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PHYTOCHEMICAL SCREENING OF *Ceratophyllum demersum* L. EXTRACTS AND EVALUATION OF THEIR ANTIOXIDATIVE AND ANTIMICROBIAL POTENTIALS

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ABSTRACT. Phytochemical screening, antioxidant and antimicrobial activities of water, ethanol and ethyl-acetate extracts of *Ceratophyllum demersum* L. were tested. In order to define a chemotype of *C. demersum* chemical composition of ethyl-acetate extract was determined. The major constituents of the ethyl-acetate extract are: hexahydrofarnesyl acetone (15.8%), ethyl hexadecanoate (11.7%), 1-hexadecanol (10.5%), neophytadiene (isomer I) (10.2%), (*E*)-Phytol (8.8%) and (*Z*)-9-Octadecen-1-ol (=Oleic alcohol) (7.0%). Antioxidant activity, which were determined by the DPPH free radicals assay and the ferric reducing antioxidant power (FRAP) showed weak antioxidant activity. Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) have been determined by microdilution method. The tested extracts showed stronger inhibitory effects against Gram positive than Gram negative bacteria, especially against *Bacillus subtilis* for the ethyl-acetate extract. Ethyl-acetate and ethanol extracts showed better antifungal than antibacterial activity, especially against *Penicillium digitatum* and *Aspergillus restrictus*. The most effective extract was ethyl-acetate, and it should be investigated further.

Keywords: aquatic plant, phytochemical screening, antioxidant, antimicrobial activity.

INTRODUCTION

Ceratophyllum demersum L. (*Ceratophyllaceae*), one of the important primary producers of aquatic ecosystems, is a cosmopolitan hydrophyte that lives completely under water (submerged), floats freely in the water column, does not produce roots, and can form denscovers just below the surface. It is found in slow flowing waters (TAS *et. al.*, 2024).

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In recent years, activities of the extracts of *C. demersum* have been studied, to some extent. The macrophyte *C. demersum*, sampled from Skadar Lake in Montenegro has been used as a bioindicator. The methanolic extract of this plant exhibited significant efficacy against all bacterial strains tested and the phytochemical analysis revealed the presence of a variety of secondary metabolites (LONE *et. al.*, 2023). There are differences in the sequences of the metal content in the plant, compared to the sequences of their bioaccumulation (BCF) ability. BCF for Mn is several times higher compared to other metals (KASTRATOVIĆ *et. al.*, 2014.). *In vitr*o free radical scavenging potential of aqueous and methanol extracts from *in vitro* proliferated *C. demersum* utilizing DPPH were researched (AWATI *et al*., 2020). This plant exhibits antioxidant properties due to the bioactive compounds it contains (ELIAŠOVÁ *et al*., 2021). The antimicrobial activity of the *C. demersum* were tested with three different extracts like acetone, butanol and methanol and determined antibacterial and antifungal activity (MALATHY and STANLEY 2015).

The present study elucidated phytochemical screening, antioxidant and antimicrobial activities of water, ethanol and ethyl-acetate extracts of *Ceratophyllum demersum* L. In this way, we wanted to complete the recent research of this plant.

MATERIALS AND METHODS

Chemicals

Organic solvents (methanol, ethanol, ethyl acetate) and concentrated hydrochloric acid (HCl) were purchased from Zorka Pharma (Šabac, Serbia). 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and Folin-Ciocalteu phenol reagent were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Gallic acid, rutin hydrate, aluminium chloride hexahydrate, Na_2HPO_4, NaH_2PO_4 and trichloroacetic acid (TCA) were purchased from Acros Organics (New Jersey, USA). Sodium carbonate (Na_2CO_3) was obtained from MP-Hemija (Belgrade, Serbia) while anhydrous magnesium sulfate was from Aldrich (USA). Dimethyl sulfoxide (DMSO), n-butanol, ferric chloride ($FeCl_3$), potassium ferricyanide ($K_3Fe(CN)_6$) and ferric ammonium sulfate ($NH_4Fe(SO_4)_2$ x $12H_2O$) were purchased from Centrohem (Stara Pazova, Serbia). Resazurin was obtained from Alfa Aesar GmbH & Co. (KG, Karlsruhe, Germany). Nutrient agar, Mueller-Hinton and Sabouraud dextrose broth were purchased from Liofilchem (Italy). Doxycycline and fluconazole were from Galenika A.D. (Belgrade, Serbia) and crystal violet stain was from Fluka AG (Buchs, Switzerland).

Plant materials

C. demersum was collected in April 2013, from the region of Ovčar-Kablar Gorge, in the Western Serbia, part of the composite valley of the West Morava River. Identification and classification of the plant material was performed at the Faculty of Science, University of Belgrade. Voucher specimens (16756) are deposited in Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU) (THIERS, 2013). The collected plant material (stems and leaves) was air-dried in darkness at an ambient temperature of (20°C). The dried plant material was cut up and stored in paper bags until needed.

Preparation of plant extracts

Dried, ground plant material was extracted by maceration with water, ethyl acetate and ethanol. Briefly, 50 g of plant material was soaked with 250 mL of solvent. The plant material was macerated three times at room temperature using fresh solvent every 24 h. The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40° C.

The amount of extract is determined by the difference in the weight of the bottle with the extract and the empty bottle. The amounts of crude extracts are presented in Table 1. The obtained extracts were kept in sterile sample tubes and stored at 4° C. The results were statistically evaluated with Microsoft Excel.

Phytochemical screening

The chemical composition of the extract was investigated by GC and GC-MS. The GC-MS analyses were performed in triplicate on a Hewlett-Packard 6890N gas chromatograph equipped with a DB-5 phenyl methyl siloxane capillary column (30 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5975C mass selective detector from the same company. The injector and interface were operated at 250 and 320° C, respectively. Oven temperature was raised from 70 to 315° C at a heating rate of 5° C/min and then isothermally held for 10 min. As a carrier gas, helium at 1.0 mL/min was used. The samples (1 μL of the extract solutions) were injected in a pulsed split mode (split ratio 40:1). The MS conditions were as follows: ionization energy 70 eV, acquisition mass range 35–650 amu and scan time 0.34 s. GC-FID analysis was carried out under the same experimental conditions using the same column as described for the GC-MS.

The extract constituents were identified by comparison of the their linear retention indices (determined relative to the retention times of C_8–C_{29} *n*-alkanes on the DB-5 column (VANDENDOOL and KRATZ, 1963) to those reported in the literature (ADAMS, 2007), and by comparison of their mass spectra to those of authentic standards, as well as those from Wiley 6, NIST05 and MassFinder 2.3 libraries. Also, a homemade MS library with the spectra corresponding to pure substances was used, and finally, wherever possible, the identification was achieved by coinjection with an authentic sample. The percentage composition was computed from the GC-FID peak areas without the use of correction factors.

The total phenolic content was determined using Folin-Ciocalteu method (WOOTTON-BEARD *et. al.*, 2011) The reaction mixture was prepared by mixing 0.2 mL of methanolic solution of extracts (1 mg/mL) and 1.5 mL of 10% Folin-Ciocalteu reagent dissolved in water. The mixture was allowed to equilibrate for 5 min and then it was mixed with 1.5 mL 6% Na_2CO_3 solution. After, being incubated for 90 min at room temperature and in darkness, the absorbance of the mixture was read at 725 nm against a blank using spectrophotometer. The blank was prepared with methanol instead of extract solution. The samples were prepared triplicate, and the mean value of absorbance was obtained. The same procedure was repeated for gallic acid which was used for calibration of standard curve. Total phenol content is reported as gallic acid equivalent by reference to linear equation of the standard curve. Then the total phenolic content was expressed a miligram of gallic acid equivalent per gram of extract (mg GAE/g of extract).

The concentrations of flavonoids were established by using aluminum chloride method (QUETTIER-DELEU *et. al.*, 2000). The mixture contained 2 mL of methanolic solution of extract (1 mg/mL) and 2 mL of 2% methanolic $AlCl_3x6H_2O$ solution. The mixture was vigorously shaken, and after 10 min of incubation at room temperature, the absorbance versus prepared blank was read at 430 nm using spectrophotometer. The samples were prepared triplicate, and the mean value of absorbance was obtained. Rutin was used as a standard for calibration of standard curve. The concentrations of flavonoids were calculated from the linear equation of standard curve. Then the concentrations of flavonoids were expressed as milligram of rutin equivalent per gram of extract (mg of RUE/g of extract).

Condensed tannins were determined by using the butanole HCl method (PORTER *et. al.*, 1986). The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of extract (1 mg/mL) with 3 mL of the butanol-HCl reagent (butanoleHCl 95:5 v/v) and 0.1 mL of the ferric reagent (2% ferric ammonium sulfate in 2N HCl). The covered tubes were heated in a water bath at 100° C for 60 min. After cooling the absorbance, versus a prepared blank

was read at 550 nm using spectrophotometer. The samples were prepared in triplicate, and the mean value of absorbance was obtained. Cyanidin chloride was used as a standard for calibration of standard curve. The concentrations of proanthocyanidins were calculated from the linear equation of standard curve. Then the concentrations of proanthocyanidins were expressed as milligram of cyanidin chloride equivalent per gram of extract (mg of CChE/g of extract).

DPPH radicals scavenging capacity assay

The ability of the plant extract to scavenge DPPH free radicals was assessed using the method (TAKAO *et. al.*, 1994), along with an antioxidant activity index (AAI) (PORTER *et. al.*, 1986). The stock solution of the plant extract was prepared in methanol to achieve the concentration of 2000 mg/mL. Further, two-fold dilutions were made to obtain concentrations from 1000 mg/mL to 15.6 mg/mL. Diluted solutions of extract (2 mL each) were mixed with 2mL of DPPH methanolic solution (80 mg/mL). After 30 min in darkness at room temperature, the absorbance was read in a spectrophotometer at 517 nm. The control samples consisted of 2 mL of methanol added to 2mL of DPPH solution. Ascorbic acid was used as a positive control. The experiment was performed in triplicate. Scavenging activity is expressed as the inhibition percentage calculated using the following equation:

$$\text{Scavenging activity (\%)} = 100 \times [(A_{control} - A_{sample}) / A_{control})]$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extract. The IC_{50} value is the effective concentration at which 50% of DPPH radicals were scavenged. It was obtained from the graph of scavenging activity (%) versus concentration of samples. Low IC_{50} value indicates strong ability of the extract to act as DPPH scavenger. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated using the following equation:

$$AAI = \text{final concentration of DPPH } (\mu g/mL) / IC_{50}\,(\mu g/mL)$$

The estimation of AAI was: if AAI < 0.5 - poor antioxidant activity; AAI > 0.5 - 1 - moderate antioxidant activity; AAI > 1 - 2 - strong antioxidant activity and AAI > 2 - very strong antioxidant activity.

Ferric reducing antioxidant power (FRAP)

Ferric reducing power of *C. demersum* extracts were determined using FRAP assay. This method is based on the reduction of colourless ferric complex (Fe^{3+}tripyridyltriazine) to blue-colored ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. Phosphate buffer was prepared by dissolving 2,76 g NaH_2PO_4 in 1000 mL of water (solution A) and 3.56 g Na_2HPO_4 in 1000 mL of water (solution B). Then was mixed 62.5 mL of solution A and 37.50 mL of solution B which was phosphate buffer. Potassium ferricyanide solution (1%, w/v) was prepared by dissolving 1 g $K_3Fe(CN)_6$ in 100mL water. Ferric chloride solution (0.1%, w/v) was prepared by dissolving 0.1 g of $FeCl_3$ in water and diluting to 100mL with H_2O. Trichloroacetic acid (TCA) solution (10%, w/v) was prepared by dissolving 10 g of TCA in water and diluting to 100 mL with H_2O.

Tested antioxidant solutions were prepared in methanol in the concentration of 1mg/mL; 0.5 mg/mL; 0.25 mg/mL; 0.125 mg/mL; 0.062 mg/mL. The same preparation procedure was for the positive control (vitamin C). 1 mL of a certain concentration of the test substance weighed and transferred to a test tube and added 1.25 mL of phosphate buffer and 1.25 mL of ferricyanide. The mixture was incubated at 50 °C in a water bath for 20 min. The incubated mixture was in to cool at room temperature, and 1.25 mL of TCA (10%) was added. Then the resulting mixture was pipetted 1.25 mL into a new tube and added 1.25 mL of

distilled water and 0.25 mL of $FeCl_3$. Read three absorbances of the room temperature-cooled analyte solutions at 700 nm and mean values were searched. Control (blank) was prepared in the same way as the tested sample, but the test substance was not added, but methanol (BENZIE and STRAIN, 1996, OU *et. al.*, 2002, MAGALHÃES *et. al.*, 2008).

Antimicrobial activities

Antimicrobial activities of water, ethyl-acetate and ethanol extract were tested against 15 microorganisms including seven strains of bacteria which are listed in Table 4. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Antimicrobial assay

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbiocidal concentration (MMC) using microdilution method with resazurin (SARKER *et. al.*, 2007). Microbial suspensions were prepared by direct colony method. The turbidity of initial suspension was adjusted by comparing it with 0.5 McFarland's standard on densitometer. Initial microbial suspensions contain about 10^8 colony forming units (CFU)/mL for bacteria and then 1:100 when diluted in sterile 0.85% saline. Twofold serial dilutions of plant extract were made in a concentration range of 5 mg/mL to 0.0195 mg/mL in sterile 96-well plates containing Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi. A 10 µl of diluted microbial suspension was added to each well to give a final concentration of 5 x 10^5 CFU/ mL. Finally, 10 µl of resazurin solution, as an indicator of microbial growth, was added to each well. The inoculated plates were incubated at 37° C for 24 h for bacteria, 28° C for 48 h for yeasts, and 28° C for 72 h for molds. MIC was defined as the lowest concentration of tested compounds that prevented resazurin color change from blue to pink. MMC was determined by plating 10 µl of samples from wells, where no indicator of color change was recorded, on nutrient agar. At the end of the incubation period, the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration.

Doxycycline and fluconazole were used as a positive control. A solvent control test was performed in order to study the effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Antibiofilm activity

The antibiofilm activity on *Escherichia coli* ATCC 25922*, Proteus mirabilis* and *Pseudomonas aeruginosa* ATCC 27853 was tested with the method described in O'TOOLE *et al.* (2000). In 96-well microtiter plates 50 µl of Mueller–Hinton broth was added. 50 µl of the tested extract was added into the first row of the plates, with an initial concentration of 10000 µg/ml, and serial two-fold dilutions were made down to 39 µg/ml. Subsequently, 10 µl of bacterial suspension (0.5 McFarland) was added into each well. The microtiter plates were then incubated for 48 hours at 37º C. The rest of the experiment was done as described in MURUZOVIĆ *et al.* (2016).

The difference in the procedure of measuring the effect on the formed biofilm is that varying concentrations of the extract were added to the microtiter plates with the biofilm formation of the tested bacteria. The results were expressed as biofilm inhibitory concentration, (BIC_{50}), defined as the lowest concentration of extract that showed 50%

inhibition on the biofilm formation (CHAIEB *et. al.*, 2011). All tests were performed in duplicate and BIC_{50} was calculated. Tetracycline was used as a positive control.

RESULTS AND DISCUSSION

Phytochemical screening

Qualitative and quantitative analysis of the ethyl-acetate extract of *C. demersum* are listed in Table 1. In this extract, 83.7% of compounds were identified. The major constituents of the extract are: hexahydrofarnesyl acetone (15.8%), ethyl hexadecanoate (11.7%), 1-hexadecanol (10.5%), neophytadiene (isomer I) (10.2%), (*E*)-Phytol (8.8%) and (*Z*)-9-Octadecen-1-ol (=Oleic alcohol) (7.0%).

Table 1. Volatile constituents of *C. demersum* ethyl-acetate extract.

RI calc.[a]	Compound	Content [%][b]	Identification Method
1103	Nonanal	t	RI,MS,Co-GC
1535	Dihydroactinidiolide	1.4	RI,MS
1700	Heptadecane	t	RI,MS,Co-GC
1705	Pristane	t	RI,MS
1772	Loliolide	t	MS
1809	Phytane	2.1	RI,MS
1842	Neophytadiene (isomer I)	10.2	RI,MS
1846	Hexahydrofarnesyl acetone	15.8	RI,MS
1866	Neophytadiene (isomer II)	1.4	RI,MS
1884	Neophytadiene (isomer III)	4.1	RI,MS
1885	1-Hexadecanol	10.5	RI,MS,Co-GC
1928	Methyl hexadecanoate	2.2	RI,MS,Co-GC
1996	Ethyl hexadecanoate	11.7	RI,MS,Co-GC
2068	(*Z*)-9-Octadecen-1-ol (=Oleic alcohol)	7.0	RI,MS
2088	1-Octadecanol	5.8	RI,MS
2106	γ-Hexadecalactone	t	RI,MS
2117	(*E*)-Phytol	8.8	RI,MS,Co-GC
2161	Unidentified component	5.8	
2167	Unidentified component	3.4	
2220	(*E*)-Phytyl acetate	t	RI,MS
2353	5-Methyl-5-(4,8,12-trimethyltridecyl)dihydro-2(3*H*)-furanone	2.7	RI,MS
2900	Nonacosane	t	RI,MS,Co-GC
	Identified	83.7	
	Unidentified (>0.5%)	9.2	
	Total	92.9	

[a] Linear retention indices experimentally determined on the DB-5 column; [b] Values are means of three individual analyses; RI – Retention indices matching with literature data; MS – mass spectra matching; Co-GC – co-injection with pure reference compound; [c] MS, 70eV, 230 ºC: RI 2161, *m/z* (rel. int.): 96 (37), 95 (69), 82 (42), 81 (96), 79 (47), 69 (34), 68 (36), 67 (100), 55 (61), 41 (48); RI 2167, *m/z* (rel. int.): 95 (54), 93 (26), 91 (26), 81 (52), 79 (100), 69 (21), 67 (69), 55 (38), 43 (20), 41 (33); t – Trace amounts (<0.05%).

Since there are various possibilities of the extraction of biological active plant compounds, we used different solvents (water, ethanol and ethyl-acetate) in this study. Concentrations of total phenols, flavonoids and tannins in extracts of *C. demersum* are shown in Table 2.

The phenolic compounds are secondary metabolites in plants and are considered as important natural molecules due to their bioactive properties. Phenolics are eliminators of free radicals and metal chelators. They can inhibit the lipid peroxidation and exhibit various physiological activities as antioxidants. In plants, these substances contribute to the defense mechanisms, and adaptation and pigmentation processes. Concerning human health, they have potential in the prevention and treatment of certain chronic diseases such as cardiovascular disease, diabetes and cancer (TAKÓ *et. al.*, 2020).

The total phenolic content in plant extracts of the species *C. demersum* depends on the type of extract, i.e. the polarity of solvent used in extraction (Table 1). Differences in total phenol, flavonoids content in ethanol extract, regarding to earlier research (MASLYC *et. al.*, 2024), are probably due to different ecological conditions.

Flavonoids have been known to be synthesized by plants in response to microbial infection, so it is not surprising that they have been found, *in vitro*, to be effective antimicrobial substances against a wide array of microorganisms (COWAN *et. al.*, 1999; STEFANOVIĆ *et. al.*, 2012;). Their activity is probably the result of their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Lipophilic flavonoids may also disrupt microbial membranes (STEFANOVIĆ *et. al.*, 2012). The concentration of flavonoids in various plant extracts of the species *C. demersum* was determined using spectrophotometric method with aluminum chloride. Ethyl-acetate extract contains the highest flavonoid concentration, 46.31 mg GA/g. In previous research, two flavonoid glycosides were isolated and one of them was identified as apigenin-7-O-glucoside (BANKOVA *et. al.*, 1995). The concentration of flavonoids in ethyl-acetate and ethanol extract are shown in table 2.

Tannins, a group of polymeric phenolic substances, are found in almost every plant part: bark, wood, leaves, fruits and roots. They are divided into two groups, hydrolysable and condensed tannins. In plant tissue, tannins have been synthesized and accumulated after microbial attack. Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, because of a property known as astringency (OJALA, 2001; STEFANOVIĆ *et. al.*, 2012). In determining the amount of tannins, a method with butanol-HCl reagent was used. The obtained results show that ethyl-acetate extract has a higher concentration of tannin than the ethanol extract (Tab. 2).

Table 2. Yield, total phenolic, flavonoid and tannin content in the extracts of *C. demersum.*

Type of extract	Yield (g/50 g dw)	Total phenolic content (mg GA/g)	Flavonoid content (mgRU/g)	Tannin content (mgCCh/g)
Water	1.44	11.03±0.83	/	/
Ethyl-acetate	0.49	26.39±0.13	46.31±0.08	6.96±0.05
Ethanol	1.17	11.23±0.13	43.62±0.09	2.99±0.03

Each value shown is the mean value standard deviation; / - not measured.

Antioxidant activity

Antioxidants are substances or nutrients which can prevent or slow oxidative damage by scavenging free radicals created in the body during standard metabolic functions or introduced from the environment. Free radicals take part in several health conditions such as

aging process, cancer, atherosclerosis, *etc.* Antioxidants might be present in plant sources such as vitamins, minerals, flavonoids, phenolics, and carotenoids, *etc* (LONE *et. al.*, 2023). The antioxidant activity of different plant extracts of *C. demersum* was determined using methanol solution of DPPH reagent. DPPH is a very stable free radical, unlike *in vitro* generated free radicals, such as hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metalion chelation and enzyme inhibition, brought about by various additives. Freshly prepared DPPH solution exhibited a deep purple color. This purple color generally fades/disappears when antioxidant molecules can quench DPPH free radicals (that is by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and converts them into a colorless/bleached product (that is 2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease of absorbance at 517 nm band (EMSEN *et. al.*, 2018; AWATI *et. al.*, 2021).

The antioxidant activity of plant extracts of *C. demersum* is expressed in terms of IC_{50} (mg/mL) values (Table 3). The obtained values for antioxidant activity examined by DPPH radical scavenging activity ranged from 10017.82 to 13662.6 µg/mL. Ethyl-acetate extract showed better antioxidant activity than ethanol extract determined using the methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. Antioxidant potential was demonstrated most noteworthy in ethanol extracts dependent on the test performed (AWATI *et. al.*, 2021).

Table 3. Antioxidant activity of the extracts from *C. demersum* (IC_{50} and AAI).

	IC_{50} [1]	AAI value
Ethyl acetate	10017.82±1960.219	0.13
Ethanol	13662.6±1433.83	0.08
Ascorbic acid	5.25	15.24

[1] value (µg/mL)

Results from Figure 1 point to a weak antioxidant activity of the extracts from *C. demersum* determined by the ferric reducing antioxidant power (FRAP). Y- Axis showed absorbances and X- Axis showed concentrations (µg/mL). Based on the obtained graphic it can be concluded that better antioxidant activity was showed by ethyl-acetate extract, and the lowest antioxidant activity was showed by water extract.

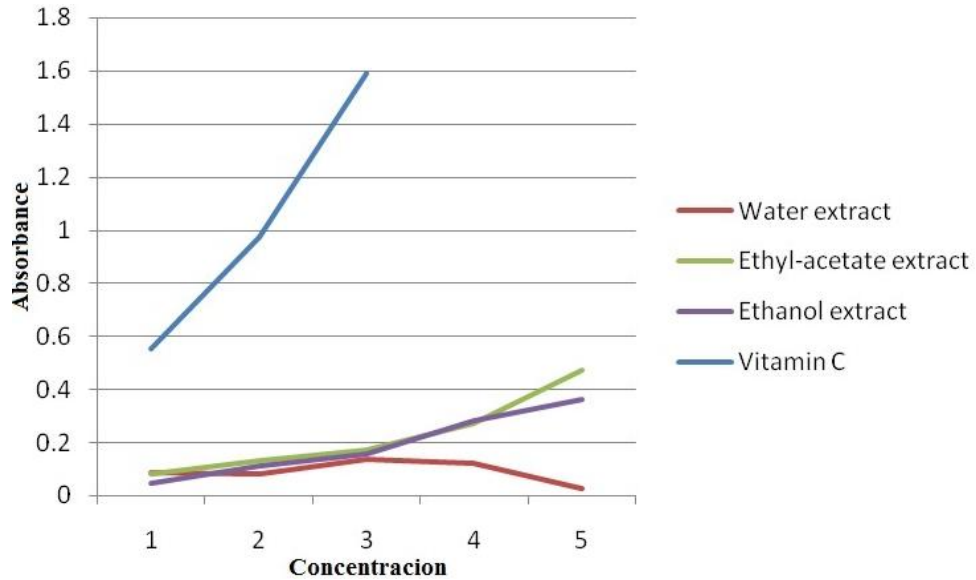

Figure 1. Antioxidant activity of the extracts from *C. demersum* determined by the ferric reducing antioxidant power (FRAP).

The higher concentrations of phenols, flavonoids and tannins were measured in ethyl-acetate extract (Table 2) than in other extracts and this may be the reason for better antioxidant activity. Results of antioxidant activity of ethanol extract confirmed previous research and showed low antioxidant activity, by the DPPH free radicals assay and the ferric reducing antioxidant power (FRAP) (KARTAL *et. al.*, 2009).

Antimicrobial activity

The results of *in vitro* antibacterial and antifungal activities of water, ethyl-acetate and ethanol extracts of *C. demersum* are shown in Table 4. For comparison, the tables also give the results of the activities of doxycycline and fluconazole. Antimicrobial activities of tested extracts were assessed by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values for 15 species of microorganisms. Efficacy of antimicrobial activity depends on the species of microorganism. The values were in the range of 0.312 to > 5 mg/mL. The tested extracts demonstrated selective and moderate antimicrobial activity and showed stronger inhibitory effects against G+ than G^- bacteria, especially against *Bacillus subtilis* for the ethyl-acetate extract. Water extracts showed generally weak antimicrobial activity. Ethyl-acetate and ethanol extracts showed better antifungal than antibacterial activity, especially against *Penicillium digitatum* for ethyl-acetate extract (0.312 mg/mL), and against *Aspergillus restrictus* for ethanol extract (0.312 mg/mL).

Table 4. Antimicrobial activity of water, ethyl acetate and ethanol extract of *C. demersum*.

Species	Water extract		Ethyl acetate extract		Ethanol extract		Doxycycline/ Fluconazole	
	MIC[1]	MMC[2]	MIC	MMC	MIC	MMC	MIC	MMC
Bacillus subtilis	>5	>5	0.625	0.625	5	>5	0.112	1.953
Staphylococcus aureus	>5	>5	1.25	>5	>5	>5	0.448	7.81
S. aureus ATCC 25923	>5	>5	2.5	>5	>5	>5	-	-
Proteus mirabilis ATCC 12453	>5	>5	>5	>5	>5	>5	-	-
Pseudomonas aeruginosa	>5	>5	>5	>5	>5	>5	62.5	125
P. aeruginosa ATCC 27853	>5	>5	>5	>5	>5	>5	250	> 250
Candida albicans ATCC 10231	2.5	>5	2.5	5	5	5	31.25	1000
Saccharomyces boulardii	5	>5	5	>5	5	>5	31.3	1000
Aspergillus restrictus	5	>5	2.5	5	0.312	1.25	500	2000
Aspergillus flavus	0.312	5	2.5	>5	0.625	5	1000	1000
Aspergillus niger	>5	>5	>5	>5	>5	>5	62.5	62.5
A. niger ATCC 16404	1.25	>5	0.625	5	5	>5	62.5	62.5
Penicillium digitatum	0.625	>5	0.312	0.312	1.25	5	-	-
P. italicum	>5	>5	5	5	5	5	1000	1000
P. verucosum	5	>5	5	>5	5	>5	-	-

[1]MIC values for plant extracts are given as mg/mL, for antibiotic as µg/mL – means inhibitory activity.
[2]MMC values for plant extracts are given as mg/mL, for antibiotic as µg/mL – means microbicidal activity.
"-" – not tested

Inhibition growth zone of ethanol extract was most effective against some strain *Bacillus subtilis* and *Bacillus cereus* (33±00 mm), and water extract against *Bacillus cereus* (28.00±0.01 mm) and *Pseudomonas aeruginosa* (24.00±0.003 mm). Ethanol extract showed inhibition growth zone against all tested organisms except *Aspergilus niger,* but water extract

showed very good inhibition growth zone against *Aspergilus niger* (53.00±0.003 mm) (FAREED *et. al.*, 2008). Comparing the research results, differences are probably due to different habitats, ecological conditions and various methods were used.

Antibiofilm activity

The examined strains (*Escherichia coli* ATCC 25922*, Proteus mirabilis* and *Pseudomonas aeruginosa* ATCC 27853) were non-biofilm-forming and biofilm inhibitory concentration of water, ethanol and ethyl-acetate extracts were irrelevant (>10 mg/mL expressed as BIC_{50}). Present results suggested the need for additional research into the use of this genre of compounds for biofilm containment.

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