



Helichrysum arenarium as a source of flavonoids: Evaluation of antimicrobial activity and flavonoid content of extracts of Helichrysum flowers in vitro

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ABSTRACT

Background: Helichrysum (family Asteraceae, kind of *H. armenium*, subfamily of Asteroideae) is known in Armenian traditional medicine for its diuretic, antioxidant, choleric and anti-inflammatory properties. Studies have shown that Helichrysum flowers have a high content of flavonoids. In this study, we investigated the impact of various extraction process parameters on the yield of flavonoids obtained from Helichrysum flowers in Armenia. Subsequently, a comprehensive antimicrobial assay was conducted on the ethanol and aqueous extracts derived from the dried flower heads of Helichrysum.

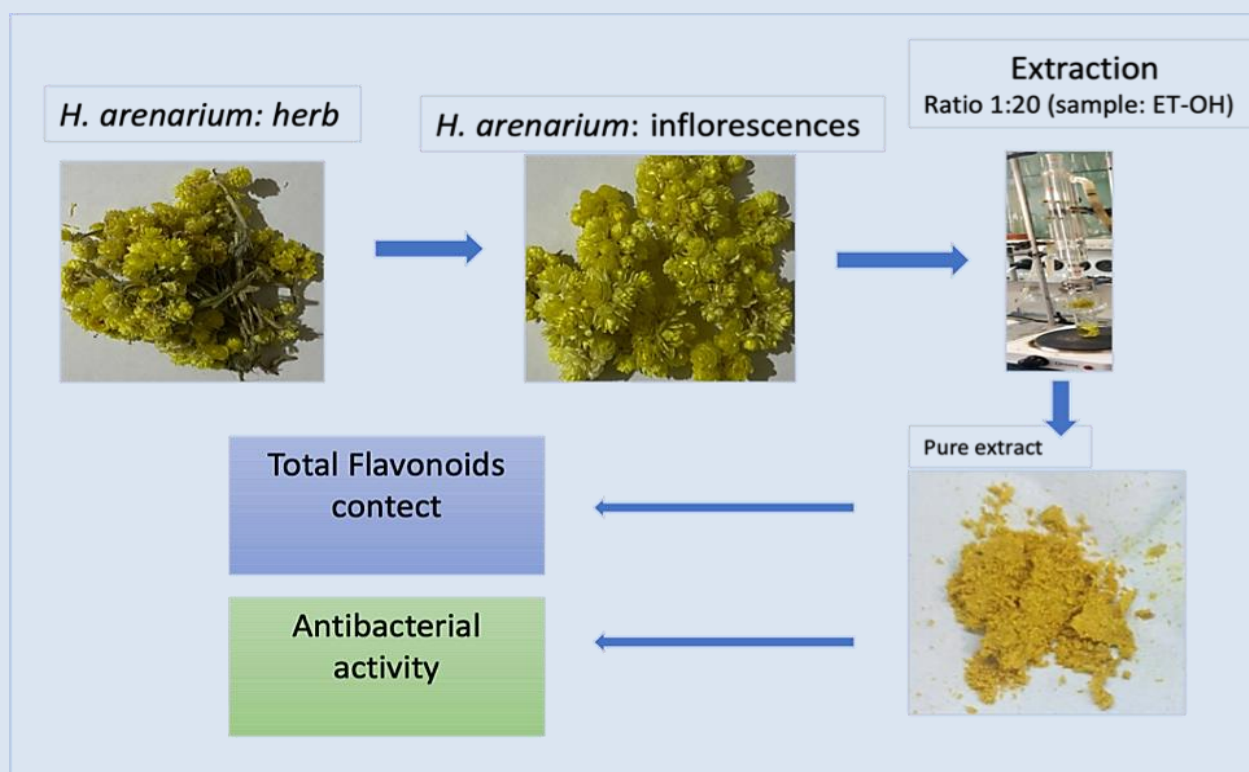
Objective: The objective of this study is to explore the influence of extraction process parameters on the yield of flavonoids from *H. arenarium* flowers and to determine their antibacterial activity.

Methods: Antibacterial activity was determined using the agar-well diffusion method against four bacterial species (*E. coli*, *Salmonella enterica*, *Staphylococcus aureus* and *S. typhimirium*). The assessment of the total flavonoid content in the ethanol extract of *Helichrysum* was conducted using the AlCl₃ method, following the procedure outlined by Andreeva and Kalinkina.

Results: The ethanolic and aqueous extracts demonstrated antibacterial activity against *Staphylococcus aureus* ATCC 6538 and *Bacillus Subtilis* 168. However, no significant activity was observed against *Escherichia coli* ATCC 8739 and *Salmonella Typhimurium* ATCC 14028. The research findings affirm that the extract derived from everlasting flowers exhibits antimicrobial properties, particularly targeting Gram-positive bacteria.

Conclusion: The results of our tests indicate that the extract of *Helichrysum* belonging to Armenian flora, is rich in flavonoids and has significant antimicrobial activity and may find application in the food industry, as well as in organic agriculture.

Key words: *Helichrysum*, plant extracts, flavonoids, antimicrobial activity, spectrophotometry



INTRODUCTION

As is well understood, food products serve as primary sources of various biologically active substances (BAS), including essential vitamins, minerals, and flavonoids, crucial for sustaining the normal biodiversity of the organism [1]. Flavonoids are ubiquitous and widely distributed throughout the plant kingdom. Flavonoids, found in enormous quantities in plants, are usually included in the human diet. However, the current use of any modern methods of processing and maintaining food products leads to a deficiency of biologically active substances in the human organism. Their deficiency in the organism can be filled with the help of biologically active food additives [2-3]. They serve as plant pigments, responsible for the colors of flowers and fruits [4]. It is confirmed that the composition of extracts from immortelle flowers is almost identical, however, in the budding phase, the amount is higher than during the flowering period, in contrast to macro- and microelements, where, for example, immortelle has a high content of calcium and potassium at any stage of development [5]. In this regard, the studied vegetation was collected in the phase of flower budding. Flowers of Helichrysum can be extracted with water and alcohol. It was found that the activity of aqueous extracts is equal to that of ethanol extract, which confirms the potential

of helichrysum. This study provides essential information for the selection of the best extract for further research into the biological activity of Helichrysum [6].

Flower heads of the plants have used as a daily herbal tea in Armenia. The effects attributed to Helichrysum species are a result of the flavonoids they contain. Plants belonging to the Helichrysum (Asteraceae) family are widely used in traditional medicine all over the world. Interest in Helichrysum, also known as immortelle, has been driven by its traditional therapeutic applications in inflammatory and allergy-related conditions, including asthma and skin inflammations [7]. Helichrysum has also been used for centuries as spices in a variety of foods and popular medicines, as well as for cosmetic purposes [8].

In addition, Helichrysum spp. has potential pharmaceutical uses due to their antioxidant, antioxidant, and anti-obesity properties [9]. Flavonoids are also secondary metabolites of plants with a wide range of pharmaceutical effects (antioxidants, gastrointestinal tract, antibiotics, etc.) [10-15]. Within the flavonoid family, flavones, which occur both as O- and C-glycosides, are a prominent class. The most common aglycones in this class are apigenin and luteolin (Figure 1).

Figure 1. Examples of representative flavones and flavonols.

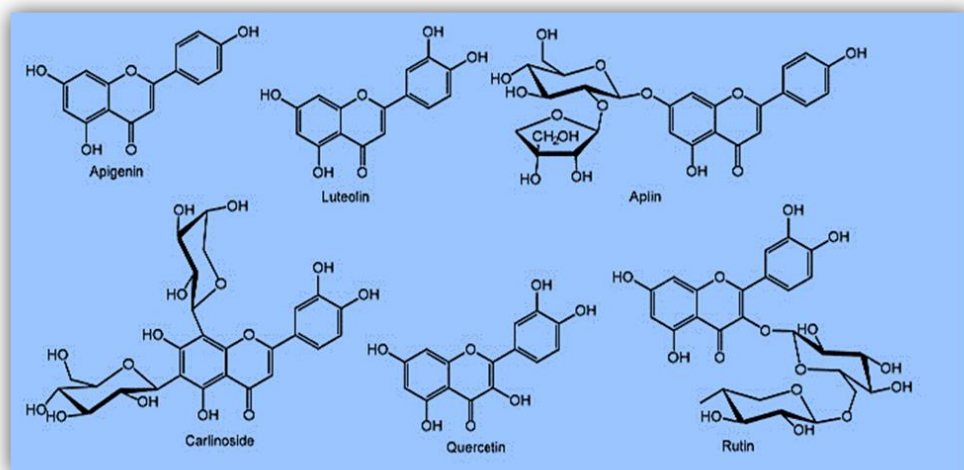


Table 1. The content of the sum of flavonoids

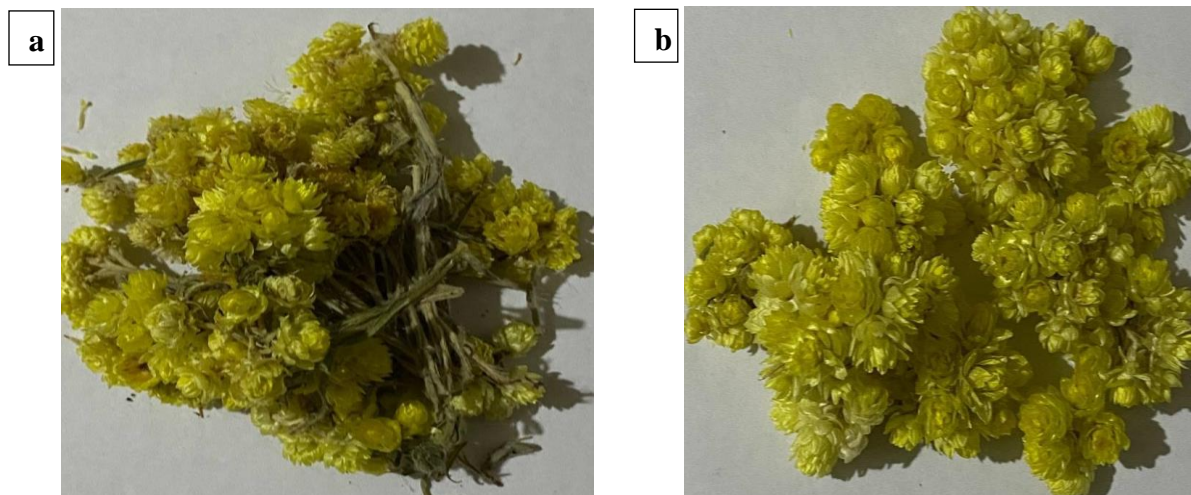
1. GF USSR, 11th edition Not less than 6% in terms of isosalipurposide
2. GF RF, 13th edition Not less than 6% in terms of isosalipurposide
3. GF of the Republic of Belarus Not less than 2.5% in terms of Rutin
4. German Pharmacopoeia Not less than 0.5% in terms of Quercetin

According to literary primary sources, the main active ingredients of raw materials and preparations of immortelle sand are flavonoids, among which the predominant is the chalconic glycoside – isosalipurposide (isohelichrysin) [4]. They serve as active markers in quantitative analysis of flavonoids. The total flavonoid content in raw materials and substances varies depending on the selected marker (Table 1). The normalization of the sum of flavonoids in everlasting flowers is performed using different markers [19]. Scientific databases including ISI Web of Knowledge, ResearchGate, SciFinder, PubMed and Scopus as well as

several traditional texts and books were searched to collect the data.

MATERIALS AND METHODS

Plant Materials: The object of the study was the flowers of the immortelle of 2022 collected in the budding phase. Helichrysum was collected in Jermuk–Vayots Dzor region, Armenia (at ~2500 m above sea level). The plants were identified in the Department of Botany and Mycology, Yerevan State University (YSU), Armenia and deposited in the Takhtadjan Herbarium of the Department of Botany and Mycology, YSU.

**Figure 2.** Raw materials from *H. arenarium*: herb (a), inflorescences (b).

Plant Extraction: The plant material underwent a comprehensive cleansing process with distilled water to eliminate any adhering soil or extraneous dust particles. Subsequently, the samples were air-dried at room temperature (18 ± 2 °C) in the shade. The mosses were

then carefully arranged in a flask and subjected to extraction using ethanol at various concentrations (96%, 80%, 70%, 50%) or water (utilizing 0.5 g of moss per 15 mL of solvent). The flask, securely attached to the reverse refrigerator, was subjected to moderate boiling

in a water bath for 60 minutes. Following extraction, the resulting solution was filtered through a paper filter and allowed to cool for 30 minutes.

Determination of Extract Yield: To determine the weight of pure extracted plant material (1 g), extraction was carried out using a Soxhlet-type extractor with 20 mL of methanol (MeOH) at 80 °C. After three extraction cycles, the extract was filtered and evaporated to dryness under vacuum at 40 °C using a rotary evaporator.

Microbial Strains: Antibacterial activity was testing in vitro against Gram-negative bacteria- *Escherichia coli* (ATCC 8739) and *Salmonella typhimarium* (ATCC 14028), as well as against the Gram-positive bacteria - *Staphylococcus aureus* (ATCC 6538) and *Bacillus subtilis*168. *Escherichia coli*, *Salmonella typhimarium* and *Staphylococcus aureus* have acquired within the frames of the research project N21T-4D091, *Bacillus subtilis* has provided by the Department of Epidemiology and Parasitology of the Armenian National Agrarian University. Maccon agar (*E. Coli*), Simmons agar (*Salmonella T.*), Manit agar (*Staphylacoccosa.*) and Meat-peptone agar (*B. subtilis*) used as a nutrient environment for microorganisms.

Determination of Antibacterial Activity: The antibacterial activity was assessed using the agar well diffusion method [16]. Plant extracts were tested for their antibacterial effects on microorganisms previously inoculated on agar plates (9 cm in diameter). Fifty microliters of the extract solutions were introduced into wells on the infected plates. Extracts were prepared at concentrations of 50,000, 25,000, 10,000, and 5000 µg/mL in absolute ethanol. Absolute ethanol without extract served as a control. The plates were chilled to facilitate sample diffusion for 1-1.5 hours and then

incubated at 37°C for 24 hours. Following incubation, the plates were examined for zones of growth inhibition, and the diameters of these zones were measured in millimeters. Dimethyl sulfoxide (DMSO) was employed as a positive control.

Determination of Total Flavonoids Content: The total flavonoid content in the ethanol extract of *Helichrysum* was assessed using the AlCl₃ method [17-18], following the procedure outlined by Andreeva and Kalinkina (14, 20). In a test tube, 500 µL of plant extract was combined with 100 µL of 10% AlCl₃, followed by the addition of 100 µL of 1 M CH₃COONa and 2800 µL of distilled H₂O. The tubes were then left at room temperature for 30 minutes to allow the reaction to proceed. Subsequently, the absorbance of the solutions was measured at a wavelength of 415 nm using a GENESYS 10S UV-VIS spectrophotometer (Thermo Fisher Scientific, USA). The total flavonoid content (TFC) was determined using a quercetin calibration curve [20-21], with quercetin as the reference standard. TFC was expressed as quercetin equivalents (QE) in mg/g of plant material's dry weight.

Statistical analysis: The results of the research were subject to statistical analysis. The normal distribution of the variation series was tested by the Kolmogorov-Smirnov test, The validity of differences between different series of experiments was evaluated by Student P-test: the value $P < 0.05$ was considered significant at 95% level confidence. Comparative analysis of the results was carried out using parametric analysis of ANOVA data. Bonferroni's accuracy has been applied to more than two groups. In cases where the data distribution deviated from normality, the Mann-Whitney test was employed for statistical analysis. The statistical calculations were made with SPSS Version 16.

RESULTS AND DISCUSSION

Antibacterial Activity of ethanolic extracts of

Helichrysum: To assess the antibacterial activity of Helichrysum, various concentrations of the flower extract were employed. Our research has shown that

extracts caused different inhibition zones among the tested microorganisms. The antimicrobial effects of the ethanolic extracts of *H. arenarium* are shown in Figure 3, at 5000, 10.000, 25.000 and 50.000 $\mu\text{g/mL}$ concentration.

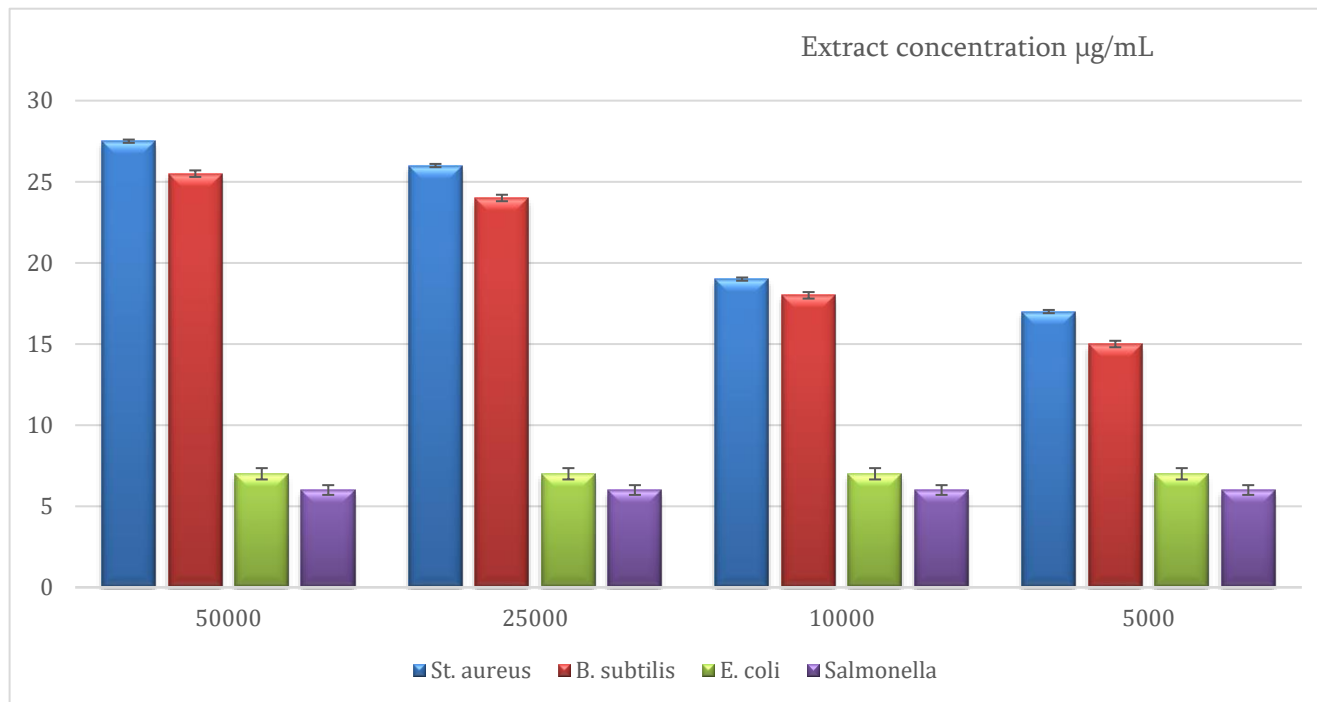


Figure 3: Antimicrobial activity of plant ethanolic extracts as mean of inhibition diameter zone (mm). Values are as mean values \pm standard deviation ($n=3$). No significant inhibition zones were observed with DMSO, used as a positive control. The ethanolic extracts at the lowest concentration exhibited the least effectiveness.

The findings from the assessment of antimicrobial activity, as depicted in Figure 3, revealed that methanol extracts of Helichrysum exhibited notable antimicrobial efficacy against *Staphylococcus aureus* (inhibition zone: 17-27.5 mm) and *Bacillus Subtilis* (inhibition zone: 15-25.5 mm). The assessment of antibacterial properties was conducted by magnitude zones of lack of growth of microorganisms. A zone diameter of less than 10 mm indicated that microorganisms are not sensitive to the test sample; up to 10 mm - for low antimicrobial activity; 10–20 mm – for medium antimicrobial activity; more than 20 mm - on high sensitivity. It should also be noted that among microorganisms *E. coli* and

Salmonella were more sensitive to Helichrysum, but this activity also depended on solvents (Water extracts no activity was found against *E. coli*, *Salmonella*). Therefore, it can be concluded that the tested Helichrysum extracts only exhibited an inhibitory effect against *B. subtilis* and *S. aureus*.

Total Flavonoids Content: In their pure state, flavonoids are crystalline compounds with specific melting points, displaying colors ranging from light yellow, yellow, or yellowish green (flavones, flavonols) to orange or orange red (aurons) and even red or blue (anthocyanins). Colorless flavonoids, isoflavones, are also quite common: catechins, flavanones, flavanols.

Spectrophotometric determination, utilizing self-maxima absorption in either direct or differential spectrophotometry, stands as one of the prevalent methods for analyzing flavonoid compounds. The spectrophotometric analysis method relies on the selective absorption of monochromatic light by the substances being studied. This absorption occurs due to electron transitions from the donor orbit to the vacant orbit of the benzene ring or acceptor Deputy [22]. Flavonoids exhibit distinct characteristics in the UV spectrum, featuring two prominent absorption bands.

The first band (Band I) is observed in the long-wave region, typically ranging from 320 to 380 nm, while the second band (Band II) is found in the short-wavelength region, spanning 240 to 270 nm. In the case of flavonols, these compounds display absorption bands at 350-390 nm (Band I) and 250-270 nm (Band II), with an additional maximum observed at 300 nm. Spectral analyses of helichrysum flower ethanolic extracts with different concentration indicate the presence of flavonoids (Figure 4).

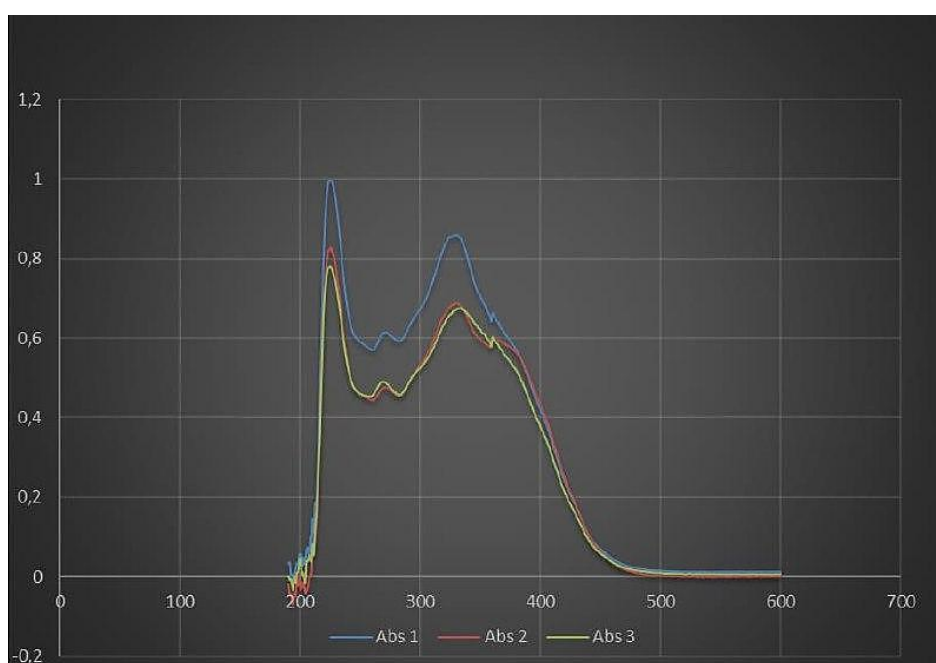


Figure 4: Flavonoids in the UV Spectrum

It is well-established that plants synthesize flavonoids in response to bacterial infections. This indicates their potential effectiveness as antibacterial substances against a wide range of microorganisms [23]. Flavonoids have the capability to inhibit or kill numerous bacterial strains, hinder crucial viral enzymes like reverse transcriptase and protease, and eliminate certain pathogenic protozoans [24]. However, their toxicity to animal cells is low, indicating that the antibacterial activity of Helichrysum extracts is likely attributed to the

presence of flavonoids. It is known that the antibacterial efficacy of flavonoids is structure dependent. The plant extracts displaying antibacterial properties have shown a correlation with elevated flavonoid levels, indicating their role as antibacterial agents [25]. Flavonoids, among the diverse phytochemical compounds found in nature, hold a significant place in traditional medicine and pharmacology. Research has highlighted the effectiveness of plant-derived products as reliable sources of antioxidants, combating diseases caused by

reactive oxygen species [26]. Research has demonstrated that phenolic compounds, including flavonoids and phenolic acids found in plants, contribute to their antioxidant properties [18, 26]. These studies have also established a connection between the total flavonoid and phenol contents and the antioxidant activity of plants [18-19]. Our studies also show that

Helichrysum flowers contain flavonoids. Studies have shown that Helichrysum flowers are notable for their high flavonoid content. In this work, we have also studied the effects of parameters on the process of extracting flavonoids from Helichrysum flowers (Table 2).

Table 2. Total flavonoid content in ethanolic and water extracts of *H. arenarium*

N	V (ml)	Extractant		Temperature (°C)	Dry weight (g)	Net weight (%)	Total Flavonoids (%)
		ET-OH	H ₂ O				
1	4.5	95	5	80±3	0.1002	13.07	0.45±0.02
2	4.5	80	20	80 ±3	0.1002	14.12	1.46±0.09*
3	4.5	70	30	80 ±3	0.1004	18.23	1.51±0.08*
4	4.5	50	50	80 ±3	0.1005	15.70	0.71±0.13
5	4.5	0	100	95±5	0.1004	10.76	1.9±0.44
6	4.5	0	100	20±3	0.1003	9.07	0.40±0.01

*P<0.05

Water extraction of raw materials has been implemented at different temperatures. Shown that with the growth of temperature increases the yield of flavonoids (20°C-0.40%, 95°C-1.9%). In addition to alcohol extraction, it is advisable to extract flavonoids from the flowers of the *H. arenarium* with 70% ethyl alcohol (1.51%).

Bioactive compounds with antioxidant capacity such as flavonoids, are the major bioactive compound found in guava leaves (*Psidium guajava*) [26], maize

(staple food in Malawi) [27], in the stem bark of *Tetrapleura tetraptera* [28] and in many other plants. Plant derived bioactive ingredients have good potential for the management of disease.

Flavonoid-rich meals have an anti-inflammatory effect in obese people who are likely to have chronic inflammation [29]. However, humans usually consume flavonoids from meals comprising a variety of basic foodstuffs and which are cooked in various ways, such as boiling, grilling, frying, and steaming. These meals

also comprise ingredients such as fats and dietary fibers, which may influence the internal change of flavonoids. Therefore, it is thought that the absorption Rate differs among the intake of flavonoids from a typical meal, supplements, or single type of food. Thus, in cases where internal changes in flavonoids are being assessed using actual eating habits, other nutrients also need to be taken into consideration [30].

CONCLUSION

The article evaluates the antimicrobial activity of everlasting flower extracts against both Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative (*E. coli*, *Salmonella*) bacteria. The findings confirm that the everlasting flower extract exhibits antimicrobial properties specifically targeting Gram-positive bacteria. Based on the results, the 70% ethanol extract of *H. arenarium* represents a significant source of flavonoids. Common *H. arenarium* species studied in Armenia exhibit antibacterial properties, suggesting their potential application as versatile herbal antibacterial agents. Particularly, extracts from *H. arenarium* can be found in utility in medicine, pharmaceuticals, and the food industry. They can serve various purposes, including acting as natural pigments and serving as alternative preventive agents against foodborne diseases. This application not only preserves food products but also avoids the use of chemical antibacterial agents.

List of abbreviations: BAS: biologically active substances; DMSO: Dimethyl sulfoxide; *H. arenarium*: *Helichrysum arenarium*; Et-OH: ethanol; RA: Republic of Armenia; USA: United State of America; USSR: Union of Soviet Socialist Republics; UV: Ultraviolet; YSU: Yerevan State University.

Authors Contributions: All authors contributed to this study and wrote this paper.

Competing interest: The authors declared that there is no competing interest.

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