Flavonoids of *Helichrysum chasmolycicum* and its antioxidant and antimicrobial activities

S. Süzgeç-Selçuk\textsuperscript{a,*}, A.S. Birteksöz\textsuperscript{b}

\textsuperscript{a} Faculty of Pharmacy, Department of Pharmacognosy, Istanbul University, 34116 Beyazıt, Istanbul, Turkey

\textsuperscript{b} Faculty of Pharmacy, Pharmaceutical Microbiology, Istanbul University, 34116 Beyazıt, Istanbul, Turkey

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Abstract

From the aerial parts of *Helichrysum chasmolycicum* P.H Davis, which is an endemic species in Turkey, the flavonoids apigenin, luteolin, kaempferol, 3,5-dihydroxy-6,7,8-trimethoxyflavone, 3,5-dihydroxy-6,7,8,4′-tetramethoxyflavone, apigenin 7-O-glucoside, apigenin 4′-O-glucoside, luteolin 4′,7-O-diglucoside, kaempferol 3-O-glucoside, kaempferol 7-O-glucoside and quercetin 3-O-glucoside were isolated. The methanol extract of the aerial parts of *H*. *chasmolycicum* showed antioxidant activity by DPPH method (IC\textsubscript{50} 0.92 mg/mL). Antimicrobial activity test was performed on the B, D, E extracts and also 3,5-dihydroxy-6,7,8-trimethoxyflavone and kaempferol 3-O-glucoside which were the major flavonoid compounds obtained from aerial parts of *H. chasmolycicum* by microbroth dilutions technique. The E (ethanol–ethyl acetate) extract showed moderate antimicrobial activity against *Pseudomonas aeruginosa*, B (petroleum ether–60% ethanol-chloroform) extract and 3,5-dihydroxy-6,7,8-trimethoxyflavone and kaempferol 3-O-glucoside which were the major flavonoid compounds obtained from aerial parts of *H. chasmolycicum* by microbroth dilutions technique. The E (ethanol–ethyl acetate) extract showed moderate antimicrobial activity against *Pseudomonas aeruginosa*, B (petroleum ether–60% ethanol-chloroform) extract and 3,5-dihydroxy-6,7,8-trimethoxyflavone showed moderate antifungal activity against *Candida albicans*.

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1. Introduction

*Helichrysum* Gaertner (Asteraceae) is a very large genus consisting of approximately 500 species in the world (Engler, 1964). The *Helichrysum* species are also found in southern Europe, south-west Asia, southern India, Sri Lanka and Australia, most species occur in Africa, including Madagascar (Lourens et al., 2008). In Turkey, the genus *Helichrysum* is represented by 26 taxa belonging to 20 species in Turkish flora of which 14 are endemic to Turkey (Davis, 1975; Davis et al., 1988; Erek, 2000; Kupicha, 1975).

Species from the genus *Helichrysum* are widely used as traditional medicine to cure various ailments (Hutchings and Van Staden, 1994; Lourens et al., 2008; Sezik et al., 2001). It is given in Dioscorides records, Materia Medica that the heads of *H. siculum* (*H. stoechas* subsp. *barrei*) are used to cure biliousness. In Turkey, several *Helichrysum* spp. are used in folk medicine for removing the kidney stones and as diuretics. The diuretic and bile regulatory effects of the *Helichrysum* spp. are due to the flavonoids they contain (Çubukçu, 2002).

Various biological activities of the members of this genus were reported elsewhere; choleretic (Delapuerta et al., 1993), chologogue (Çubukçu, 2002), antimicrobial (Meyer and Afolayan, 1995), antimalarial (Van Vuuren et al., 2006), antioxidant, antidiabetic (Aslan et al., 2007), antinflammatory (Jäger et al., 1996), antiproliferative (Yagura et al., 2008), antiviral and antituberculous (Lall et al., 2006).

The chemistry of *Helichrysum* genus is complex with a wide variety of chemical classes which are flavonoids, chalcones, phloroglucinol derivatives, essential oils, α-pyrones and diterpenes (Jakupovic et al., 1986; Lourens et al., 2008).

Increasingly, flavonoids are becoming the subject of medicinal research. In the Department of Pharmacognosy of Istanbul University, investigations are in process dealing with the Anatolian *Helichrysum* spp., especially on their flavonoids (Çubukçu and Bingöl, 1984; Çubukçu and Meriçli, 1977; Çubukçu and Yüksel, 1982; Meriçli et al., 1984; Süzgeç et al.,...
2. Materials and methods

2.1. Plant material

_H. chasmolyicum_ P.H. Davis (Asteraceae), aerial parts were collected on Dedegül Mountain, Isparta-Aksu, Turkey in August 1997 and identified by Professor Hasan Özçelik. A voucher specimen was deposited in the Herbarium of the Faculty of Science and Literature, Süleyman Demirel University (No: Özçelik 7838), Isparta, Turkey.

2.2. General experimental procedures

The UV spectra were measured on a Shimadzu UV-1601 spectrophotometer in MeOH. ^1^H NMR spectra were recorded on a Bruker 500 MHz spectrophotometer using CDCl₃ as a solvent. Paper chromatography (PC) were applied on Whatman No. 1. For preparative TLC, silica gel 60 F₂₅₄ plates (0.25 mm, Merck) were used. For column chromatography silica gel 60 (0.063–0.200 mm; Merck) (0.2–0.5 mm; Merck), polyamide 6 (Fluka Chemical) and Sephadex LH-20 (Sigma-Aldrich) were used. For antioxidant activity 2,2-Diphenyl-1-picrylhydrazyl and ascorbic acid were purchased from Fluka Chemical. All other chemicals used were of analytical grade.

2.3. Extraction and isolation of flavonoid compounds

The dried aerial parts (500 g) of _H. chasmolyicum_ was first extracted with petroleum ether and then with EtOH (95%) in a Soxhlet apparatus. The petroleum ether extract was concentrated (A) (yield 3.36 %) and extracted with 60% ethanol. The 60% ethanol extract was concentrated and extracted with chloroform (B) (yield 2.05 %). The concentrated 95% EtOH extract was diluted with H₂O and extracted with toluene (C) (yield 1.87 %), chloroform (D) (yield 1.53 %) and ethyl acetate (E) (yield 2.17 %) successively for fractionation. A qualitative evaluation of the extracts by TLC and PC were performed in order to select the extracts which are rich in flavonoids. For TLC, solvent systems toluene:ethyl acetate:formic acid (5:4:1), benzene:diethyl ether:di chloromethane (1:1:1), ethyl acetate: ethyl methyl ketone:formic acid:water (5:3:1:1), benzene: ethanol (2:1), benzene:acetone (8:2) and for PC: butanol:acetic acid:water (4:1:5), acetic acid:water: conc.HCl (30:10:3), butanol:acetic acid:water (3:1:1), acetic acid:water (15:85) were used. Thus the extract B, D and E were selected to be investigated for their flavonoids. The extract B and D were chromatographed over silica gel. The extract E was chromatographed over silica gel and polyamide columns. Fractions were collected in 40 mL volumes and combined according to TLC analysis.

The extract B (2 g) was separated on a column of silica gel eluting with toluene:acetone (100:0, 98:2, 97:3, 96:4, 95:5, 90:10, 80:20). From combined fraction 15–18 (224 mg) (toluene:acetone—96:4), 3,5-dihydroxy-6,7,8-trimethoxyflavone (15 mg) was isolated using preparative TLC developed with benzene:acetone (8:2). From combined fraction 19–22 (91 mg) (toluene:acetone—95:5) and fraction 23–31 (164 mg) (toluene:acetone—95:5), 3,5-dihydroxy-6,7,8′-tetramethoxyflavone (7 mg) was isolated using preparative TLC developed with petroleum ether:chloroform:methanol (7:4:1).

The extract D (1.5 g) was separated on a column of silica gel eluting with toluene:acetone (from 7:1 to 1:1 gradually). From combined fraction 8–9 (12 mg) (toluene:acetone—7:1), kaempferol (3 mg) was isolated using preparative TLC developed with benzene:acetone (8:2). From combined fraction 10–11 (36 mg) (toluene:acetone—6:1), and 12–13 (9 mg) (toluene:acetone—6:1), apigenin (6 mg) was isolated using preparative TLC developed with benzene:ethanol (8:2). From combined fraction 29–40 (79 mg) (toluene:acetone—4:1) and 41–47 (25 mg) (toluene:acetone—4:1), luteolin (5 mg) was isolated using preparative PC developed with acetic acid:water (40:60).

The extract E was separated on a column of silica gel eluting with toluene:acetone (from 7:1 to 1:1 gradually) and on a column of polyamide eluting with distilled water:methanol (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100). For each column 1 g of extract E was used. From fraction 82 (72 mg) (toluene:acetone—50:50), apigenin 7-O-glucoside (7 mg) and kaempferol 3-O-glucoside (astragalin) (15 mg) were isolated using preparative TLC developed with benzene: ethanol (2:1). From combined fraction 82–90 (60 mg) (water:methanol—60:40), apigenin 4′-O-glucoside (6 mg) was isolated using preparative TLC developed with benzene:ethanol (2:1). From combined fraction 91–97 (62 mg) (water:methanol—60:40), quercetin 3-O-glucoside (10 mg) and kaempferol 7-O-glucoside (6 mg) were isolated using preparative TLC developed with benzene:ethanol (2:1). From combined fraction 98–103 (27 mg) (water:methanol—60:40) and fraction 104–109 (21 mg) (water: methanol—50:50), luteolin 4′,7-O-diglucoside (7 mg) and luteolin 4′-O-glucoside (6 mg) were isolated using preparative TLC developed with benzene:ethanol (2:1). Further repeated purification over Sephadex LH-20, eluting with methanol for all flavonoid glucoside compounds.

The glycosides were subjected to acid hydrolysis. The structures of the pure compounds were elucidated by Rf values, colour reactions and spectroscopic methods (UV, ^1^H NMR) on comparison with authentic samples and with their data.
apigenin 7'-glucoside (7 mg) were isolated from extract B; apigenin (6 mg), luteolin (5 mg) kaempferol (3 mg) were isolated from extract D; apigenin 7'-O-glucoside (7 mg), apigenin 4'-O-glucoside (6 mg), luteolin 4'-O-glucoside (6 mg), luteolin 4',7-O-digluco- side (7 mg), kaempferol 3-O-glucoside (15 mg), kaempferol 7-O-glucoside (6 mg), quercetin 3-O-glucoside (10 mg) were isolated from extracts E.

2.3.1. Acid hydrolysis and sugar analysis

Glycoside was mixed with 6% aqueous hydrochloric acid (5 mL) using a minimum of methanol to effect complete solution. The solution was heated at 110 °C on a steam bath for 6 h and then cooled and extracted thoroughly by shaking with ether. Evaporation of the aqueous layer yielded glucose. PC was used to identify sugars by comparison of the RI values with authentical samples. For PC solvent systems ethyl acetate: pyridine:water (12:5:4) and butanol:acetic acid:water (4:1:5) were used.

2.4. DPPH radical-scavenging activity

The antioxidant activity test was performed on the extract obtained from the aerial parts of H. chasmolycicum (1 g) using methanol (100 mL) in Soxhlet apparatus for 8 h. The assay was done according to the DPPH assay (Blois, 1958; Suja et al., 2005).

Extract solution (0.1 mL) in methanol at different concentrations (1–0.61 mg/mL) was added to 3.9 mL of methanol solution (0.025 g/lt) of DPPH. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 515 nm against methanol. All measurements were made in triplicate and averaged. Two controls were used for this test, a negative control (containing all reagents except the test sample) and positive controls using the reference antioxidant as ascorbic acid (1–0.61 mg/mL) for comparison. The ability to scavenge DPPH radical was calculated by the following equation:

\[
\text{Inhibition} \% = \left( \frac{\text{Absorbance of the control at 515 nm} - \text{Absorbance of the sample at 515 nm}}{\text{Absorbance of the control at 515 nm}} \right) \times 100
\]

The antioxidant activity of the methanol extract from aerial parts of H. chasmolycicum was expressed as IC50. The IC50 value was defined as the concentration (in mg/mL) of the extract that inhibit the formation of DPPH radicals by 50%. The IC50 value was calculated from the data obtained using GraphPad Prism software. The DPPH assay was repeated four times for each concentration.

2.5. Antimicrobial activity

Antimicrobial activity test was performed on the B, D, E extracts obtained from aerial parts of H. chasmolycicum and also on the pure compounds 3,5-dihydroxy-6,7,8-trimethoxyflavone and kaempferol 3-O-glucoside.

Antimicrobial activity against Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 4352, Pseudomonas aeruginosa ATCC 27853, Proteus mirabilis ATCC 14153 and Candida albicans ATCC 10231 were determined by the microbroth dilutions technique using the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI, 2002, 2006). Mueller–Hinton broth for bacteria, RPMI-1640 medium buffered to pH 7.0 with MOPS for yeast strain were used as the test medium. Serial two-fold dilutions ranging from 5000 µg/mL to 4.9 µg/mL were prepared in medium. The inoculum was prepared using a 4–6 h broth culture of each bacteria and 24 h culture of yeast strains adjusted to a turbidity equivalent to a 0.5 McFarland standard, diluted in broth media to give a final concentration of 5×10^3 cfu/mL for bacteria and 0.5×10^6 to 2.5×10^3 cfu/mL for yeast in the test tray. The trays were covered and placed in plastic bags to prevent evaporation. The trays containing Mueller–Hinton broth were incubated at 35 °C for 18–20 h and the trays containing RPMI-1640 medium were incubated at 35 °C for 46–50 h. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth. As control, antimicrobial effects of the solvents were investigated against test microorganisms. According to values of the controls, the results were evaluated. All the microorganisms were obtained from American Type Culture Collection (ATCC), Manassas (VA), USA.

Ciprofloxacin was used as positive control for tested bacteria whereas fluconazole was used as positive control for yeast. Each treatment was replicated three times.

3. Results and discussion

Twelve known flavonoids were isolated from the aerial parts of H. chasmolycicum and shown in Table 1. H. chasmolycicum flavonoid content has been found different from the other Anatolian Helichrysum species in comparison. The helichry- sins, characteristic Helichrysum flavonoids, were not detected in this species. The flavonoid composition of H. chasmolycicum appears to be similar to H. compactum in their major flavonoids (3,5-dihydroxy-6,7,8-trimethoxyflavone, kaempferol 3-O-glucoside) (Süzgeç et al., 2005).

The results from this study are in accordance with previous biological activity reports on the genus Helichrysum (Albayrak et al., 2010; Cushnie and Lamb, 2005). The compounds responsible for the antimicrobial and antioxidant activity of H. chasmolycicum are possibly the flavonoids found in the aerial parts.

The methanol extract of aerial parts of H. chasmolycicum possessed DPPH radical-scavenging activity with an IC50 value of 0.92 mg/mL.
The flavonoid compounds isolated from aerial parts of *H. chasmolycicum*.

<table>
<thead>
<tr>
<th>Flavonoid compounds</th>
<th>Exports</th>
<th>Flavonoid amounts (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>D</td>
<td>6</td>
</tr>
<tr>
<td>Luteolin</td>
<td>D</td>
<td>5</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>D</td>
<td>3</td>
</tr>
<tr>
<td>3,5-dihydroxy-6,7,8-trimethoxyflavone</td>
<td>B</td>
<td>12</td>
</tr>
<tr>
<td>3,5-dihydroxy-6,7,8,4′-tetramethoxyflavone</td>
<td>B</td>
<td>7</td>
</tr>
<tr>
<td>Apigenin 7-O-glucoside E</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Apigenin 4′-O-glucoside E</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Luteolin 4′-O-glucoside E</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Luteolin 4′,7-O-diglucoside E</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Kaempferol 3-O-glucoside E</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Kaempferol 7-O-glucoside E</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Quercetin 3-O-glucoside E</td>
<td>10</td>
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</tr>
</tbody>
</table>

Extracts: B (petroleum ether–60% ethanol-chloroform extract); D (ethanol-chloroform extract); E (ethanol–ethyl acetate extract).

In a previous study, it was reported that the methanolic extracts of 16 Helichrysum species collected from Turkey were investigated for their antioxidant activity by DPPH method and also antimicrobial activities except *H. chasmolycicum*. When compared with the antioxidant activity results which were done according to DPPH method, the other Helichrysum species showed more activity than *H. chasmolycicum*.

The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Ćetković et al., 2008; Hernández et al., 2009). The main phenolic compounds of the genus *Helichrysum* are flavonoids which have remarkable antioxidant activity (Czinner et al., 2000). Based on this report, antioxidant potential of the extract studied here could be attributed to flavonoids.

The antimicrobial activity results are shown in Table 2. The antimicrobial activity of extracts from *Helichrysum* species have been widely reported (Çoşar and Çubukcu, 1990; Meyer and Afolayan, 1995; Rios et al., 1991; Tomás-Barberan et al., 1990). The antibacterial activity has been demonstrated for a number of *Helichrysum* species as *H. armenium* subsp. *armenium*, *H. pallasi*, *H. graveolens*, *H. orientale*, *H. plicatum* subsp. *plicatum* and their methanol extracts were found to be active against *Aeromonas hydrophila*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Bacillus brevis*, *B. cereus*, *B. subtilis*, *S. aureus*, by the agar-well diffusion method in a previous study. Also methanol extracts of these species have showed antifungal activity against *C. albicans* (Albayrak et al., 2010).

In our previous study the extracts D and E of *H. compactum* showed antibacterial and antifungal activity with a MIC value of 40 μg/mL against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* by the serial dilution method (Süzgeç et al., 2005).

In this study, extracts B and E from *H. chasmolycicum* showed moderate antibacterial and antifungal activity against *P. aeruginosa* and the yeast *C. albicans*, while no activity was observed against *S. aureus* and *E. coli*, *K. pneumoniae* or *P. mirabilis* for any of the extracts. Extract D didn’t show antibacterial activity against any of the organisms tested. Extract E showed antibacterial activity against *P. aeruginosa* with a MIC value of 625 μg/mL. But kaempferol 3-O-glucoside which is isolated from E extract has no activity against all microorganisms tested. The flavonoid glucosides or other compounds could be responsible for the antimicrobial activity of extract E.

Both extract B and its major flavonoid compound 3,5-dihydroxy-6,7,8-trimethoxyflavone showed antifungal activity against *C. albicans* with a MIC value of 312 μg/mL. Afolayan and Meyer (1997) reported that galangin (3,5,7-trihydroxy-flavone) possessed antimicrobial activity and the antimicrobial property of galangin might be associated with its lack of hydroxyl groups on the B ring. Also our study, the antimicrobial property of 3,5-dihydroxy-6,7,8-trimethoxyflavone might be associated with its lack of hydroxyl groups on the B ring as galangin. On the other hand, the other flavonoid compounds were isolated in a limited amount in *H. chasmolycicum* and could not be tested antimicrobial activity in this study.

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