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Investigation on total phenolic content, antibacterial, and antioxidant activity of ethanolic extract of *Helichrysum leucocephalum* Boiss

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Abstract
Because of undesirable side effects of synthetic antioxidants and antibiotics, there is considerable interest in the use of phytomedicines. For this purpose, the present study evaluated total phenolic content, antibacterial, and antioxidant effect of ethanolic extract of *Helichrysum leucocephalum* for the first time in the world. Ethanolic extract of *H. leucocephalum* was analyzed for its total phenols according Rutin standard. DPPH assay was used to discover its antioxidant properties. Based on disc diffusion, MIC, and MBC the antibacterial activity was studied at several concentrations on five bacteria as Gram-negative and Gram-positive. The results demonstrated high contents of total phenols (449.2 mg Rutin equivalent/g dry weight). IC50 value for free radical-scavenging capacity was 69.94±0.17 µg/mL. Evaluation the antibacterial activity indicated moderate activity against all the Gram-positive bacteria tested with the minimum inhibitory concentration (MIC) ranging from 125 to 250 mg/ml. None of the concentrations of the extract was effective against *E. coli*.

Keywords: *Helichrysum leucocephalum*, Disc diffusion, MIC, MBC, DPPH, Total phenol

1. Introduction
In recent years, there has been a worldwide trend towards the use of the natural phytochemical that are present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables [1]. *Helichrysum* belongs to Asteraceae family and includes about 600 species widespread through in Eurasia, Africa and Australia [2, 3]. It is represented, in flora of Iran, by 19 species, eight of which are endemic [4]. *Helichrysum* species are herbaceous, perennials or shrubs, their leaves are dense, oblong to lanceolate, simple entire. The bracts are numerous white or colored [2]. This genus naturally occurs in the Mediterranean areas, and its extracts are used in popular medicine in this region [5]. Different compounds like phenolics, e.g., flavonoids and chalcones, phthalides, α-pyron derivatives, terpenoids, essential oils, volatiles and fatty acids have been found in *Helichrysum* [6]. Different studies reported that phenolic compounds have protection against a wide range of diseases such as coronary heart disease, stroke, and certain types of cancers [7, 8] and exhibit biological activities such as anti-inflammatory, immune-stimulating agents, antiallergenic, anti-artherogenic, and antimicrobial, antithrombotic, anti-stress, anti-hyperglycemia and vasodilator effects [9].
It could be mentioned that, the interest in *Helichrysum* has been motivated by their traditional therapeutic applications. Traditionally, the wound caused by circumcision is bandaged with mashed leaves of *H. pedunculatum* Hilliard & Burtt., *H. appendiculatum* Hilliard & Burtt. or *H. longifolium* DC. However, traditional circumcision has a high risk of infection. *H. longifolium* is a plant that has shown potential as a source of chemotherapeutic compounds [10, 11]. Phytochemical studies have revealed that *Helichrysum* is rich in flavonoids and other water soluble polyphenolic compounds [12]. The antibacterial potentials of *H. longifolium* extracts have been studied and reported [13]. *H. arenarium* has been reported for its antiseptic, coleretic and spasmolytic properties [14], while *H. graveolens* traditional applications in controlling the symptoms of diabetes mellitus, wound healing and as a diuretic have been reported in Turkey [15]. *H. stoechas* is particularly referred in Spanish folk medicine for its anti-inflammatory and wound healing properties as well as uses for toothache, and urologic conditions [16]. Furthermore, *Helichrysum* had a great reputation in traditional medicine as anti-inflammatory [17], anti-oxidant [18], antimicrobial [19], antiviral [20] and anti-HIV [21] properties.

The other studied species of this genus are *H. arenarium* (L.) Moench [22], *H. stoechas* (L.) Moench [23], and *H. graveolens* (M.Bieb.) Sweet [15].

This information on *H. leucocephalum* is scanty in available literatures thus suggesting that not much work has been done on it. The aim of the present study was to investigate total phenol, the *in vitro* antimicrobial, and antioxidant activity of the aerial part of *H. leucocephalum* collected from the middle of Iran. These compounds represent novel leads and future studies may allow the development of a medicine and a pharmacologically acceptable agent.

2. Materials and methods

2.1 Plant Materials

The aerial part of *H. leucocephalum* was collected at flowering time in Mehriz, Yazd, Iran. The samples were identified in Herbarium of Yazd University, Yazd, Iran. The materials were dried and kept in the shadow at laboratory temperature. After drying all material were powdered and kept for ethanolic extract.

2.2 Isolation the Ethanolic Extract

For preparation of the ethanolic extract, 100 g of plant powder were extracted overnight with 500 ml of ethanol, in a mechanical shaker at room temperature. The extraction method was continued for three days. The extraction was filtered with Whatman No. 2 and dried by rotary evaporation. Finally, it stored in a dark vial at 4 °C.

2.3 Determination of Total Phenolic Content (TPC)

TPC of the extract was determined spectrophotometrically using folin–ciocalteu and rutin as a reagent and standard combination, respectively. For this purpose, 1 ml of rutin at dissimilar concentration (20, 40, 60, 80, and 100 µg/ml) was mixed with 5 ml Folin Ciocalteu (diluted proportionally 1 to 10) that incubated at laboratory temperature. After 10 minutes, 4 ml of Na_2CO_3 (75 mg/ml) was added with the mixture and incubated for 30 minutes. Finally, the total phenols were determined at 765 nm. All of these processes were done for the ethanolic extraction, too. In other words, 1 ml of rutin replaced with 1 ml of the extraction with 250 µg/ml concentration. Measurements were carried out in triplicate and calculations were based on calibration curve obtained with rutin.

2.4 DPPH Radical Scavenging Activity Assay

For determination the radical scavenging activity, rutin (the concentration was 5, 10, 20, and 40 µg/ml), vitamin C (the concentration was 5, 10, 20, and 40 µg/ml), and 1 ml of DPPH were added with 2.5 ml of different concentration (3.12, 6.25, 12.5, 25, 50, and 100 µg/ml) of the extract. Blank (mixture without DPPH) and control (mixture without the extract) were prepared, too. All the samples were kept at darkness situation and laboratory temperature for 30 minutes. Finally, all of them were measured by spectrophotometer at 518 nm.

$$Ip = \frac{ODC - (ODS - ODB)}{ODC} * 100 \quad (1)$$

ODC: the absorbance value of control
ODS: the absorbance value of control
ODB: the absorbance value of blank

2.5 Antibacterial Screening

Staphylococcus aureus (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aeruginosa* (ATCC 9720), *Bacillus subtilis* (ATCC 6051), and *Escherichia coli* (ATCC 8739) were test strains derived. All microorganisms were bought from Scientific and Industrial Research Organization of Iran. The antibacterial activity was determined by disc diffusion method. Briefly, bacteria were grown to 0.5 standard of McFarland's tube on Mueller-Hinton equivalently. 100 ml of different concentration (62.5, 125, 250, and 500 mg/ml) were added to the well. After that, the plates were incubated at 37°C for 24 h and zone of inhibitions were measured in millimeters. A series of dilutions of the extract (0.97, 1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, and 250) were prepared in Muller-Hinton Broth to evaluate minimal inhibitory concentration (MIC). From the 24-hour culture of microorganisms, microbial suspensions were prepared with a half-McFarland equivalent of turbidity and diluted at the ratio of 1:15. Then 100 ml of microbial suspension was added to each tube. The positive control contained Muller-Hinton Broth and microbial suspension while the negative one contained the ethanol extract and Muller-Hinton Broth. The samples incubated at 37°C for 24 hours. The last dilution with visible microbial opacity was recorded as the minimum microbial concentration. After these steps, all samples that did not grow in the MIC were cultured on the Muller Hinton Agar. The tubes containing the lowest concentration of the extract and 99.9% of the initial bacterial inoculation were considered as minimum bactericidal activity (MBC).

2.6 Statistical Analysis

For the present study, all tests were done in triplicate. The total phenol results were reported as MEAN ± SEAM. Data were analyzed by one-way ANOVA with Graph pad Prism 3.02. Statistically significant effects were determined using Tukey post test at $p < 0.05$.

3. Results & Discussion

3.1 Total Phenol Contents (TPC)

Based on rutin calibration curve as a standard, TPC was calculated. As can be seen in figure 1, the horizontal and vertical axis show the rutin concentration (µg/ml) and absorption intensity, respectively. According to rutin, the phenolic content of the species (dry extract) was estimated 449.20 ± 10.04 mg/g. The TPC of *H. leucocephalum* ethanolic extract was more than four MeOH extracts of *Helichrysum* in previous study. Total phenol content of *H. arenarium* subsp. *erzincanicum*, *H. arenarium* subsp. *rubicundum*, *H. armenium* subsp. *araxinum*, *H. plicatum* subsp. *pseudoplicatum* were 125.57 ± 1.0, 71.81 ± 1.0, 86.01 ± 0.6, and 144.50 ± 1.2 mg GAE/g, respectively [24]. The total phenolic content of the methanolic extract of *H. chasmolycicum* was 108.33 ± 0.88 mg GAE/g [25]. The total phenolic contents of the ethanolic and water extracts of *H. plicatum* subsp. *plicatum* collected from eastern Anatolia were 113.5 ± 8.6 and 75.9 ± 3.7 mg GAE/g extract, respectively [26]. It has been determined that the total phenolic contents of the methanolic extracts of *H. pamphylicum*, *H. sanguineum*, and *H. chasmolycicum* were 119.85 ± 2.0, 63.8 ± 0.6, and 71.51 ± 0.5 mg GAE/g extract, respectively [27]. In addition, it could be said that phenolic compounds contribute to quality and nutritional value in terms of taste, aroma, flavor, and providing health beneficial effects [28].

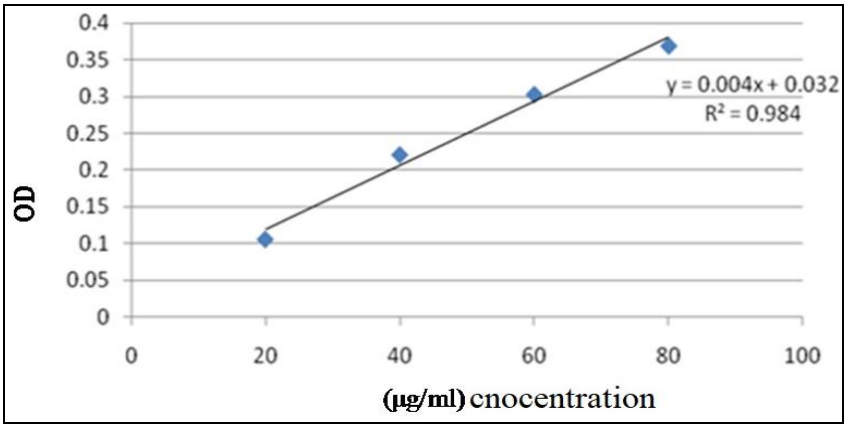

Fig 1: Rutin calibration curve

3.2 DPPH Radical Scavenging Activity Assay

Based on the calculation and concentration curve, IC_{50} value was estimated for the extract, rutin, and vitamin C. All DPPH results are summarized in table 2. The results indicated definite scavenging activity of the extracts towards DPPH radicals, which depends on acceptable dose. Furthermore, there are many reports dealing with the antioxidant activity of different *Helichrysum* species. For instance, the extracts of *H. chasmolycicum*, *H. chionophilum*, *H. plicatum* subsp. *plicatum*, and *H. arenarium* subsp. *aucheri* have been reported to have antioxidant activity in which the IC_{50} value were 246.83 ± 1.23 mg AAE/g, 40.5, 48.0, and 47.6 µg/mL, respectively [25, 29]. The methanolic extracts of 16 *Helichrysum* species were investigated for their *in vitro* antioxidant, radical scavenging in Turkey. All the extracts showed strong antioxidant and radical scavenging activity. The highest IC_{50} value (7.95 µg/ml) was observed for the extract of *H. stoechas* subsp. *barellieri* [30]. Briefly, as can be resulted the DPPH radical scavenging activity results of the previous study are better than *H. leucocephalum*. The antioxidative effect of phenolic in functional foods is due to a direct free radical scavenging activity, reducing activity and an indirect effect arising from chelation of metal ions [31]. Hence, it appears that differences in antioxidant activity could be related to the nature of the phenolic and variances in genus. Finally, it should be mentioned that the present results showed direct relationship between the property and concentration.

Table 1: IC_{50} value of *H. leucocephalum*, Vitamin C, Rutin

Extract/control	IC_{50} (µg/mL)
H. leucocephalum	69.94 ± 0.17
Vitamin C	2.12 ± 0.11
Rutin	5.53 ± 0.06

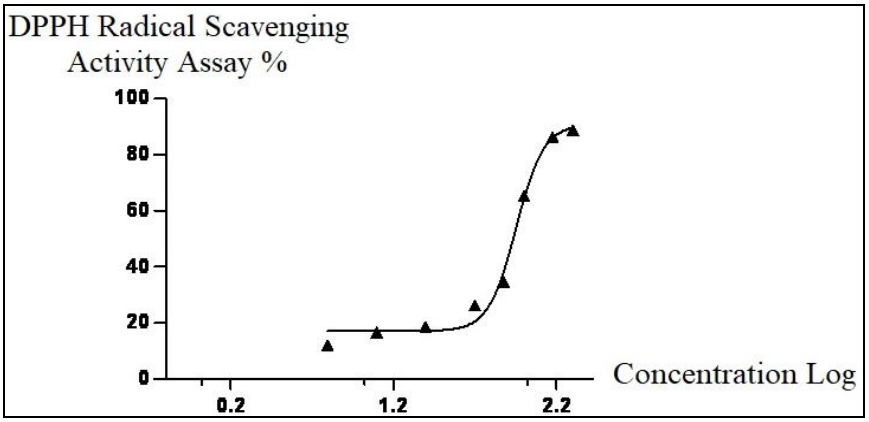

Fig 2: Concentration curve of the extract versus percentage DPPH radical scavenging activity

3.3 Antibacterial Activity

As can be seen in figure 3, the results discovered that the extract from aerial part of *H. leucocephalum* had different inhibitory activities against pathogenic bacteria. Briefly, the suitable extract concentration (500 mg/ml ≤) had positive property on *S. aureus*, *S. epidermidis*, and *B. subtilis*. The acceptable concentration for *P. aeruginosa* was 1000 mg/ml. There is an important point that non-concentration of the ethanolic extract of *H. leucocephalum* had effect on *E. coli*. The result of minimum inhibitory concentration (MIC) for *S. aureus*, *S. epidermis* were 125 mg/mL. MIC result for *P. aeruginosa* and *B. subtilis* were 250 mg/mL. MBC levels for *S. epidermis*, *P. aeruginosa*, and *B. subtilis* were also recorded 500 mg/mL. MBC level of *S. aureus* was 125 mg/mL. The antibacterial activities of *Helichrysum* species have been reported elsewhere. The methanolic and hexanic extracts of *H. stoechas* showed moderate antibacterial activity against *Bacillus cereus*, *Acinetobacter baumannii*, *Proteus mirabilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* [32]. The extracts from *H. italicum* have the ability to inhibit the growth of *Staphylococcus* sp [33]. The methanolic extract of *H. chasmolycicum* demonstrated antibacterial activity and *Y. enterocolitica* was the most resistant bacteria [25]. It was also determined that the acetone extract of *H. dasyanthum* was active against *S. aureus* (MIC = 15.63 µg/mL) [12]. Investigation on *H. italicum* diethyl ether extract showed inhibited *Staphylococcal* growth and some of its enzymes [19]. Study the antibacterial activity of *H. aureonitens* confirmed its positive effect against *S. aureus* [34]. The methanolic extract of *H. foetidum* revealed inhibited the

growth of *S. aureus*, *Streptococcus pyogenes*, *E. coli*, and *P. aeruginosa* (MIC > 4 mg/mL) [35]. The acetone extract of *H. cymosum* subsp. *cymosum* was active against *Enterococcus faecalis*, *B. cereus*, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *Y. enterocolitica*, *K. pneumoniae*, *Cryptococcus neoformans*, and *C. albicans* (MIC = 0.078-0.313 mg/mL) [36].

In the present study, the findings had some differences with the observations of previous studies. In other words, *H. leucocephalum* showed moderate activity against Gram-positive bacteria and it had no activity against *E. coli* as a Gram-negative bacteria.

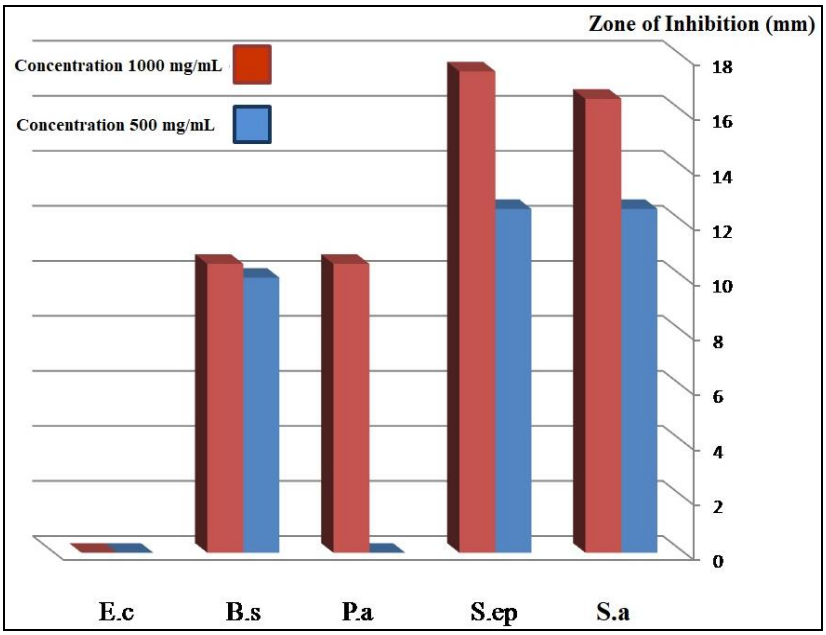

Fig 3: Comparison in different concentrations of the extract

4. Conclusions

The present study showed that the ethanolic extract of *Helichrysum leucocephalum*, which contain high amount of phenolic compounds, exhibited sufficient antioxidant activity. The current data indicate that further studies are needed to evaluate the *in vivo* potential of this extract in animal models. The isolation and characterization of the active antioxidant and phenolic compounds are desirable, too. Determination of the antioxidant compounds of plant extracts and essential oils will help to develop new drug supplement for antioxidant therapy. Because of moderate antibacterial activity of *H. leucocephalum*, it could not be recommended as natural source of bactericidal. From this point of view, the other *Helichrysum* could be better for antibacterial studies.

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