening, the other most abundant peak, 326.22906 m/z, is understood to be a compound of aromatic origin. Figure 3 displays the m/z data acquired from the ESI ion scan of the crude extract analysis. Nonetheless, further studies are needed for comprehensive characterization.

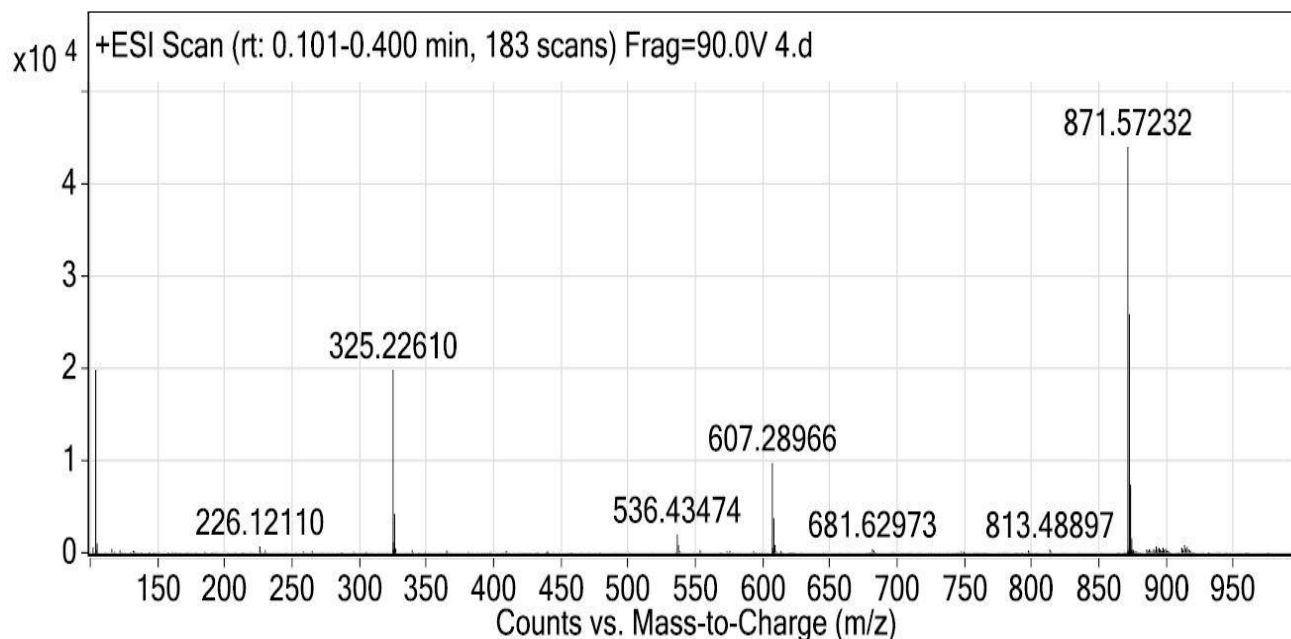


Figure 3: ESI-MS positive ionization for the major peaks in *A. gummifer* crude extract

Table 2: Chemical Compounds identified in the *A. gumnifer* extract through Q-TOF-LC/MS analysis.

Compounds	Molecular Formula	ions	m/z	Score
Pheophytin a	C ₅₅ H ₇₄ N ₄ O ₅	(M+H) ⁺	871.57239	97,82
Glabin C	C ₄₁ H ₆₄ N ₈ O ₉	(M+H) ⁺	813.48901	62,45
Euphornin	C ₃₃ H ₄₄ O ₉	(M+Na) ⁺	607.28967	94,1
Desmethylnaproxilone glucuronide	C ₂₅ H ₂₉ N O ₆	(M+H) ⁺	440.20584	76,51
Histidyl-histidyl-phenylalanine	C ₂₁ H ₂₅ N ₇ O ₄	(M+H) ⁺	440.20584	76,26
Linalyl anthranilate	C ₁₇ H ₂₃ N O ₂	(M+Na) ⁺	296.16275	46,03
alpha-Terpinyl anthranilate	C ₁₇ H ₂₃ N O ₂	(M+Na) ⁺	296.16275	46,03
1-(p-Methoxyphenyl)-5-piperidino-1-penten-3-one	C ₁₇ H ₂₃ N O ₂	(M+Na) ⁺	296.16275	46,03
Alpha-Pyrrolidinopropiophenone	C ₁₃ H ₁₇ N O	(M+Na) ⁺	226.12109	82,69

Discussion

Plants produce a wide variety of metabolites that enable their survival and adaptation to environmental stress factors. These compounds are generally divided into two groups: primary and secondary metabolites. Primary metabolites are directly involved in essential physiological processes such as growth, development, and reproduction, while secondary metabolites mostly function in environmental interactions [22], [23]. Secondary metabolites include various structural groups such as alkaloids, flavonoids, terpenoids, phenolic compounds, and glycosides. These compounds fulfill many vital roles in plants, such as defense against herbivores, resistance to pathogens, protection from ultraviolet radiation, competitive advantage, and signaling. Moreover, many secondary metabolites are of great importance to humans, as they are widely used in drug development, food additives, cosmetic products, and agricultural biotechnology. Therefore, a detailed understanding of the structural diversity and biological functions of secondary metabolites is crucial for both basic science and applied research. In the present study, we initially investigated the antimicrobial and antibiofilm properties of *A.*

gumnifer collected from Erzurum Palandöken Mountain. Locally known as 'geven', *A.* [24], [25], [26] *gumnifer* was first evaluated for antimicrobial activity using agar well diffusion (data not shown) and microdilution assays. No antimicrobial activity was observed against the reference microorganisms tested in this study. This could be attributed to the insolubility of the antimicrobial compounds in the methanolic extract. Another possible explanation is the degradation of compounds in the extract at 62°C. Additionally, the timing of plant collection might have influenced the results, as the plant materials used in this study were collected during September and October [27], [28].

Although the crude extract of *A. gumnifer* had no antimicrobial results, interestingly it showed antibiofilm activity against some bacterial and yeast strains. This situation suggests that antimicrobial and antibiofilm properties are independent of each other. In fact, there are many studies addressing this issue. The antibiofilm property is attributed to the prevention of microorganism adhesion to surfaces without killing them. In this context, *A. gumnifer* extract may have

inhibited the quorum sensing mechanism or interfered with the motility mechanisms responsible for initial adhesion [28], [29], [30], [31]. Inhibition of biofilm is very valuable in drug-resistant strains such as *C. albicans* and *S. aureus*. Slightly different from this observation, methanol extracts of *Astragalus membranaceus* enriched with flavonoid fractions have been shown to exhibit potent antibacterial and antibiofilm activity against *Bacillus cereus*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were reported as 1.56 mg/mL and 6.25 mg/mL, respectively, and biofilm formation was significantly suppressed as shown by crystal violet test [32].

Destroying the biofilms is as important as inhibiting pathogens. In fact, 80% of infectious diseases today are caused by biofilms. The fact that biofilms are so effective necessitates the discovery of compounds with antibiofilm properties. In the current study, upon analyzing the chemical composition of the crude extract of *A. gummifer*, the presence of aromatic compounds and alkanes was identified. Library screening of the Q-TOF-LC/MS results led to the identification of a total of nine compounds. A list of

these compounds is presented in Table 2. Upon examination, none of these compounds correspond to previously identified substances known to exhibit antibiofilm properties. This highlights the need for further investigation of the pure forms of these compounds for their potential antibiofilm activity in future studies.

Author Contributions

RT, AE, MKC and AY were made the experiments. AY designed and supervised the experiment. RT and AY contributed to the writing.

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Declaration of Interest

There is no conflict of interest between the authors. All authors have read and approved the manuscript.

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