



Solvent-Dependent Antioxidant Profiles of *Helichrysum arenarium* (L.) Moench Extracts: A Comparative In Vitro Assessment

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Abstract

Aim: *Helichrysum arenarium* (L.) Moench is perennial herbaceous plant belongs to the Asteraceae family and is widely distributed in Europe and Asia. It is used in medicine due to its phytochemicals.

Material and Methods: In the current study, extracts were obtained using ethanol, water and chloroform solvents, and the antioxidant potentials of the obtained extracts were determined by various in vitro tests.

Results: In the DPPH radical scavenging activity test the ethanol extract showed higher inhibition than the other extract samples. The Ec50 values for ethanol, chloroform, and water was determined as 16.60 µg/mL, 85.67 µg/mL and 175.86 µg/mL respectively. This is thought to be related to the ratio of phenolic compounds released during extraction. Also the obtained extract samples has moderate Nitric oxide, superoxide and hydrogen peroxide scavenging activity.

Conclusion: These findings suggest that *H. arenarium* may serve as a potential natural source of antioxidants.

Keywords: Helichrysum arenarium, antioxidant, extract, medicine

INTRODUCTION

The name Helichrysum derives from the Greek words “helios” (sun) and “chrysos” (gold), referring to its flowers. There are over 600 species of Helichrysum worldwide found in Africa, Madagascar, Australasia and Eurasia. Belonging to the Asteraceae (Compositae) family, Helichrysum species are considered traditional medicinal plants. Studies indicate that decoctions of Helichrysum species are used in various Turkish and South African traditional medicines. Traditional preparations often have stomachic, diuretic, and anti-asthmatic properties as well as activity against kidney stones. The capitulum are

ground into a powder and mixed with barley flour to make a wound healing ointment [1]. *Helichrysum arenarium* (L.) Moench a perennial herbaceous plant belonging to the Asteraceae family and native to Europe, Central Asia, and China [2]. The main components of extracts obtained from *H. arenarium* flowers are polyphenols and flavonoids, generally present as glycosides [3, 4]. Studies have reported that it exhibits potent antioxidant, anti-inflammatory, and antimicrobial activity due to its chemical content [5, 6]. Studies have reported that it has bile-extracting, liver protective and tumor-necrosis inhibitory effects due to the phenolic compounds it contains [2, 7]. Due

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to its appearance and healing properties, the species is protected and cultivated in some European and Asian countries such as Sweden, Poland, Kazakhstan and Serbia [8].

Free radicals are known reactive oxygen and reactive nitrogen species. When we look at the chemical structure of free radical has unpaired electron. Free radicals can participate reaction occur in the cell metabolism. Therefore free radicals play a dual role as beneficial or deleterious [9]. Especially the high amount of free radicals can cause oxidative stress. Oxidative stress causes various pathogenesis diseases, DNA damage etc.. Therefore it is important that the prevention of the deleterious effect on the metabolism. In the current study we investigated the antioxidant potential of *H. arenarium* extract samples.

The novelty of the present study derives from its solvent-dependent comparative design, which has not been systematically applied to *Helichrysum arenarium* extracts in previous reports. Earlier studies generally examined single-solvent extracts (typically ethanol or methanol), limiting the ability to assess how extraction polarity influences antioxidant distribution. By conducting a harmonized comparison of ethanol, water, and chloroform extracts across multiple ROS/RNS models (DPPH, hydrogen peroxide, nitric oxide, superoxide) and ferric-reducing activity, this study provides new insights into solvent-specific biochemical behavior. Moreover, differential NO and superoxide scavenging patterns observed here have not been previously documented. These solvent-based comparative profiles offer original contributions and establish a reference point for future mechanistic and phytochemical investigations.

MATERIAL AND METHODS

Hydrogen peroxide (H_2O_2), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), potassium ferrocyanide ($K_3Fe(CN)_6$), potassium dihydrogen phosphate (KH_2PO_4), sodium nitroprusside, N-(1-naphthyl)ethylenediamine dihydrochloride and phosphoric acid (H_3PO_4) were supplied by Merck Millipore (Germany). Ascorbic acid, butyl hydroxytoluene (BHT), sodium phosphate monobasic, sodium phosphate dibasic, Nitro blue tetrazolium (NBT), trichloroacetic acid (TCA) and iron (III) chloride ($FeCl_3$) were supplied by Sigma-Aldrich (USA). Sulfanilamide was supplied by Fluka (Switzerland). All chemicals were of analytical grade and used without any additional purification. All plants were collected fresh from Ardahan Province and studied.

Extraction

H. arenarium were collected from the East Anatolian region in Turkey (Ardahan). For extraction, 10 grams of *H. arenarium* powder was placed in a round flask and 100 mL of ethanol [10], water, and chloroform solutions were added. The mixture was stirred at 25°C for 24 hours. They were then filtered, oven dried to remove organic solvents and stored in the refrigerator for later use (Figure 1-2).



Figure 1. General view of *H. arenarium* flower and *H. arenarium* powder



Figure 2. *H. arenarium* leaf extraction process

All extraction procedures were performed under controlled conditions. Plant powders were macerated at 25 °C for 24 h using a solid-to-solvent ratio of 1:10 (w/v). After filtration, solvents were removed using a rotary evaporator at 40 °C (for ethanol and chloroform) or a freeze-dryer (for water extracts). The final extracts were weighed, yield percentages were calculated, and samples were stored at -20 °C until analysis.

Antioxidant Activity

All in vitro antioxidant assays were performed in triplicate ($n=3$). For each assay, calibration curves and reagent blanks were prepared according to the referenced protocols. The absorbance measurements were obtained using a UV-Vis spectrophotometer, and all results were expressed as mean \pm standard deviation (SD). Statistical differences between extracts were evaluated using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

DPPH radical Scavenging activity

The DPPH radical scavenging activity of the samples was assessed following the method described by Kahraman et al. [11]. Different concentrations of the samples were mixed with a DPPH solution (19mg/50mL). Then this mixture was incubated at 30 min in the dark medium. Afterwards, the absorbance was measured at 517 nm. The DPPH radical scavenging activity of the samples was determined at equation 1.

$$\text{DPPH radical inhibition} = (A1 - A2) / A1 \times 100 \quad (1)$$

A1 and A2 refer to the control and sample absorbance values respectively.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the samples was investigated to the reported method by Unal et al. [12]. Briefly: The hydrogen peroxide solution (43 μM) was mixed to the various concentration of samples and 2 mL PBS (pH 7.4) solution. Then this mixture was incubated at 10 min and absorbance was measured at 230 nm against blank. The hydrogen peroxide scavenging activity of the samples was determined at equation 1.

Ferric reducing Activity

The ferric reducing antioxidant power of the samples was assessed following the method described by Kahraman et al. with slight modifications [11]. Briefly, different concentrations of the samples were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] solution. The mixture was incubated at 50 °C for 20 minutes in a water bath. After incubation, 2.5 mL of % trichloroacetic acid (TCA) was added to terminate the reaction, and the mixture was centrifuged at 3000 rpm for 10 minutes. Subsequently, 2.5 mL of the resulting supernatant was transferred to a clean test tube and mixed with 2.5 mL of distilled water and 500 μL of 1% ferric chloride (FeCl_3). The mixture was allowed to stand at room temperature for 10 minutes. The absorbance of the resulting solution was measured at 700 nm

Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity of the samples was evaluated with slight modifications to the method reported by Mehrous et al. [13]. Different concentrations of the samples were mixed with 2 mL of alkaline DMSO solution (20 mg dissolved 1 mL distilled water and complete to the 100 mL distilled water) and 200 μL of nitroblue tetrazolium (NBT) solution. The mixture was incubated for 10 minutes at room temperature. Afterwards, the absorbance was measured at 560 nm. The superoxide radical scavenging activity was calculated using Equation 1.

Nitric Oxide Scavenging Activity

The nitric oxide scavenging activity of the samples was evaluated with some modifications to the method reported by Patel et al. [14]. Different concentrations of the samples were mixed with 10 mM sodium nitroprusside solution in phosphate buffered saline (PBS, pH 7.4) and 500 μL PBS. Then samples were incubated at 25 °C for 150 minutes. Afterwards 1 mL sulfanilamide (1% sulfanilamide in 2.5% phosphoric acid) was added to the samples and stand for five minutes. Afterwards 1 mL N-(1-naphthyl)ethylenediamine dihydrochloride (0.1% in 2.5% phosphoric acid) and stand for 30 minutes. The absorbances was recorded at 546 nm. The nitric oxide scavenging activity was determined by equation 1.

RESULTS

DPPH Radical Scavenging Activity

DPPH is a nitrogen based free radical and is used to determine the antioxidant potential of compounds. In this study, the radical scavenging activity of DPPH was investigated using extracts prepared in different solvents. Figure 3 shows the DPPH radical scavenging activity of the extracts. The ethanol extract had greater scavenging activity than the water and chloroform extracts. At 400 $\mu\text{g}/\text{mL}$ ethanol, water, chloroform extracts and ascorbic acid gave scavenging to the 87.84 %, 77.78 %, 50.91 % and 92.18 % respectively. This depends on the solvents used in the extraction. In the recent studies reported that the phenolic compounds are known to have high antioxidant potential. It is thought that extracts prepared using water, ethanol, and chloroform release varying amounts of phenolic compounds. The Ec_{50} value is defined as the value at which 50 % of the initial DPPH radical is inhibited. The ascorbic acid equivalent value is the equivalent of a compound in terms of ascorbic acid. Ascorbic acid equivalent values are determined using the equation $\text{Ec}_{50\text{ascorbic}}/\text{Ec}_{50\text{sample}}$ (10). The Ec_{50} and ascorbic acid equivalent values are shown at Table 1.

Table 1. The Ec_{50} and AAEQV values

Samples	Ec_{50} value ($\mu\text{g}/\text{mL}$)	AAEQV
Ethanol extract	16.60	0.73
Water extract	85.67	0.14
Chloroform extract	175.86	0,069
Ascorbic acid	12.15	-----

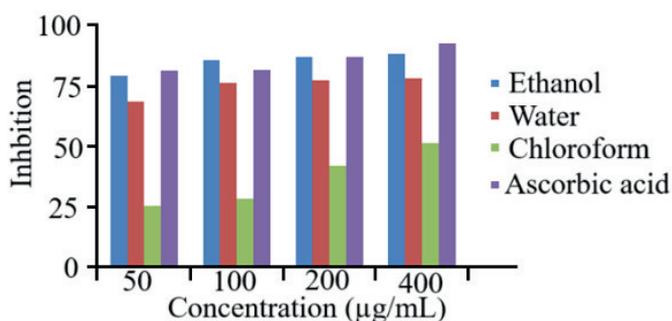


Figure 3. DPPH radical scavenging activity

Hydrogen peroxide scavenging activity

Hydrogen peroxide is not active free radical. However it can participate to the Haber weiss and Fenton based reaction. Haber Weiss and fenton based reaction make formation hydroxyl and superoxide radical [15]. Hydroxyl and superoxide radical are toxic properties at cell. Therefore it is important that the deleterious of hydrogen peroxide at metabolism. In this purpose in the current study investigated to the hydrogen peroxide scavenging activity. The obtained extracts has moderate hydrogen peroxide scavenging activity. At 400 $\mu\text{g}/\text{mL}$, ethanol, water, chloroform and ascorbic acid gave scavenging to the 60.82%, 53.11%, 34.29% and 73.86% respectively (Figure 4).

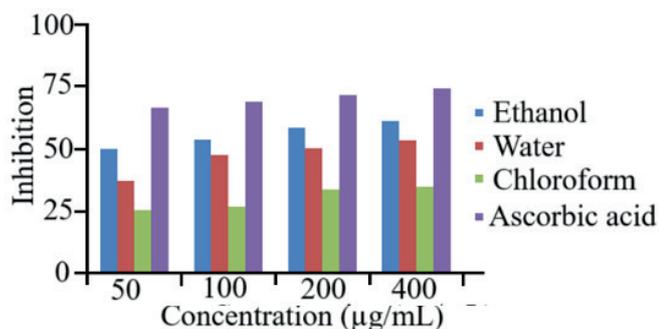


Figure 4. Hydrogen peroxide radical scavenging activity

Ferric reducing Activity

The ferric reducing activity one of the useful test to the determinant of secondary antioxidant potentials. In the ferric reducing activity, the compounds reducing capacity is an important indicator of its potential antioxidant activity. Compounds with reducing power are electron donors and can reduce oxidized intermediates of lipid peroxidation processes, thus acting as primary and secondary antioxidants [16]. Table 2 seen to the absorbance values at 700 nm of samples. The increase absorbance values refers to the increase reducing activity.

Table 2. Absorbance values at 700 nm.

Concentration (µg/mL)	Ethanol extract	Water extract	Chloroform extract	Ascorbic acid
50	0.76	0.74	0.099	0.81
100	0.95	0.78	0.114	0.98
200	1.08	0.84	0.128	1.14
400	1.15	0.87	0.151	1.21

(Values are expressed as mean \pm SD (n = 3). Different letters indicate statistically significant differences between samples (p < 0.05).)

Nitric oxide Scavenging Activity

The water extract of *H. arenarium* exhibited low moderate NO scavenging activity than ethanol and chloroform extracts (Figure 5). NO scavenging capacity was concentration dependent, with 400 µg/ml scavenging being most efficient. At 400 µg/mL ethanol, water, chloroform extracts and BHT gave nitric oxide scavenging to the 33.82%, 47.22%, 24.49% and 50.14% respectively.

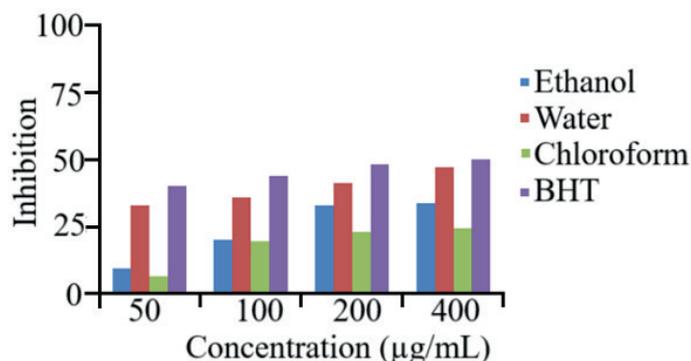


Figure 5. Nitric oxide scavenging activity

Superoxide Radical Scavenging Activity

The superoxide radical has toxic properties in metabolism. In the present study, superoxide radical formation was achieved

by reacting DMSO with NaOH. The ethanolic extract shown strong superoxide radical scavenging activity than water and chloroform extracts. At 400 µg/mL ethanolic, water, chloroform extracts and BHT gave scavenging to the 66.47 %, 58.21 %, 34.19 % and 61.42 % (Figure 6).

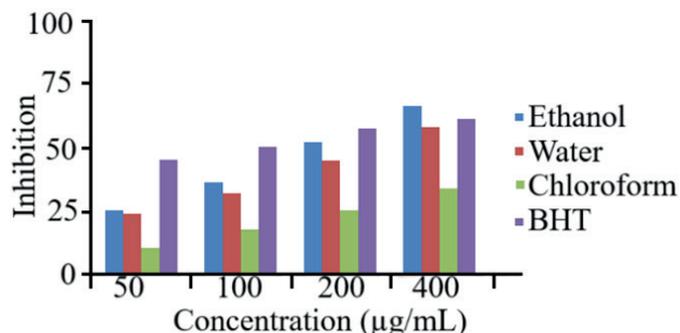


Figure 6. Superoxide radical scavenging activity

Data presented in Table 1 and Table 2 represent mean \pm SD values obtained from three independent experiments. Statistical differences among extracts were analyzed using one-way ANOVA. Different letters or asterisks in Figures 3–6 indicate statistically significant differences between sample groups (p < 0.05).

DISCUSSION

The antioxidant activity of the plant extracts are important to the deteriorate oxidative stress and disease related oxidative stress. Oxidative stress act cause development of the diseases such as cardiovascular, neurodegenerative etc[17]. Antioxidant molecules act deleterious effect of the free radicals. Butylated hydroxyanisole (BHA), butylated hydroxy toluene (BHT) and tertiary butylated hydroquinone (TBHQ) are frequently used synthetic antioxidants. However in the recent studies have reported that long term utilize synthetic antioxidant has side effects such as cell toxicity and cancerogenic. Also synthetic antioxidant has low dispersibility at water and it can not showed antioxidant activity in human tissues [18]. Studies have investigated the toxicity of the synthetic antioxidants BHA, BHT, and TBHQ under various experimental conditions. A study by Efsa et al. showed that BHA has low acute toxicity, with a lethal dose 50 (LD50) value of > 2000 mg/kg bw in mice and rats [18]. Another study showed that BHA has a potent cytotoxic effect on human astrocytes [19]. Therefore it is important that the find to the natural antioxidant compound. In the current study that the antioxidant potential of different extract was evaluated by various in vitro antioxidant test. In the current study that the *H. arenarium* extracts has strong DPPH radical scavenging activity. Specifically ethanolic and water extract of *H. arenarium* has strong DPPH radical scavenging activity. The DPPH scavenging activity of the ethanolic extract may have increased due to the phenolic compounds released during extraction. Ethanolic extract may contain more phenolic compounds than extracts obtained us-

ing chloroform and water solvents. Therefore it may have exhibited higher DPPH radical scavenging activity. The reducing power test is used to determine the Fe+3 reduction potential of a compound. The reducing power test results are consistent with the DPPH radical results. The ethanolic extract has a higher reducing potential than the other groups. Reactive oxygen species (ROS) naturally occurring during the respiratory process are closely related to cellular energy production and include components such as the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$) [20]. Superoxide radical occurs through the gradual reduction of molecular oxygen (O_2). Superoxide radical is a weaker oxidant compared to other species such as hydroxyl radicals and peroxynitrite. However this does not mean that superoxide radicals are harmless. On the contrary superoxide radicals facilitate the formation of much more reactive and potentially toxic species through secondary reactions. For example while it can be converted to hydrogen peroxide via the enzyme superoxide dismutase an oxidative chain reaction can be triggered that progresses to the hydroxyl radical via the Fenton or Haber-Weiss reactions. Furthermore they can react with nitric oxide (NO) to form highly harmful compounds such as peroxynitrite ($ONOO^-$) [21]. These secondary and more potent oxidant species are capable of initiating lipid peroxidation on lipid components in cell membranes. Lipid peroxidation can lead to serious cellular consequences such as disruption of membrane integrity, disruption of cellular signaling pathways and even apoptosis. Therefore it is important that prevent to the harmful effect of superoxide radical at cell metabolism. Ethanolic and water extract of *H. areniarum* has strong superoxide radical scavenging activity. The IC_{50} values of ethanolic, water and chloroform extract was determined 167.45 $\mu g/mL$, 199.96 $\mu g/mL$ and 417.35 $\mu g/mL$ respectively.

Nitric oxide (NO) is produced from the amino acid L-arginine by vascular endothelial cells, phagocytes and some brain cells. Nitric oxide is one of the important bioregulatory molecules with physiological effects including blood pressure control, nerve signal transmission, platelet function, antimicrobial and antitumor activities [22]. Nitric oxide is classified as a reactive nitrogen species due to its unpaired electron and reacts significantly with certain types of proteins and other free radicals [23]. The toxicity of NO increases significantly when it reacts with superoxide to form the peroxynitrite anion ($ONOO^-$) [24]. In the current study that NO released from SNP has a strong NO^+ character capable of altering the structure and function of many cellular components. "Therefore, preventing the negative effects of nitric oxide (NO) on metabolic processes is of great importance. In this study, it was observed that *H. arenarium* extract samples had low nitric oxide scavenging activity.

Our findings are consistent with previous reports demonstrating that ethanolic extracts of *Helichrysum* species contain higher levels of phenolic constituents, which are primarily responsible for free radical scavenging activity (Czinner et al., 2000; Albayrak et al., 2010). In particular, the EC_{50} value of the ethanolic extract in our study (16.60 $\mu g/mL$) falls within the range reported for *Helichrysum* species with strong antioxidant potential. These results reinforce the importance of solvent polarity in phenolic extraction efficiency.

Although the present findings clearly indicate strong in vitro antioxidant activity, in vitro outcomes do not always translate directly into clinical efficacy. Factors such as bioavailability, metabolic stability, plasma binding, tissue distribution and interaction with endogenous antioxidant systems can significantly alter the in vivo effectiveness of plant-derived antioxidants. Therefore, future studies employing cellular and in vivo models are required to validate whether the antioxidant potential observed in vitro can be translated into clinically meaningful outcomes.

CONCLUSION

Antioxidants derived from natural sources may be an alternative to synthetic antioxidants in combating diseases associated with oxidative stress. Studies have determined that phytochemicals found in plants possess antioxidant properties. Therefore in this study, extracts were obtained using different solvents at different concentrations, and their antioxidant potential was evaluated using various in vivo tests. In general the ethanolic extract demonstrated higher antioxidant potential than other extracts. The results of this study indicated that *H. areniarum* possesses strong antioxidant potential. However we believe that more comprehensive and systematic studies are necessary to determine the potential of this plant for medical use.

The solvent-dependent comparison provided in this study contributes new insight into how extraction polarity modulates antioxidant distribution. Although the strongest activity was observed in the ethanolic extract, distinctive NO and superoxide patterns suggest that different solvents preferentially extract different redox-active constituents. This multidimensional dataset offers foundational information for future mechanistic, phytochemical and pharmacological studies.

Author Contributions

U.Y (first author) & Z.Y; analysis, methodology, validation; İ.G & A.İ & O.K & B.S & U.K & F.U; investigation, resources, writing and editing;

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Conflicts of Interest

The author declares no conflict of interest.

Ethics Approval: This study did not involve human participants, patient data, or experimental animals. Therefore, ethics committee approval was not required.

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