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Phenolic Profile and Antioxidant Capacity of *Helichrysum arenarium* Extracts: A Comprehensive LC-MS/MS and Antioxidant Analysis

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ABSTRACT

Helichrysum arenarium, commonly known as the immortal or everlasting flower, is a member of the Asteraceae family celebrated for its potential medicinal properties. This study aims to elucidate the phenolic profile and antioxidant properties of *H. arenarium* using advanced analytical techniques. Methanol extract of *H. arenarium* was analyzed using Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) to identify and quantify various phenolic compounds. The phenolic profile revealed high concentrations of luteolin (744.57 mg 100^{-1} g^{-1}), quercetin (113.13 mg 100^{-1} g^{-1}), and naringenin (229.60 mg 100^{-1} g^{-1}), while other compounds were below the limit of quantification. The antioxidant capacity was evaluated using DPPH, ABTS, CUPRAC, and FRAP assays, showing moderate activity compared to standard antioxidants such as BHA, BHT, and Trolox. The methanol extract exhibited DPPH and ABTS radical scavenging activities of 19.14% and 26.91%, respectively, with FRAP and CUPRAC absorbance values of 0.441 and 0.653. These findings highlight the potential of H. arenarium as a source of natural antioxidants and pave the way for future research to optimize its therapeutic applications, especially in combating oxidative stress-related conditions.

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Helichrysum arenarium Ekstraktlarının Fenolik Profili ve Antioksidan Kapasitesi: Kapsamlı LC-MS/MS ve Antioksidan Analizi

ÖZET

Helichrysum arenarium, halk arasında ölümsüz veya sonsuz çiçek olarak bilinen, Asteraceae familyasına ait bir bitkidir ve potansiyel tıbbi özellikleriyle tanınmaktadır. Bu çalışmanın amacı, *H. arenarium*'un fenolik profilini ve antioksidan özelliklerini ileri düzey analitik tekniklerle açıklığa kavuşturmaktır. *H. arenarium*'un metanol ekstraktı, çeşitli fenolik bileşenleri tanımlamak ve miktarlarını belirlemek üzere Sıvı Kromatografi-Tandem Kütle Spektrometrisi (LC-MS/MS) kullanılarak analiz edilmiştir. Fenolik profil, luteolin (744.57 mg 100^{-1} g^{-1}), kuersetin (113.13 mg 100^{-1} g^{-1}) ve naringenin (229.60 mg 100^{-1} g^{-1}) gibi yüksek konsantrasyonlarda bileşenler içerirken, diğer bileşenler tespit sınırının altındadır. Antioksidan kapasite, DPPH, ABTS, CUPRAC ve FRAP testleri kullanılarak değerlendirilmiş ve standart antioksidanlar olan BHA, BHT ve Trolox ile karşılaştırıldığında orta derecede aktivite gözlenmiştir. Metanol ekstraktı, DPPH ve ABTS radikal temizleme aktiviteleri sırasıyla %19.14 ve %26.91, FRAP ve CUPRAC absorbans değerleri ise sırasıyla 0.441 ve 0.653 olarak belirlenmiştir. Bu bulgular, H. arenarium'un doğal antioksidan kaynağı olarak potansiyelini vurgulamakta ve özellikle oksidatif stresle ilgili koşullarla mücadelede terapötik uygulamalarını optimize etmek için gelecekteki araştırmaların yolunu açmaktadır.

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INTRODUCTION

Helichrysum arenarium, commonly known as the immortal or everlasting flower, is a species of flowering plant in the Asteraceae family, renowned for its diverse pharmacological properties and traditional medicinal uses. It has been traditionally utilized in various cultures for its medicinal properties, including its purported anti-inflammatory, antimicrobial, and antioxidant activities (Eroğlu et al., 2010; Umaz et al., 2023). Recent research has increasingly focused on the biochemical constituents of this plant, particularly its phenolic compounds, which are known for their significant antioxidant properties and potential health benefits. The presence and concentration of these phenolic compounds are critical in understanding the therapeutic efficacy of *H. arenarium*.

Phenolic compounds, a diverse group of secondary metabolites, play a crucial role in protecting plants against oxidative stress. They are also recognized for their ability to scavenge free radicals, thereby mitigating oxidative damage to cells and tissues. In the context of human health, phenolics have been linked to a reduced risk of chronic diseases associated with oxidative stress, such as cardiovascular diseases and cancer (Miller & Rice-Evans, 1997; Prior & Cao, 2000; Boudet, 2007; Halliwell & Gutteridge, 2007; Necip et al., 2021; Kaygısız et al., 2024; Uğur et al., 2024; Zengin et al., 2024). Understanding the profile of phenolic compounds in *H. arenarium* and their antioxidant efficacy is essential for harnessing its full therapeutic potential. In this context, Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) has emerged as a powerful analytical technique for the precise identification and quantification of phenolic compounds in plant extracts. LC-MS/MS provides high sensitivity and specificity, allowing for the detailed characterization of complex mixtures (Gao & Hu, 2015). This method is particularly valuable for profiling the diverse array of phenolic compounds present in *H. arenarium* and determining their relative concentrations.

To evaluate the antioxidant capacity of *H. arenarium*, several assays were employed, including ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), and CUPRAC (Cupric Reducing Antioxidant Capacity), tests. These assays are widely used to assess different mechanisms of antioxidant activity, providing a comprehensive overview of the plant's potential to neutralize oxidative species.

This study aims to elucidate the phenolic profile of *H. arenarium* and its antioxidant properties. This information will not only enhance understanding of the plant's medicinal value but also contribute to the development of natural antioxidants for therapeutic applications.

METHODS and MATERIALS

Chemicals

The compounds listed below were employed as standards in the LC-MS/MS analysis: acetohydroxamic acid (98%), vanillic acid (≥97%), catechin hydrate (≥ 99%), resveratrol (99%), thymoquinone (≥97%), caffeic acid (98%), gallic acid (98%), salicylic acid (99%), p-hydroxybenzoic acid (99%), phloridzin dihydrate (≥ 99%), oleuropein (≥ 80%), myricetin (≥96%), 2-hydroxy-1,4-naphthoquinone (97%), kaempferol, quercetin (98%) (≥97%), and alizarin (97%) from Sigma-Aldrich (Darmstadt, Germany); protocatechuic acid (97%), naringenin (≥95%), butein (≥98%), luteolin (≥98%), and silymarin (≥95%) from Merck (Darmstadt, Germany), ellagic acid (95%) and syringic acid (97%) from Fluka (Buchs, Switzerland); curcumin (≥99.5%) from Supelco (USA). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), trolox, trichloroacetic acid (TCA), and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were obtained from Sigma-Aldrich (Germany), and potassium persulfate ($K_2S_2O_8$), $CuCl_2$, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and potassium ferricyanide [$K_3Fe(CN)_6$] were obtained from Merck (Darmstadt, Germany).

Plant Samples

Helichrysum arenarium L. Moench plant was collected in May at Çelikhan town, Adiyaman, Turkey. The herb was washed with pure water and then dried in the shade at room temperature.

The Plant Sample Extraction

A 5 g portion of the powdered sample was subjected to extraction using 50 mL methanol as the extraction solvent, employing the maceration technique at ambient temperature for 24 hours. Following the extraction, the solution was filtered and evaporated to yield a dry extract. This extract was then reconstituted to a 1 mg mL^{-1} concentration and utilized for assays evaluating antioxidant capacity as well as for LC-MS/MS analysis.

LC-MS/MS Analyses

The analysis of 24 phytochemicals was conducted using a Shimadzu Nexera HPLC system coupled with a dual mass spectrometer (Kyoto, Japan). The liquid chromatograph was outfitted with LC-30AD binary pumps, a DGU-20A3R degasser, a SIL-30AC autosampler, and a CTO-10AS column oven. Separation of the analytes was achieved on an Inertsil ODS4 C18 reversed-phase analytical column (150 mm × 4.6 mm, 3 µm particle size). Gradient elution was performed at a flow rate of 0.5 mL min^{-1} and a column temperature of 40°C, with an injection volume of 4.0 µL. The mobile phase consisted of solvent A (water containing 5.0 mM ammonium formate and 0.1% formic acid) and solvent B (methanol containing 5.0 mM ammonium formate and 0.1% formic acid). The gradient elution program was as follows: 40-90% B from 0 to 20 minutes, 90-99% B from 20 to 23 minutes, 99-40% B from 23 to 24 minutes, and 4% B from 24 to 29 minutes. Mass spectrometric detection was carried out using a Shimadzu LCMS 8040 triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source, operating in positive and negative ionization modes. Data acquisition and analysis were performed with Shimadzu LabSolutions software (Kyoto, Japan). Quantification of the analytes was achieved using multiple reaction monitoring (MRM) mode. Phenolic compounds were analyzed with two or three transitions per compound: the primary transition was used for quantification, while the additional transitions provided confirmation of the results.

Antioxidant Capacity

To determine the antioxidant capacity, the following tests were applied: DPPH free radical, ABTS cation radical scavenging activity, cupric reducing (CUPRAC), and ferric reducing (FRAP) methods (Miller, 1971; Elmastaş et al., 2006; Apak et al., 2004). UV/VIS Spectrophotometer (Shimadzu 2000S Model, Japan) was used for the detection of antioxidant capacity.

Statistical Analysis

The results were expressed as arithmetic mean ± standard error of the mean (sem); n = 3. Subsequently, the Tukey test was conducted to compare the antioxidant activity (DPPH, ABTS, FRAP, and CUPRAC) between the methanol extract, BHA, BHT, and Trolox. The results indicated significant differences across the treatments for all assays ($p < 0.001$).

Multiple linear regression analyses (Shimadzu LabSolutions software, Kyoto, Japan) were conducted to detect the concentrations of phenolic compounds identified in the LC-MS/MS data.

RESULTS and DISCUSSION

The LC-MS/MS system, noted for its high selectivity and sensitivity, was utilized to analyze phytochemicals present in *Helichrysum arenarium*. Key analytical parameters such as limits of detection (LOD), limits of quantification (LOQ), linear ranges, and coefficient of determination (R^2) for the studied analytes were determined, as summarized in Table 1.

Linear regression analyses were conducted to detect the concentrations of phenolic compounds identified in the LC-MS/MS data. Notably, all compounds exhibited a strong relationship ($R^2 > 0.99$), underscoring the high explanatory power of the regression models and the accuracy of the employed methodology. For instance, the linear regression equation derived for luteolin, y = 1389x - 40923, yielded an R^2 value of 0.9988. This correlation between luteolin concentration and peak area highlights the robustness of the analytical approach and the reliability of the quantification results.

For the phytochemical determination, a methanol extract of *H. arenarium* was utilized with the LC-MS/MS technique. Acetohydroxamic acid, catechin hydrate, syringic acid, fumaric acid, caffeic acid, phloridzin dihydrate, myricetin, quercetin, butein, naringenin, luteolin, and kaempferol from phenolic compounds were quantified in the methanol extract of *H. arenarium*. The concentration of luteolin was notably high at 744.57±4.21 mg 100^{-1} g^{-1}, while quercetin and naringenin concentrations were 113.13±1.22 mg 100^{-1} g^{-1} and 229.60±3.15 mg 100^{-1} g^{-1}, respectively. p-hydroxybenzoic acid, thymoquinone, curcumin, protocatechuic acid, salicylic acid, resveratrol, 2-hydroxy-1,4-naphthoquinone, ellagic acid, silymarin, and alizarin were under LOQ (Table 2).

The antioxidant capacity of the methanol extract of *H. arenarium* plant was assayed with the following tests: DPPH free radical scavenging, ABTS cation radical scavenging, cupric reducing (CUPRAC), and ferric reducing (FRAP). Results of antioxidant capacity are given in Table 3. DPPH and ABTS results were expressed as percentage radical scavenging activity, and CUPRAC and FRAP results were expressed as absorbance.

Table 1 Analytical parameters for LC-MS/MS analysis (Uğur & Güzel, 2023)
Çizelge 1. LC-MS/MS analizi için analitik parametreler

Compounds	Retention time (min)	Precursor ion (m z^{-1})	Product ion (m z^{-1})	Linear regression	LOD (µg L^{-1})	LOQ (µg L^{-1})	R^2	Linear range (µg L^{-1})
Acetohydroxamic acid	0.406	76.15	58	y = 216.91x + 6165.8	6.90	23.01	0.9989	20-750
Syringic acid	3.001	199.1	140.1	y = 112.03x + 1316.1	2.88	9.61	0.9994	10-500
Vanillic acid	2.762	168.95	65	y = 48.343x + 662.5	84.78	282.61	0.9993	250-1000
Resveratrol	3.606	229	135	y = 733.34x - 69955	41.83	139.43	0.999	250-1000
Thymoquinone	3.337	165	137	y = 349.23x - 2887.4	7.64	25.47	0.9971	20-500
Caffeic acid	2.836	179	135	y = 1227.2x - 5396.5	2.87	9.58	0.9948	10-100
Gallic acid	1.278	169.1	124.9	y = 305.07x - 1859.3	3.92	13.06	0.9981	10-100
Protocatechuic acid	3.556	181	108	y = 1382.2x - 4393.1	2.76	9.20	0.9967	10-500
p-hydroxybenzoic acid	3.555	137.2	93.1	y = 3831.2x - 94423	8.92	29.74	0.9996	40-500
Oleuropein	3.567	539.1	377	y = 324.26x - 5388.8	7.17	23.90	0.9997	40-750
Salicylic acid	3.558	137.2	93	y = 3838.2x - 149277	22.88	76.25	0.9977	75-1000
2-Hydroxy-1.4-naphthoquinone	3.664	173.1	145	y = 461.45x - 4553.8	2.07	6.91	0.9989	10-500
Phloridzin dihydrate	3.594	435.1	273.1	y = 120.23x - 9479.5	81.80	272.67	0.9989	250-1000
Ellagic acid	3.681	301.1	228.9	y = 18.841x + 911.46	23.74	79.14	0.9967	100-1000
Myricetin	3.644	317	179.1	y = 588.4x - 4990.6	4.34	14.45	0.9987	20-500
Butein	3.935	271	134.9	y = 62.943x - 2793	38.50	128.20	0.996	100-1000
Quercetin	3.891	301.1	150.9	y = 150.09x - 422.87	7.79	25.98	0.9997	20-500
Silymarin	3.996	481.1	453.1	y = 199.91x + 950.97	8.00	26.70	0.9997	40-750
Naringenin	3.952	271	150.9	y = 700.8x - 26469	68.40	228.10	0.9997	250-1000
Kaempferol	4.298	285	117	y = 62.513x - 821.08	3.90	13.00	0.9982	20-1000
Luteolin	4.069	285	150.9	y = 1389x - 40923	6.40	21.40	0.9988	40-1000
Catechin hydrate	2.532	291	139.1	y = 1717.9x - 563.99	2.05	6.84	0.9988	10-750
Alizarin	4.594	239	211	y = 26.512x - 1721	15.30	51.10	0.9991	60-2000
Curcumin	4.672	367.1	216.9	y = 1908.9x - 8252.1	12.80	42.70	0.9994	40-1000

The methanol extract of *H. arenarium* exhibited moderate antioxidant activity in comparison to synthetic antioxidants (BHA and Trolox). The DPPH radical scavenging activity of the extract was 19.14±2.13%, significantly lower than that of BHA (47.03±6.39%) and Trolox (57.81±4.09%). The ABTS assay revealed similar patterns, with the extract showing 26.91±2.60%, compared to BHA (93.65±4.71%) and Trolox (90.03±3.07%). Statistical analysis using the Tukey test indicated significant differences in antioxidant activity between the extract and the standards, $p < 0.001$. The test confirmed that the activity of the extract was significantly lower than both BHA and Trolox ($p < 0.001$, Table 3).

Table 2 Results of qualitative and quantitative determination of phytochemicals in *H. arenarium* extract by LC-MS/MS

Çizelge 2. H. arenarium ekstrakındaki fitokimyasalların LC-MS/MS ile kalitatif ve kantitatif tayin sonuçları

Compounds	Means ± sem (mg 100^{-1} g^{-1})	Compounds	Means ± sem (mg 100^{-1} g^{-1})
Acetohydroxamic acid	23.23±0.17	Kaempferol	202.63±7.90
Catechin hydrate	3.09±0.10	2-Hydroxy-1,4-naphthoquinone	< LOQ
Syringic acid	5.22±0.13	Curcumin	< LOQ
Fumaric acid	50.28±5.72	Ellagic acid	< LOQ
Caffeic acid	136.24±0.42	Thymoquinone	< LOQ
Phloridzin dihydrate	190.87±6.93	Protocatechuic acid	< LOQ
Myricetin	10.87±0.83	Salicylic acid	< LOQ
Quercetin	113.13±1.22	Silymarin	< LOQ
Butein	10.83±0.21	Resveratrol	< LOQ
Naringenin	229.60±3.15	*p*-hydroxybenzoic acid	< LOQ
Luteolin	744.57±4.21	Alizarin	< LOQ

Data represent arithmetic mean ± standard error of the mean (sem) of three independent samples.

Table 3 Antioxidant capacity of 0.2 mg/mL concentration of *H. arenarium* extracts, BHA, BHT, and trolox

Çizelge 3. 0.2 mg/mL H. Arenarium ekstraktı, BHA, BHT ve Trolox'a ait antioksidan kapasite sonuçları

	DPPH	ABTS	FRAP	CUPRAC
	(%)		(Absorbance)	
Methanol extract	19.14±2.13^{b}	26.91±2.60^{c}	0.441±0.020^{c}	0.653±0.011^{d}
BHA	47.03±6.39^{a}	93.65±4.71^{a}	0.669±0.034^{b}	1.995±0.010^{b}
BHT	21.71±4.21^{b}	58.21±2.66^{b}	1.232±0.034^{a}	2.278±0.021^{a}
TROLOX	57.81±4.09^{a}	90.03±3.07^{a}	1.265±0.028^{a}	1.812±0.032^{c}
p	*0.001*	*0.001*	*0.001*	*0.001*

Data represent average values ± standard deviation of three independent samples. Different letters $^{[a-d]}$ indicate significant differences according to a Tukey test. ($P < 0.001$).

The growing interest in natural products as therapeutic agents is evident in the increasing use of these substances to address various diseases. As a result, research into the effectiveness of plant-derived compounds has gained significant traction, highlighting their potential as novel treatment options. Among these compounds, phenolic substances are particularly noteworthy. These secondary metabolites, produced by plants, play crucial roles in their defense mechanisms, protecting them from herbivores, pests, pathogens, and a range of environmental stressors. Phenolic compounds are known for their diverse biological and pharmacological activities. They exhibit potent antioxidant properties, which help neutralize harmful free radicals in the body (Çuhacı et al., 2021). Additionally, they possess anti-inflammatory effects that can mitigate chronic inflammation, a key factor in many diseases. Their anti-tumor and anti-cancer properties are also of considerable interest, as they could potentially play a role in cancer prevention and treatment. Moreover, phenolic compounds have demonstrated antiviral and anti-allergic activities, further underscoring their potential therapeutic benefits (Sarker & Oba, 2020; Gülçin et al., 2002). Recent studies have highlighted the effectiveness of these phenolic compounds, particularly in the prevention and management of chronic conditions. They are increasingly recognized for their potential to address neurodegenerative diseases, such as Alzheimer's and Parkinson's, as well as metabolic disorders like diabetes (La Fata et al., 2014). Furthermore, their role in combating cardiovascular diseases and cancer underscores their importance in modern therapeutic strategies. As research continues, the full range of benefits offered by these plant-derived compounds is likely to become even more apparent.

In the present study, different amounts of acetohydroxamic acid, catechin hydrate, syringic acid, fumaric acid, caffeic acid, phloridzin dihydrate, myricetin, quercetin, butein, naringenin, luteolin, and kaempferol in the methanol extract of *H. arenarium* were detected. In terms of phytochemical content, the high concentration of

luteolin aligns with previous studies (Sroka et al., 2004; Babota et al., 2018), which identified luteolin as a major phenolic compound in *H. arenarium*. And also, the results of luteolin, kaempferol, naringenin, caffeic acid, syringic acid, protocatechuic acid, and quercetin were comparable to those previously reported (Gradinaru et al., 2014; Grinev et al., 2016; Babota et al., 2018; Pljevljakušić et al., 2018). In one study conducted in Poland, Sroka et al. (2004) quantified caffeic acid, syringic acid, p-hydroxybenzoic acid, kaempferol, quercetin, and protocatechuic acid by HPLC in different solvent extracts of *H. arenarium*. On the contrary, p-hydroxybenzoic acid and protocatechuic acid were under LOQ in the present study. Babota et al. (2018), low amounts of luteolin and kaempferol in *H. arenarium* were found between 5.76-9.98 and 7.16-181.23 mg 100^{-1} g^{-1} using different extraction solvents, respectively. On the other hand, Jarzycka et al. (Jarzycka et al., 2013) found high amounts of naringenin (1740 mg 100^{-1} g^{-1}) by HPLC in *H. arenarium*. The differences between the phenolic compounds in *H. arenarium* plant depend on the soil, the processes after harvesting, the extraction process, and the analysis methods.

As shown in Table 3, the antioxidant capacity of *H. arenarium* extracts was compared with those of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and trolox. *H. arenarium* extract showed lower activity than BHA, BHA, and trolox in all test results, too. The results of this study confirm the moderate antioxidant activity of *Helichrysum arenarium* methanol extracts, which were less potent compared to synthetic antioxidants such as BHA and Trolox. The significant difference in activity, as indicated by the Tukey test results ($p < 0.001$), suggests that while the extract has potential as a natural antioxidant, its efficacy is not as high as that of commercial alternatives. In previous studies, Babota et al. (2018) reported that the antioxidant capacity of *H. Arenarium* extract was 4.04 mg mL^{-1} (ABTS) and 4.91 mg mL^{-1} (DPPH). Umaz et al. (2023), the antioxidant activity results in two different populations of the *H. Arenarium*, found as 114.8-118.8 mg 100^{-1} g^{-1} and 0.642-0.766 absorbance according to DPPH and CUPRAC tests, respectively. The results obtained by Babota et al. (2018) using the DPPH and ABTS assays are significantly lower compared to the findings of the present study (Tablo 3). In addition, the DPPH assay results reported by Umaz et al. (2023) are also lower than those observed in the present study. On the other hand, the results from the CUPRAC assay conducted by Umaz et al. align closely with the results of the present study (Tablo 3). Antioxidant activity is primarily associated with phenolic compounds, which exhibit their effects through various mechanisms such as scavenging free radicals, providing reducing power, and chelating metal ions (Gülhan & Yangılar, 2022). The effectiveness of these compounds in combating oxidative stress is largely due to their phenolic hydroxyl groups. These hydroxyl groups are crucial, as they significantly enhance the compounds' ability to neutralize free radicals, thus contributing to their overall antioxidant potential (Işık, 2020; Güzel & Elmastaş, 2020).

The major phenolic compounds reinforce the potential of *H. arenarium* as a source of natural antioxidants. However, the moderate antioxidant activity observed may be due to factors such as extraction methods or environmental influences on the phytochemical content. Future research should focus on optimizing extraction methods to enhance the bioavailability of these phenolic compounds and further explore the therapeutic potential of *H. arenarium* extracts.

CONCLUSION

The analysis of *Helichrysum arenarium* using LC-MS/MS successfully identified and quantified several key phenolic compounds, including luteolin, quercetin, and naringenin, which are known for their antioxidant properties. Despite the moderate antioxidant activity of the methanol extract, as compared to synthetic antioxidants such as BHA, BHT, and Trolox, the presence of high levels of phenolic compounds suggests that *H. arenarium* has significant potential for further research and application as a natural antioxidant. The variations in antioxidant activity observed in different studies highlight the impact of extraction methods and plant sources on the efficacy of *H. arenarium*. Future studies should prioritize refining extraction processes and further elucidating the bioactive mechanisms of its phenolic components. Such efforts are critical to fully unlocking the therapeutic potential of *H. arenarium*, particularly in combating oxidative stress-related disorders and supporting the development of novel, natural antioxidant therapies.

Conflict of Interest

The authors declare they have no conflict of interest.

Contribution of the Authors as Summary

The authors declare the contribution of the authors is equal.

Ethics Statement

This study doesn't require an ethics committee decision.

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