Antimicrobial activity of *Helichrysum plicatum* DC.

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Abstract

Dry flower heads of *Helichrysum plicatum* were characterized by HPLC-DAD and a detailed antimicrobial assay of its ethanol extract was performed. Identification of phenolic compounds indicated the presence of apigenin, naringenin and kaempferol as free aglycones, glycosides of apigenin, naringenin, quercetin and kaempferol as well as chlorogenic acid and chalcone derivate. Antimicrobial activity of the extract was evaluated against various bacteria and fungi as well as yeast *Candida albicans* using microdilution method. Gram-positive bacteria were more sensitive to the tested extract (*MIC* values were to 0.02 mg/mL) than Gram-negative bacteria (the greatest *MIC* was 0.055 mg/mL). Regarding pathogenic fungi, our tests demonstrated that fungi were more sensitive to the tested extract than bacteria. The growth of the majority of the tested fungi was inhibited by concentration of 0.005 mg/mL. Moreover, the extract was significantly more active than commercial fungicide, fluconazole. The results of our tests indicate that the extract of *H. plicatum* has significant antimicrobial activity and may find application in the pharmaceutical and food industry and organic agriculture.

Keywords: *Helichrysum plicatum*, antimicrobial activity, naringenin, kaempferol, apigenin.

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Helichrysum plicatum DC. Prodr. is perennial plant which belongs to the section *Helichrysum* of the genus *Helichrysum* (fam. Asteraceae) which comprises approximately 500–600 species [1,2]. It is native to Balkan Peninsula (Serbia, FYR Macedonia, Albania and Greece), Anatolian Peninsula (Turkey, Armenia and Caucasus) and Iran [3]. Its distribution in Serbia is limited only to southern regions (Metohija, Bosilegrad and Rudina Mountain) [4]. This species can be distinguished from sandy everlasting, widely used in traditional medicine, *H. arenarium* (L.) Moench, by its densely glandular stem and leaves [5]. According to the literature data, plants from this genus are widely used in traditional medicine for decoctions and infusions due to their choleretic and cholagogue activities, and moreover for stimulation of the secretion of gastric juice [6]. Also, some members of this genus are known for their anti-inflammatory and anti-allergic properties [7]. These properties are mainly attributed to the presence of phenolic compounds [8–11]. Chemical profile of *H. plicatum* has been previously described in detail and it is characterized by the presence of apigenin, naringenin, kaempferol, quercetin and luteolin glycosides as well as free apigenin, naringenin, kaempferol and luteolin [12,13]. The plant has been used in Serbian and FYR Macedonian traditional medicine for treatment of gastric and hepatic disorders [12]. Furthermore, antidiabetic, antioxidant, antibacterial [14–16], and spasmolytic activities [17] of this species were also reported. However, only few studies have been undertaken to investigate the antimicrobial activity of *H. plicatum* [18].

The aim of the present study was to estimate antimicrobial activity of *H. plicatum* ethanol extract on ten bacteria and nine fungal strains, together with one yeast strain.

EXPERIMENTAL

Material

Plant material and extract preparation

Air-dried raw material (flower heads) of *H. plicatum* was purchased from a commercial supplier (Agroherbal, Albania), in July 2015. Flower heads of *H. plicatum* were extracted with aqueous ethanol (1:1) by triple percolation, at room temperature, DER 1:5 *m*/*m*. The water–ethanol extract was concentrated under reduced pressure at 80 °C, to one third of its volume. After cooling at 8 °C, the water extract was decanted and re-extracted with ethyl acetate-ethanol (96%, 9:1) and then the extract was evaporated under vacuum at 60 °C to obtain a yellow-orange dried powder.

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Chemical profile of extract

Obtained dry extract (100 mg) was subjected to acid hydrolysis with H_2SO_4 (20 mL, 5 mass%) in a water bath for 2 h, with reflux condenser. After heating, solution was cooled and neutralized with 10% $NaHCO_3$ solution to pH 7.0. Methanol from neutral solution was evaporated under vacuum at 50 °C and obtained solution re-extracted with diethyl ether (tree times per 50 mL). Diethyl ether extract was evaporated to dry extract and dissolved in 10 mL of methanol and prepared for determination of flavonoid aglycones (naringenin, apigenin and kaempferol). The quantification of flavonoid aglycones (naringenin, apigenin and kaempferol) in hydrolyzed extract was carried out using a high-performance liquid chromatography with diode-array detection (HPLC-DAD) method. Analyses were carried out on Agilent 1200 RR with DAD detector, on a reverse phase Lichrospher RP-18 analytical column 250 mm×4 mm i.d., particle size 5 µm (Agilent). Mobile phase A (H_2O containing 1% H_3PO_4) and B (MeCN), elution by gradient according to the following scheme: 70% A 0–18 min, 70–30% A 18–25 min, 30–0% A 25–30 min, 0% A 30–35 min. The injection volume was 10 µL, the flow rate 1mL/min, and detection was at 260 and 340 nm. Naringenin, apigenin and kaempferol standards were purchased from Sigma, purity 95%, ≥ 95% and ≥ 96%, respectively.

Determination of the antimicrobial activity

Microbial strains

The antimicrobial activities of the *H. plicatum* ethanol extract were tested against bacteria (ATCC collection) and fungi isolated and identified from some medicinal plants. The antibacterial activity was tested against the Gram-negative bacteria *Escherichia coli* (ATCC 35218), *Salmonella typhimurium* (ATCC 13311), *Salmonella enteritidis* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 29906) as well as against the Gram-positive bacteria *Bacillus subtilis* (ATCC 10907), *Staphylococcus aureus* (ATCC 29213), *Micrococcus luteus* (ATCC 10240), *Micrococcus flavus* (ATCC 14452), and *Listeria monocytogenes* (ATCC 15313). Furthermore, to assess the broader biological activity of the extract, we have included in examination yeast *Candida albicans* (ATCC 10231) and nine fungi *Fusarium solani*, *F. subglutinans*, *F. equiseti*, *F. verticillioides*, *Curvularia lunata*, *Aspergillus flavus*, *Chaetomium sp.*, *Alternaria alternata* and *Penicillium sp*.

Microdilution test

Antimicrobial activity of extract was tested with a modified version of the microdilution technique described by Hanel and Raether [19] and Daouk *et al*. [20] by determining the minimum inhibitory concentrations (*MIC*s). Briefly, for the determination of *MIC* serial dilutions of the stock solutions of the extract (dissolved in DMSO) in broth medium (Mueller–Hinton broth for bacteria and Sabouraud broth for yeast and fungi) were prepared in a microtiter plate (96 wells). For antibacterial activity, 10 µL of each bacteria suspension (concentration 10^6 CFU/ml) was added to wells and 10 µL of resazurin solution. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 48 h. *MIC* was defined as the lowest concentration of the tested plant extract that inhibited the visible growth of microorganisms after incubation, prevented resazurin color change from blue to pink.

To investigate the antifungal activity of extract, a modified version of the microdilution technique was used [19,21]. Fungal spores were washed from the surface of malt agar (MA) plates (malt extract, 30 g; peptone, 3 g; agar, 15 g). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The microplates were incubated for 72 h at 28 °C. The lowest concentration without visible growth was defined as the minimal concentration, which completely inhibited fungal growth (*MIC*). The standard antibiotic steptomycin (1 mg/mL DMSO) was used to control the sensitivity of tested bacteria, whereas fluconazole (1 mg/mL DMSO) was used as a control against fungi. Microdilution technique used for estimation of the inhibitory activity of extract on yeast *Candida albicans*, was exactly the same as for fungal strains.

RESULTS AND DISCUSSION

Chemical profile of extract

HPLC chromatogram of the flavonoid compounds in *H. plicatum* extract is shown in Figure 1.

Flavonoid peaks were identified by comparing their UV absorption spectrum and retention time with those of commercial standards. The main constituents of the *H. plicatum* flowers were glycosides of naringenin, apigenin, kaempferol and quercetin which gave corresponding aglycones after hydrolysis [13,17].

Three major flavonoid aglycones (naringenin, kaempferol and apigenin) were analyzed after acid hydrolysis of the corresponding glycosides. The HPLC chromatogram of flavonoid aglycones in *H. plicatum* extract is shown in Figure 2.

The obtained hydrolyzed extract contained large amounts of three flavonoid aglycones, and naringenin was the most abundant (521.1 mg/g), followed by kaempferol (123.05 mg/g) and apigenin (69.3 mg/g). Flavonoid profile of *H. plicatum* in our study is in

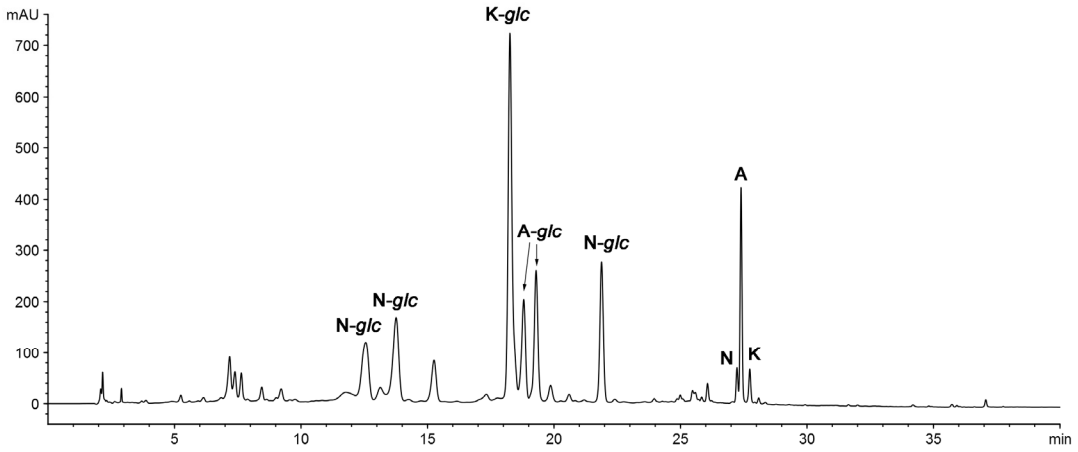

Figure 1. HPLC-DAD chromatogram of Helichrysum plicatum non-hydrolyzed ethanol extract; N-glc, K-glc, A-glc – glycoside derivatives of naringenin, kaempferol and apigenin, respectively; N, K, A – aglycones of naringenin, kaempferol and apigenin, respectively.

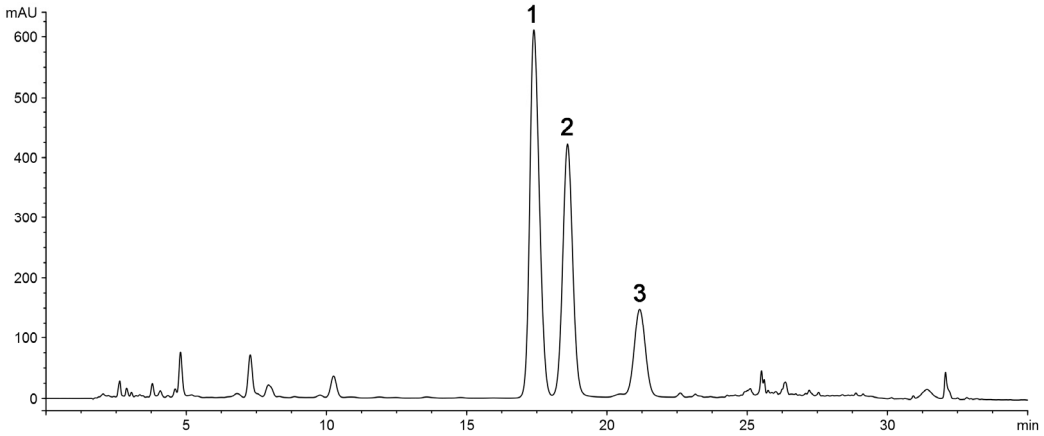

Figure 2. HPLC profile of H. plicatum hydrolyzed extract (260 nm). Aglycones peaks: 1, naringenin; 2, apigenin; 3, kaempferol.

accordance with the previous report, however, quantitative differences exist [12]. Kulevanova *et al.* [12] reported that kaempferol was the most abundant, and naringenin and apigenin were present in same quantities in hydrolyzed extract they analyzed. This discrepancy could be the result of different extraction procedure or different origin of the starting plant material. Hydrolyzed extract was produced and analyzed only for the purpose of quantification of main flavonoid components. For antimicrobial assay non-hydrolyzed extract of the *H. plicatum* inflorescence was used.

Antimicrobial activity

For the assessment of the antimicrobial activity of the extract, microdilution method in microtiter plates with system of 96 wells was used. Extract was dissolved in dimethylsulfoxide (DMSO) and serial dilutions were tested. The results of the antimicrobial activity are given in Tables 1 and 2.

The *MIC* values against bacteria growth were between 0.01 and 0.055 mg/mL of the extract (Table 1). Gram-positive bacteria (*B. subtilis*, *L. monocytegenes*, *M. flavus*, *M. luteus*) were more sensitive to the tested

Table 1. Minimum inhibitory concentration of Helichrysi flos extract (mg/ml)

Microorganism	*Helichrysi flos* extract	Antibiotic streptomycin
Escherichia coli	0.055	0.005
Salmonella typhimurium	0.050	0.038
Salmonella enteritidis	0.045	0.038
Staphylococcus aureus	0.030	0.005
Pseudomonas aeruginosa	0.050	0.016
Proteus mirabilis	0.030	0.005
Bacillus subtilis	0.010	0.005
Micrococcus flavus	0.015	0.005
Micrococcus luteus	0.015	0.016
Listeria monocytogenes	0.020	0.016
Candida albicans	0.020	0.005

extract than Gram-negative bacteria, *i.e.*, their growth was completely inhibited at the lowest tested concentrations. Soil bacteria *B. subtilis* was the most sensitive bacteria. For the inhibition of Gram-negative bacteria growth, higher concentrations of extract were needed and the most resistant was *E. coli* (*MIC* 0.055 mg/mL).

The majority of the tested fungi were sensitive to *H. plicatum* extract, with MIC value of 0.005 mg/mL (Table 2). The obtained results indicated that fungi were more sensitive to *H. plicatum* extract then bacteria. Also, extract of *H. plicatum* was significantly more active than commercial fungicide, fluconazole.

Table 2. Minimum inhibitory concentration of Helichrysi flos extract MIC (mg/ml)

Fungi	*Helichrysi flos* extract	Antimicotic fluconazole
Fusarium solani	0.005	1.8
Fusarium subglutinans	0.040	1.6
Fusarium equiseti	0.005	1.0
Fusarium verticillioides	0.005	1.4
Curvularia lunata	0.010	1.0
Aspergillus flavus	0.040	1.8
Chaetomium sp.	0.040	2.0
Alternaria alternata	0.005	1.6
Penicillium sp.	0.005	1.8

F. subglutinans, *A. flavus* and *Chaetomium* sp. showed higher resistance to tested extract. However, obtained results point to an important fact that for the inhibition of fungal growth, which are producers of various mycotoxins, particularly aflatoxins (*A. flavus*), are sensitive at low concentrations of tested extract.

Our results are in accordance with the literature data. Albayarak *et al*. [22,23] examined sixteen extracts of plants of the genus *Helichrysum*, including *H. plicatum subsp. plicatum* and *H. plicatum subsp. polyphyllum*. The results of this study showed that *H. plicatum subsp. plicatum* exhibited an antibacterial effect against Gram-positive bacteria *B. subtilis* and *S. aureus*. The strongest activity of all examined species of *Helichrysum* was against *C. albicans*, while on the *E. coli* was not effective. On the other hand, *H. plicatum subsp. polyphyllum* was more effective on *E. coli*, and less effective on *B. subtilis*, while other antimicrobial activities were similar. *H. arenarium subsp. aucher* exerted a stronger effect on Gram-negative bacteria (mild antimicrobial activity on *E. coli* and stronger activity on *P. aeruginosa*) comparing to gram positive bacteria (no effects on *B. subtilis* and *S. aureus*), and less effect on *C. albicans*. Stanojković *et al*. [24], reported that extracts of *Helichrysum arenarium* and *Achillea millefolium* (Asteraceae) showed the best antimicrobial activity in terms of inhibiting the growth of tested bacteria. Furthermore, in the same report, the most resistant bacteria on inhibitory activity of the extract was coliform bacteria *E. coli*, while the most sensitive was *B. subtilis*. The obtained results indicate that strong antibacterial and antifungal potential *H. plicatum* extract might be related to the presence of flavonoids. It is known that these compounds exert a strong antimicrobial potential against bacteria and fungi as well [25–27].

We can assume that a good antimicrobial activity of plant extracts examined in our study is result of the activities of the dominant components or of their synergies. Previously published reports demonstrated that flavonoids naringenin and apigenin, identified in the examined extract of *H. plicatum*, demonstrated strong inhibitory activity on the growth of both bacteria and fungi [16,25]. According to Andrade *et al*. [28] and Mandalari *et al*. [29] naringenin exhibited inhibitory effect (*MIC* from 0.25 to 2 mg/mL) to *Escherichia coli*, *Salmonella enterica*, *S. typhimurium*, *Pseudomonas putida*, *Bacillus subtilis*, *Listeria innocua*, *Lactococcus lactis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Saccharomyces cerevisiae*, while apigenin strongly inhibited *Staphylococcus epidermidis*, *S. aureus*, *E. coli*, *S. typhimurium* and *Stenotrophomonas maltophilia* [30].

According to some studies it has been shown that naringenin exerts an antibacterial effect through the impact on the alteration of cytoplasmic membrane fluidity in hydrophilic and hydrophobic regions, suggesting that it reduced the fluidity of outer and inner layers of membranes of bacterial cells. Another group of researchers has indicated that naringenin significantly inhibited bacterial motility that is thought to be important in virulence as they guide bacteria to their sites of adherence and invasion [30].

Ohemeng *et al.* [31] screened apigenin for inhibitory activity against DNA gyrase, an enzyme involved in DNA synthesis.

The obtained results, along with published data, indicate that the extract of *H. plicatum* possess significant antimicrobial activity and may find application in the food industry as a natural preservative to suppress growth of bacteria that causes food spoilage, such as *L. monocytogenes* or *S. typhimurium*. In Russia, dry extract of *H. arenarium* is already in use in the form of granules in preparation called “arenarin”, which has application in the suppression of phytopathogenic bacteria [24].

Considering that extract of *Helichrysum plicatum* tested in this study showed also a strong antifungal potential against phytopathogenic fungi isolated and identified in dried medicinal herbs (so-called drugs), one possibility is the use of this extract in the protection of medicinal plants against plant pathogenic fungi as biological control. Biological control involves the use

of natural origin products, plant extracts and essential oils, as well as useful microorganisms and their extracts in plant protection [32]. From this aspect, it is possible to apply extract of *Helichrysum plicatum* in combined treatment of the soil where the plant will grow in combating soil phytophatogenic microorganisms, and with foliar spraying. Also, extracts and essential oils of some medicinal plants are already in use for protection and prevention of decay of fruit and vegetables by phytopathogenic fungi in the warehouse where they are stored [33,34].

CONCLUSION

The results of the present study indicate a promising antimicrobial potential of *H. plicatum* extract which inhibited the growth of the tested bacteria, yeasts and fungi with *MIC* values ranging from 0.005 to 0.055 mg/mL. In that sense, this extract could find its application in food industry as a natural preservative to suppress growth of bacteria. Pathogenic fungi were more sensitive than bacteria to the extract of *H. plicatum* and it could be recommended for combat plant pathogenic fungi as biological control, for soil sterilization and against phytopathogenic fungi in the warehouses.

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IZVOD

ANTIMIKROBNA AKTIVNOST *Helichrysum plicatum* DC.

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(Naučni rad)

Etanolski ekstrakt osušenih cvetnih glavica *Helichrysum plicatum* je okarakterisan tehnikom HPLC-DAD i detaljno ispitivan na antimikrobnu aktivnost. Identifikacija fenolnih jedinjenja ukazuje na prisustvo apigenina, naringenina i kemferola, u obliku slobodnih aglikona, kao i glikozda apigenina, naringenina, kvercetina i kemferola zajedno sa hlorogenskom kiselinom i halkonskim derivatima. Antimikrobna aktivnost ekstrakta ispitivana je mikrodilucionom metodom na različitim bakterijama i gljivama, kao i na kvascu *C. albicans*. Gram-pozitivne bakterije su bile osetljivije na testirani ekstrakt od Gram-negativnih bakterija. Njihov porast je u potpunosti inhibiran pri nižim testiranim koncentracijama od 0,01 do 0,02 mg/mL. U pogledu osetljivosti patogenih gljiva, naši testovi su pokazali da su gljive osetljivije na testirani ekstrakt od bakterija. Porast većine ispitivanih gljiva je inhibiran pri koncentraciji od 0,005 mg/mL. Štaviše, ekstrakt je bio značajno aktivniji od komercijalnog fungicida flukonazola. Rezultati naših ispitivanja ukazuju da ekstrakt *H. plicatum* ima značajnu antimikrobnu aktivnost i može naći primenu u farmaceutskoj industriji, industriji hrane i organskoj poljoprivredi.

Ključne reči: *Helichrysum plicatum* • Antimikrobna aktivnost • Naringenin • Kemferol • Apigenin