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ANTIMICROBIAL, ANTIOXIDANT AND ANTIBIOFILM ACTIVITY OF EXTRACTS OF *MELILOTUS OFFICINALIS* (L.) Pall

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ABSTRACT

In this paper, the antioxidant and antimicrobial activity, the concentrations of total phenols, flavonoids, tannins and proanthocyanidins in the water, acetone, diethyl ether and ethanol extracts of *Melilotus officinalis* L. were analysed and their effect on the bacterial biofilm formation. The highest concentration of total phenols (36.25 mgGA/g) and tannins (21.25 mgGA/g) were detected in the water extract. The highest concentration of flavonoids (53.09 mgRU/g) was detected in the acetone extract. Proanthocyanidins were not detected in the water extract, while the highest concentration of these compounds was measured in the acetone extract (3.77 mgCChE/g). The antioxidant and reducing power of the *M. officinalis* extracts were measured by spectrophotometric method, and all results were compared to vitamin C and water extract of *Aronia melanocarpa*. The water extract showed the highest antioxidant activity, while the diethyl ether extract the lowest one. The extent of reducing power in the examined extracts was various. The water extract demonstrated the highest activity and the absorbance was from 0.03 to 0.68, while the lowest reducing power was demonstrated in the diethyl ether extract whose absorbance was from 0.04 to 0.20. *In vitro* antimicrobial activity was tested by microdilution method determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC). 25 microorganisms were examined, including 19 species of bacteria and 6 species of fungi. The extracts showed greater effect on G^+ bacteria than on the G^- bacteria. The acetone extract showed the highest antimicrobial activity. The acetone extract inhibit the biofilm formation of bacteria *Proteus mirabilis* and *Pseudomonas aeruginosa.*

Key words: *Melilotus officinalis*, antimicrobial activity, antioxidant, phenols, flavonoids, tannins, biofilm.

INTRODUCTION

Melilotus officinalis (L.) Pall., yellow sweet clover, belongs to the family Fabaceae (Tribe Trifolieae). The plant species is widespread, belonging to the Eurasian floral element. Its habitat is beside roads and fields, on walls, in vineyards, on infertile soils, especially on soils rich in quicklime. In some habitats, yellow sweet clover is considered to be an invasive species (Josifovic, 1972). This plant is known for its usage as a natural remedy in the form of a balm or an ointment. It is used as an ingredient of herbal mixtures made for compresses that are applied in order to accelerate the absorption (modifier of swelling) and open ulcers. It is, also, used for treating headaches and stomach pains. *M. officinalis* possesses a healing effect due to the presence of active substances such as coumarin 0.4-0.9%, dihydrocoumarin-melilotin, melilotozid, tannins, flavonoids, etc. (Sarić, 1989). Coumarin originated from this plant species is used in the pharmaceutical industry as an ingredient of anticoagulant drugs (Casley-Smith *et al.,* 1993).

According to the published literature data on the antioxidant activity of related species, it was noticed that the plants of the genus *Melilotus* possess antioxidant activity (Braga *et al.,* 2012). Studies showed that coumarin isolated from the water and ethanol extracts of *M. officinalis* demonstrated great antioxidant and anti-inflammatory effects; it removed free radicals from an organism exposed to stress (Braga *et al.,* 2012). It is believed that antioxidant activity originates from the presence of plants' secondary metabolites, compounds which are strictly determined by genes and not susceptible to environmental influences (Krzakowa and Grzywacz, 2010). One of the clinical studies confirmed the effect of *M. officinalis* extract, containing 0.25% of coumarin, on acute inflammation of the male rabbits (Pleşca-Manea *et al.,* 2002). In patients suffering from breast cancer, coumarin from the extracts of *M. officinalis* was used. Coumarin showed a positive impact on the recovery of patients (Pastura *et al.,* 1999). Aćamović-Djoković *et al.* (2002) have been noticed the antimicrobial activity of petroleum and ethyl acetate extracts of *M. officinalis* on the various strains of bacteria. The antibacterial activity of the water extract of *M. officinalis* on the examined strain of *Pseudomonas aeruginosa* was also discovered, using disk diffusion method (Karakas *et al.,* 2012). Du-Xiao-Feng *et al.* (2008) have been concluded that extracts of *M. officinalis* demonstrated antifungal activity.

The aims of this study were to determine the concentration of total phenols, flavonoids, tannins and

proanthocyanidins in the water, acetone, diethyl ether and ethanol extract of *M. officinalis* and the antioxidant activity of the extracts using two methods, DPPH method and reducing power. Moreover, the *in vitro* antimicrobial (antibacterial and antifungal) activity was investigated compared to the selected species of bacteria and fungi and the effect of the most active extracts on bacterial biofilm formation.

MATERIALS AND METHODS

Chemicals: A 2,2-diphenyl-1-picrylhydrazyl (DPPH), tetracycline, ampicillin, amphotericin B, gallic acid, rutin, cyanidin chloride were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Potassium ferricyanide, trichloroacetic acid, ferric chloride, ascorbic acid and dimethyl sulfoxide (DMSO) were purchased from Acros Organics (New Jersey, USA). Resazurin was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Crystal violet stain was obtained from Fluka AG (Buchs SG, Switzerland). Nutrient liquid media, a Mueller–Hinton broth and a Sabouraud dextrose broth were purchased from Torlak (Belgrade, Serbia) and an antimycotic, itraconazole, from Pfizer Inc., USA. Organic solvents (ethanol, diethyl ether and acetone) and concentrated hydrochloric acid (HCl) were purchased from Zorka Pharma (Šabac, Serbia). Polyvinylpolypyrrolidone (PVPP) was purchased from Sigma-Aldrich, St. Louis, MO, USA.

Plant material: The aerial parts of *M. officinalis* were collected on Mount Bukulja (Serbia), during the summer of 2012. Identification and classification of the plant material was performed at the Faculty of Science, University of Kragujevac (Serbia). The voucher samples were deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac (Serbia). The collected plant materials were air-dried in darkness at ambient temperature.

Preparation of plant extracts: The dried, ground plant material was extracted by maceration with water, acetone, diethyl ether and ethanol. Briefly, 30 g of the plant material was soaked with 150 ml of the solvent. The plant material was macerated three times at room temperature using fresh solvent every 24 hours. After every 24 hours, the samples were filtered through filter paper and the filtrates were collected and evaporated to dryness using a rotary evaporator (IKA, Germany) at 40 °C. The obtained extracts were kept in sterile sample tubes and stored at - 20 °C. The yield of obtained extracts based on dry weight was calculated from the formula:

$$\text{Yield (\%)} = (W1 \times 100) / W2$$

where W1 was the weight of extract after evaporation of solvent and W2 was the dry weight of the sample.

Phytochemical analysis of plant extracts

Determination of total phenol content: The total phenol content of the extracts was quantified according to the Folin-Ciocalteu's method as described by Wootton-Beard *et al.* (2011). Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the total phenolic content was expressed as milligram of gallic acid equivalents (GAE) per gram of extract (mg GAE/g of extract).

Determination of total flavonoid content: The total flavonoid content of the extracts was determined using the aluminium chloride method as described by Quettier-Deleu *et al.* (2000). Rutin (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the concentrations of flavonoids were expressed as milligram of rutin equivalents (RUE) per gram of extract (mg of RUE/g of extract).

Determination of total extractable tannin content: Total extractable tannin (TET) content was estimated indirectly by spectrophotometric measurement of the absorbance of the solution obtained after the precipitation of the tannins with polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, St. Louis, MO, USA) as described by Makkar *et al.* (1993). The TET was expressed as milligram of gallic acid equivalents (GAE) per gram of extract (mg GAE/g of extract).

Determination of proanthocyanidin content: The proanthocyanidin content was measured by the butanol-HCl method with ferric ammonium sulphate as a catalyst as described by Porter *et al.* (1986). Cyanidin chloride (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the proanthocyanidin content was expressed as milligrams of cyanidin chloride equivalents (CChE) per gram of extract (mg CChE/g of extract).

Determination of antioxidant activity

DPPH radicals scavenging capacity assay: The ability of *M. officinalis* extracts to scavenge DPPH free radicals was assessed using the method described by Takao *et al.* (1994). The tested concentrations of plant extracts were from 62.5 µg/ml to 1000 µg/ml. Diluted solutions of extract (2 ml each) were mixed with 2 ml of DPPH methanolic solution (40 µg/ml). Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and water extract of *Aronia melanocarpa* were used as a reference compounds. The plant *Aronia melanocarpa* is known as a good antioxidant, and because of a nature of tested compounds, one synthetic compound and one plant extract were chosen as standard. Radical scavenging activity is expressed as the inhibition percentage calculated using the following formula:

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

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extract.

Reducing power: The reducing power of the plant extracts was determined according to the method of Oyaizu (1986). The tested concentrations of plant extracts were from 62.5 µg/ml to 1000 µg/ml. The absorbance of the reaction mixture was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference compound.

Determination of antimicrobial activity

Test microorganisms: Antimicrobial activity of *M. officinalis* extracts was tested against 19 strains of bacteria and 6 strains of fungi. The list of tested microorganisms is presented in Table 4 and 5. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms (fungi and ATCC strains) were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation: Bacterial and yeast suspensions were prepared by the direct colony method (Andrews, 2005). The turbidity of initial suspensions was adjusted using 0.5 McFarland densitometer (BioSan, Latvia). Initial bacterial suspensions contain about 10^8 colony forming units (CFU)/ml and yeast suspensions contain 10^6 CFU/ml. 1:100 dilutions of initial suspension were additionally prepared into sterile 0.85 % saline. The suspensions of fungal spores were prepared by gentle stripping of spores from slopes with growing mycelia. The resulting suspensions were 1:1000 diluted in sterile 0.85 % saline.

Microdilution method: Antimicrobial activity was tested using microdilution method with resazurin determining the minimum inhibitory concentration (MIC) (Sarker *et al.*, 2007). Twofold serial dilutions of the plant extracts were made in sterile 96-well microtiter plates containing 0.1 ml of Mueller - Hinton broth (Torlak, Belgrade, Serbia) per well for bacteria and 0.1 ml of Sabouraud dextrose broth (Torlak, Belgrade, Serbia) per well for fungi. The tested concentration range was from 0.156 mg/ml to 20 mg/ml. The microtiter plates were inoculated with the suspensions to give a final concentration of 5×10^5 colony forming units (CFU)/ml for bacteria and 5×10^3 CFU/ml for fungi. The growth of the bacteria and the yeasts was monitored by adding resazurin (Alfa Aesar GmbH & Co., Karlsruhe, Germany), an indicator of microbial growth. Resazurin is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated microtiter plates were incubated at 37 °C for 24 h for bacteria, at 28 °C for 48 h for yeasts and at 28 °C for 72 h for moulds. MIC was defined as the lowest concentration of tested plant extracts that prevented resazurin color change from blue to pink. For moulds, MIC values of the tested plant extracts were determined as the lowest concentration that inhibited visible mycelia growth. Minimum microbicidal concentration (MMC) was determined by inoculation of nutrient agar medium by plating 10 µl of samples from wells, where no indicator color change was recorded. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration.

Ampicillin, tetracycline, amphotericin B (Sigma Chemicals Co., USA) and itraconazole (Pfizer Inc., USA), dissolved in nutrient liquid medium, were used as reference compounds. Stock solutions of crude extracts were obtained by dissolving in 10% dimethyl sulfoxide (DMSO; Acros Organics, USA). Each test included growth control and sterility control. All tests were performed in duplicate and mean values were presented.

Determination of antibiofilm activity

Tissue culture plate method (TCP): The TCP assay described by Christensen *et al.* (1985) is most widely used test for detection of biofilm formation. We screened all strains for their ability to form biofilm by TCP method with some modifications. Each test included biofilm formation control. Bacterial biofilm formation properties were well described by O'Toole *et al.* (2000).

The tissue culture 96-well plates (Sarstedt, Germany) were prepared by dispensing 50 µl of nutrient broth, Mueller–Hinton broth, into each well. A 50 µl from the stock solution of tested extracts (concentration of 20 mg/ml) was added into the first row of the plate. Then, twofold, serial dilutions were made using a multichannel pipette. The tested concentration range was from 0.16 to 20 mg/ml. A 50 µl of fresh bacterial suspension was added to each well. The inoculated plates were incubated at 37 °C for 24 h. After incubation content of each well was gently removed by tapping the plates. The wells were washed with 200 µl of sterile 0.85% saline to remove free-floating bacteria. Biofilms formed in plate were stained with crystal violet (0.1% w/v; Fluka AG, Switzerland) and incubated at the room temperature for 20 minutes. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (ODs) of stained adherent bacteria were determined with a micro ELISA plate reader (RT-2100C, Rayto, Shenzhen, China) at wavelength of 630 nm (OD630 nm). Biofilm inhibitory concentration (BIC_{50}) was defined as the lowest concentration of extract that showed 50% inhibition on the biofilm formation (Chaieb *et al.*, 2011).

Only broth or broth with extracts served as control to check sterility and non-specific binding of media. To compensate for background absorbance, OD readings from sterile medium, extracts, fixative and dye

were averaged and subtracted from all test values. All tests were performed in duplicate. Tetracycline, were used as reference compounds.

Data analysis: All data were presented as means ± standard deviations (mean ± SD) where appropriate. Pearson correlation coefficient was determined. All calculations were performed using Microsoft Excel software (Redmond, Washington, DC, USA).

RESULTS AND DISCUSSION

Concentrations of total phenols, flavonoids, tannins and proanthocyanidins: More polar solvents, such as water (36,9%) and ethanol (6,47%), provided higher yields of extracts than less polar ones, such as acetone (2,23%) and diethyl ether (1,83%). The highest concentration of total flavonoids (53.09 mgRU/g) and the highest concentration of total proanthocyanidins (3.77 mgCChE/g) were measured in the acetone extract. The highest concentration of total phenols (36.25 mgGA/g extract) and the highest concentration of total tannins (21.25 mgGA/g) were in the water extract. The results are shown in Table 1.

According to the literature data, the methanol extract of *M. officinalis* was the most examined and it demonstrated the highest concentration of flavonoids 57 ± 5.4 mgRU/g and phenols 289.5±5 mgGAE/g (Braga *et al.*, 2012).

In this paper, the concentrations of phenolic compounds in the acetone, diethyl ether and ethanol extract of *M. officinalis* were examined for the first time. Among all examined secondary metabolites, flavonoids are detected in the highest concentrations.

Antioxidant activity: The results of the antioxidant activity of the water, acetone, diethyl ether and ethanol *M. officinalis* extracts and of the positive control using DPPH method are shown in Table 2. Comparing the results of the examined samples to the results of the positive control (vitamin C and water extract of *Aronia melanocarpa*), moderate antioxidant activity of the examined plant was detected. The examined concentrations of the extracts were active in the range of 1000 µg/ml to 62.5 µg/ml. Decrease in concentrations of the examined extracts, led to decrease in antioxidant activity.

Table 1. Concentrations of total phenols, flavonoids, tannins and proanthocyanidins in the extracts of *M. officinalis*

Type of extract	Flavonoids concentration (mgRU/g extract)	Phenolic concentration (mgGA/g extract)	Tannins concentration (mgGA/g extract)	Proanthocyanidins concentration (CChE/g extract)
Water	19.66 ± 0.23	36.25 ± 0.79	21.25 ± 1.32	n.d
Acetone	53.09 ± 1.67	27.12 ± 0.49	11.87 ± 0.12	3.77 ± 0.03
Diethyl ether	33.52 ± 0.22	16.37 ± 0.59	6.12 ± 0.16	1 ± 0.13
Ethanol	34.19 ± 0.06	21.37 ± 1.09	1.37 ± 1.08	0.77 ± 0.59

Data are presented as mean ± standard deviation.
n.d – not detected

Table 2. Comparative antioxidant activity of *M. officinalis* extracts obtained by DPPH method

Concentration	Water extract	Acetone extract	Diethyl ether extract	Ethanol extract	*Aronia melanocarpa* water extract	Vitamin C
	% of activity					
1000µg/ml	93.59 ± 0.43	67.95 ± 1.90	61.28 ± 0.66	71.98 ± 0.05	91.06 ± 0.18	97.21
500µg/ml	91.94 ± 0.12	57.88 ± 1.08	39.07 ± 1.49	39.99 ± 0.17	72.66 ± 0. 08	97.18
250µg/ml	71.25 ± 0.26	36.08 ± 0.74	27.88 ± 1.43	20.51 ± 0.37	47.91 ± 0.31	97.18
125µg/ml	38.64 ± 0.13	20.88 ± 0.55	23.45 ± 0.47	14.1 ± 0.70	36.48 ± 0.07	97.18
62.5µg/ml	21.43 ± 1.02	11.72 ± 1.01	17.94 ± 0.87	13 ± 0.30	21.87 ± 0.33	97.15

Data are presented as mean ± standard deviation

The water extract demonstrated the antioxidant activity in the range of 93.59% to 21.43%; the acetone extract of 67.95% to 11.72%; diethyl ether extract of 61.28% to 17.94% and ethanol extract of 71.98% to 13%. The highest antioxidant activity, was detected in the water extract, while the lowest one was detected in the diethyl ether extract. The water extract even showed higher antioxidant activity than a positive control, and water extract of *A. melanocarpa*. Based on these results, it could be concluded that the antioxidant activity are in correlation with phenolic compounds concentration. These results were confirmed by Pearson correlation

coefficient. Positive linear correlation between DPPH scavenging activity and total phenol and tannin content was noticed (r = 0.91, 0.77, respectively). Negative linear correlation was shown for flavonoids and proanthocyanidins (r = -0.68, -0.51, respectively).

According to the literature data, it could be observed that the antioxidant activity of the acetone and diethyl ether extracts of *M. officinalis* was examined for the first time. The results of this study confirm once again, the antioxidant activity of the extracts of plants belonging to the genus *Melilotus.*

Reducing power: Extracts of *M. officinalis* demonstrated low reducing power compared to the positive control (vitamin C) (Table 3).

Table 3. Comparative reducing power of *M. officinalis* extracts.

Concentration	Water extract	Acetone extract	Diethyl ether extract	Ethanol extract	Vitamin C
	Absorbance at 700nm				
1000µg/l	0.68 ± 0.69	0.39 ± 0.39	0.20 ± 0.41	0.22 ± 0.23	2.94
500µg/ml	0.31 ± 0.32	0.21 ± 0.22	0.12 ± 0.12	0.11 ± 0.00	2.94
250µg/ml	0.18 ± 0.18	0.09 ± 0.09	0.07 ± 0.07	0.06 ± 0.00	2.84
125µg/ml	0.07 ± 0.08	0.04 ± 0.04	0.05 ± 0.05	0.05 ± 0.00	2.67
62.5µg/l	0.03 ± 0.03	0.00 ± 0.00	0.04 ± 0.04	0.01 ± 0.00	1.19

Data are presented as absorbance mean value ± standard deviation

The results showed that decrease in the concentration of the extracts, led to decrease in the reducing power of the extracts. The extent of reducing power in the examined extracts was various. The highest activity was detected in the water extract and the absorbance were from 0.03 to 0.68, while the lowest reducing power was demonstrated in the diethyl ether extract whose absorbance were from 0.04 to 0.20. Absorbance of the acetone extract ranged from 0.00 to 0.39, while absorbance of the ethanol extract showed the reducing power in the range of 0.01 to 0.22. Based on the results it can be concluded that the water extract had the highest reducing power. Strong, positive linear correlation between reducing power and total phenol and tannin content was noticed (r = 0.98, 0.95, respectively). Negative linear correlation was shown for flavonoids and proanthocyanidins (r = -0.45, -0.22, respectively).

Antimicrobial activity: The results of *in vitro* antibacterial and antifungal activity of the water, acetone, diethyl ether and ethanol *M. officinalis* extracts are shown in Tables 4 and 5. MIC and MMC values of antibiotics, ampicillin and tetracycline, and antifungals, itraconazole and amphotericin B, were used as a comparison and they are shown as well in Tables 4 and 5. It was observed that 10% DMSO did not inhibit the growth of microorganisms.

The antimicrobial activity of examined extracts was determined against 25 species of microorganisms. In this experiment, the values of MIC and MMC were in a range of <0.156 mg/ml to >20 mg/ml. The intensity of antibacterial activity depended on the type of extract and the type of bacteria. The extracts were active in the following ascending order: water <ethanol < diethyl ether <acetone.

The obtained results of *M. officinalis* extracts, demonstrated fairly weak antibacterial activity on G^- bacteria. The most active extracts were the acetone and the diethyl ether, while the water and the ethanol extracts demonstrated significantly lower activity. The examined G^-bacteria showed higher sensitivity to the acetone and diethyl ether extracts, but never in concentrations less than 10 mg/ml for MIC and MMC, except for *Escherichia coli* ATCC 25922 which was sensitive in the concentration of 5 mg/ml of the ether extract for MIC.

Bacteria *Salmonella enterica, S. typhimurium* and *Escherichia coli* were not shown sensitivity to tested concentrations (>20 mg/ml for MIC and MMC), while *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* were not shown sensitivity only to the water extract. *Klebsiella pneumoniae* was not shown sensitivity to the ethanol extract as well.

G^+ bacteria (11 were examined) were more sensitive to the tested extracts than G^- bacteria. The highest sensitivity towards the extracts showed probiotics bacteria *Bifidobacterium animalis* subsp. *lactis, Bacillus subtilis* IP5832, *Lactobacillus rhamnosus* (from <0.15 mg/ml to 10 mg/ml for MIC and MMC). Referring to the other tested G^+ bacteria, the highest sensitivity demonstrated those belonging to the genus *Bacillus* (<0.15 mg/ml to 10 mg/ml for MIC and MMC). *Enterococcus faecalis* showed great insensitivity to the examined extracts (generally above 20 mg/ml for MIC and MMC. *Staphylococcus aureus* ATCC 25923 was not shown sensitivity to the water extract, MIC and MMC were >20 mg/ml, while it was sensitive to the other extracts in the range of 0.15 mg/ml to 10 mg/ml for MIC and MMC. *S. aureus, Sarcina lutea, E. faecalis* ATCC 39212 behaved similarly. The highest concentrations of flavonoids were detected in the acetone extract of *M.*

*officin*alis, and this extract also showed the highest antibacterial activity on G^+ and G^- bacteria.

Table 4: Antibacterial activity of water, acetone, diethyl ether and ethanol extract of *M. officinalis*

Species	Water extract		Acetone extract		Diethyl ether extract		Ethanol extract		Ampicillin		Tetracycline	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
Klebsiella pneumoniae	>20	>20	20	>20	20	>20	>20	>20	>128	>128	4	32
Escherichia coli	>20	>20	>20	>20	>20	>20	>20	>20	2.1	1.2	2	6
E. coli ATCC 25922	>20	>20	10	20	5	10	20	20	0.37	0.5	4	6
Pseudomonas aeruginosa	20	>20	10	>20	10	>20	20	>20	>128	>128	>128	>128
P. aeruginosa ATCC 27853	>20	>20	10	>20	20	>20	20	>20	>128	>128	4	32
Proteus mirabilis	20	>20	10	>20	10	10	20	>20	>128	>128	>128	>128
Salmonella typhimurium	>20	>20	>20	>20	>20	>20	>20	>20	2	2	2	2
Salmonella enterica	>20	>20	20	20	20	>20	>20	>20	1	1	2	4
Enterococcus faecalis	>20	>20	20	20	>20	>20	>20	>20	4	6	1	6
E. faecalis ATCC 39212	10	>20	10	20	5	20	10	>20	0.25	0.75	1.5	3
Staphylococcus aureus	5	5	0.6	1.25	0.6	1.25	2.5	5	< 0.06	< 0.06	< 0.06	< 0.06
S. aureus ATCC 25923	>20	>20	10	20	5	10	20	20	0.25	0.75	1.5	3
Sarcina lutea	10	10	0.3	0.3	>0.15	>0.15	0.6	0.6	n.d	n.d	n.d	n.d
Bacillus suptilis	10	10	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15	16	128	< 0.06	0.25
Bacillus suptilis ATCC 6633	10	10	0,3	0.3	0.3	0.3	0.15	0.3	3	4	0.25	0.37
Bacillus cereus	10	10	<0.15	<0.15	0.3	<0.15	<0.3	0.3	4	6	0.25	0.5
Bifidobacterium animalis subsp. *lactis*	5	10	<0.15	<0.15	<0.15	<0.15	0.3	0.3	< 0.06	0.12	4	8
Bacillus subtilis IP 5832	10	10	0.3	0.3	0.3	0.3	0.6	0.6	8	16	< 0.06	< 0.06
Lactobacillus rhamnosus	5	10	<0.15	<0.15	<0.15	0.6	0.3	0.3	< 0.06	0.12	0.12	1

Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values given as mg/ml for plant extract and µg/ml for antibiotics; n.d. – not determinated

Extracts of *M. officinalis* demonstrated moderate antifungal activity. Among the extracts, the acetone extract demonstrated the greatest activity on the examined fungi (in the range of 5 mg/ml to 20 mg/ml), the diethyl ether extract was effective on *Candida albicans* in concentration of 2.5 mg/ml for MIC. Other extracts demonstrated antifungal activity in the range of 5 mg/ml to 20 mg/ml for MMC and MIC. *Aspergillus niger* and *A. flavus* showed sensitivity only in the highest concentration of 20 mg/ml for the MIC and MMC.

Table 5. Antifungal activity of water, acetone, diethyl ether and ethanol extract of *M. officinalis*

Species	Water extract		Acetone extract		Diethyl ether extract		Ethanol extract		Amphotericin B		Itraconazole	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
Aspergillus niger	20	20	10	10	20	20	20	20	0.98	0.98	15.62	15.62
Aspergillus flavus	20	20	20	20	20	20	20	20	0.98	15.62	0.98	0.98
Penicillium italicum	20	20	5	5	20	20	5	5	7.81	7.81	0.98	0.98
Penicillium chrysogenum	20	20	5	5	5	5	10	10	7.81	7.81	0.98	0.98
Candida albicans	10	10	5	10	2.5	10	5	10	0.98	1.95	1.95	1.95
Candida albicans ATCC 10231	10	10	10	20	10	10	5	10	0.49	1.95	1.95	1.95

Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values given as mg/ml for plant extract and µg/ml for antimycotic

The results of antimicrobial activities of the acetone, diethyl ether and ethanol extracts were presented for the first time in this paper, to the best of the authors' knowledge. It was observed that the antimicrobial activity was connected to the concentration of secondary metabolites in the extracts.

According to the reviewed literature, it could be observed that the antimicrobial activity of *M. officinalis* was confirmed. Aćamović-Djoković *et al.* (2002) was compared the activity of *M. albus* (white clover), *M. melissophyllum* and *M. officinalis* (yellow clover) petroleum ether and ethyl acetate extracts and concluded that ethyl acetate extracts were the most active. Karakas *et al.* (2012) performed a comparative research on the biological activities and examined 16 various plant from Turkey included *M. officinalis*, using disk diffusion method. The antibacterial activity of the *M. officinalis* water extract, presented as zone of growth inhibition, on the tested strain of *Pseudomonas aeruginosa* was 22,5 mm. Moreover, the methanol and ethyl acetate extracts *of M. officinalis* possess inhibitory effect on fungi. Antifungal activity of various *M. officinalis* extracts was tested on 12 plant pathogenic fungi *in vitro* and *in vivo*. The results showed that the antifungal activity of the ethyl acetate *M. officinalis* extract was higher than activity of ethyl acetate extracts of other plants (Feng *et al.*, 2008).

Effect on biofilm formation: In order to discover natural compounds capable of inhibition and prevention of the bacterial biofilms formation, the acetone and diethyl ether extract of *M. officinalis* were examined. The effect of the extracts on the formation of biofilms by two bacterial strains, *Pseudomonas aeruginosa* and *Proteus mirabilis*, was examined in this paper. The study demonstrated that the strains of bacteria formed a moderate biofilm *in vitro*. In the presence of the examined extracts, the possibility of biofilm formation was altered. The results are shown in Table 6.

The acetone extract demonstrated the highest inhibitory effect on the examined bacterial strains. BIC_{50} for the species *P. mirabilis* was in the concentration of 2044.5 µg/ml and BIC_{50} for *P. aeruginosa* was in the concentration of 3510 µg/ ml. The diethyl ether extract demonstrated lower activity. BIC_{50} for the species *P. mirabilis* was in the concentration of 4938.5 µg/ml and BIC_{50} for *P. aeruginosa* was in the concentration > 5000 µg/ml.

Table 6. Influence of acetone and diethyl ether extract of species *M. officinalis* on the biofilm formation

Species	Acetone extract	Diethyl ether extract	Tetracycline
	BIC_{50}		
Proteus mirabilis	2044.5	4938.5	n.d.
Pseudomonas aeruginosa	3510	>5000	2715.7

Biofilm inhibitory concentration (BIC_{50}) values given as µg/ml
n.d – not determinated

The acetone extract showed the highest inhibitory activity on the biofilm formation, probably due to the highest concentration of flavonoids measured in the extract. This study is significant for the fact that the effect of *M. officinalis* extracts on formation of biofilm bacteria was examined for the first time.

Conclusion: Based on the examination of the water, acetone, diethyl ether and ethanol *M. officinalis* extracts in this paper, it could be concluded that all extracts, especially the acetone extract, are important source of biologically active compounds, and significant for antimicrobial activity which could be used for controlling microorganisms. The acetone extract also demonstrated major inhibitory activity on biofilm, although bacteria in biofilm are known for being more resistant than planktonic cells to antimicrobial agents. Due to the fact, the further research on broader spectrum of plant extracts activity on planktonic cells and bacteria in the biofilm as well, is required. Apart from the acetone extract, the water extract of *M. officinalis* showed a high percentage of antioxidant activity. The obtained results validated the usage of *M. officinalis* in traditional medicine and indicated necessity for further research.

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