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The phenolic constituents and antimicrobial activity of *Xanthium spinosum* (Asteraceae) extracts

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ABSTRACT:
Xanthium spinosum is a cosmopolitan annual herb used in traditional medicine worldwide. Although known from ethnobotanical studies, the species is scarcely investigated from the aspects of phytochemistry and biological activity. Therefore, the phenolic composition and biological activity of *X. spinosum* were examined. Plant specialised metabolites (phenolics) extracted from the roots, leaves and fruits with dichloromethane:methanol (1:1) were analysed by liquid chromatography mass spectrometry (LC-MS). In total 10 phenolic compounds were identified and quantified. Six compounds were common to all the extracts. Chlorogenic acid was the most abundant constituent in all the extracts (4.262 mg/g in the fruit extract, 0.820 mg/g in the leaf extract, and 0.540 mg/g in the root extract). The biological activity (antimicrobial and antibiofilm) of the extracts was tested against 12 microfungi and 12 bacterial strains by the microdilution method. All the extracts exhibited moderate antimicrobial and antibiofilm activity and inhibited the growth of most of the examined microorganisms. The obtained results indicate the potential role of the tested extracts in pharmacy and medicine.

Keywords:
phenolic acids, flavonoids, antifungal activity, antibacterial activity, antibiofilm activity

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INTRODUCTION

The genus *Xanthium* L. (Heliantheae, Asteraceae) is comprised of 25 annual and perennial herbaceous species with a very wide geographical distribution in the temperate regions of America and Eurasia (Löve & Dansereau 1959; Sheng *et al.* 2019). The representatives of this genus can easily be recognized by their spiny fruiting structures which resemble burrs (Somaratne *et al.* 2019). The genus is considered complex and on the basis of morphological characteristics there are several species and local ecotypes (Somaratne *et al.* 2019). The number of species has changed from two to more than 20, and according to recent studies, five species are recognized within *Xanthium* (Tomasello 2018; Somaratne *et al.* 2019). In Serbia the species of this genus grow in arable and ruderal areas (Gajić 1975).

Xanthium spinosum L. is an annual herbaceous plant with an erect, densely branched stem. The leaves have petioles under which there are tripartite thorns. Originating from America, nowadays the species has a cosmopolitan distribution. In Serbia *X. spinosum* is widespread and grows in agricultural crops and ruderal habitats (Gajić 1975).

Xanthium spinosum is used worldwide in traditional medicine to treat various health problems (Ginesta-Peris *et al.* 1994; Martínez & Barboza 2010; Ullah *et al.* 2013; Varga *et al.* 2014, 2020; Romero *et al.* 2015; Yuan *et al.* 2018). It is traditionally used against rabies, to relieve chronic fevers, to ease the effects of di-

abetes, and even to stimulate saliva production (Yuan *et al.* 2018). In Romania it is used for urinary problems and various prostate diseases (Varga *et al.* 2020). In traditional Toba medicine, warts can be treated with thorns of *X. spinosum* (Martínez & Barboza 2010). In Pakistan, *X. spinosum* is medicinally important as a diaphoretic, diuretic and sedative agent where the root, leaves, and fruits are used. This species is also considered useful for the treatment of hydrophobia, as well as an emetic when an infusion of the root is administered (Ullah *et al.* 2013). The seeds of *X. spinosum* bear a poisonous compound carboxyatractyloside which is toxic to livestock, but animals generally avoid this plant because of its thorns (GISD 2022). Genotoxicity studies were conducted to determine the presence of any toxic effects of root infusion of *X. spinosum* and the results indicate that there was no mutagenic effect or DNA damage induced when the lowest concentration was applied (Güez *et al.* 2012). Research using BALB/C mice showed that extract of *X. spinosum* mature leaves can cause hepatic damage (Silvero-Isidre *et al.* 2016).

Sesquiterpene lactones are the most prominent class of compounds of *X. spinosum* extracts, with those of xanthanolide type being the most frequent (Omar *et al.* 1984; Abdei-Mogib *et al.* 1991; Marco *et al.* 1993; Klecáková-Karlícková & Jahodár 2005; Yuan *et al.* 2018; Varga *et al.* 2020). Two sesquiterpene lactone glycosides of 11α,13-dihydro-8-epi-desacetylxanthiuminol, three kaurene glycosides closely related to carboxyatractyloside and atractyloside, together with didesulphated derivates of carboxyatractyloside and atractyloside were isolated from the chloroform-methanol and methanol extracts of the aerial parts of *X. spinosum* (Piacente *et al.* 1996). The sesquiterpene lactone of guaianolide type, ziniolide, was isolated from the hydroalcoholic fraction of *X. spinosum n*-hexane root extract (Bader *et al.* 2013). Also, the presence of eleven new sesquiterpenoids, eight of humulane-type and three of germacrene-type, along with twelve previously described compounds was established in *X. spinosum* fruit extract (Wang *et al.* 2021). In addition to the aforementioned compounds, vanillin, coniferyl alcohol, monoterpenoid derivate dehydrovomifoliol, monoterpene lactone loliolide, sesamin, and squalene were also found in various extracts of this species (Abdei-Mogib *et al.* 1991; Marco *et al.* 1993; Yuan *et al.* 2018). The fractionation of methanolic extract of the aerial parts of *X. spinosum* resulted in the isolation of axillarin, isoquercitroside, hyperoside, and isochlorogenic acid (Sanz *et al.* 1991). A variety of phenolic compounds were found in the aqueous-methanolic extract of the flowering aerial parts of *X. spinosum* including hydroxybenzoic and hydroxycinnamic derivatives, one benzyl alcohol derivate, flavonoids and diterpenes (Varga *et al.* 2020). In addition, phytosterols, *β*-sitosterol and stigmasterol, as well as several fatty acids, namely palmitic, palmitoleic, azelaic, stearic, oleic, and arachidonic, were identified in the ethanolic extract of the aerial parts and the acetone extract of the fruits of *X. spinosum*, respectively (Klecáková-Karlícková & Jahodár 2005).

The biological activity of specialised metabolites from *X. spinosum* is scarcely investigated. A decoction of *X. spinosum* demonstrated antioxidant activity in DPPH, ABTS, and FRAP assays (Dadé *et al.* 2009). The methanolic extract of *X. spinosum* roots showed potential in the inhibition of 5-LOX, COX-1, and 12-LOX enzymatic pathways in intact pro-inflammatory cells, with the synthesis of the 15(S)-HETE, anti-inflammatory eicosanoid, being induced (Bader *et al.* 2013). Ethanol, chloroform, and hexane extracts of the stem and leaves of *X. spinosum* exhibited antimicrobial activity against phytopathogenic fungi *Pythium ultimum*, *Penicillium expansum*, and *Fusarium solani* (Hashem *et al.* 2016). Different *X. spinosum* extracts showed antifungal activity against the human-opportunistic pathogens *Geotrichum candidum* and *Candida albicans* (Hashem *et al.* 2019). However, a decoction of *X. spinosum* leaves and branches did not show activity against *Salmonella typhi*, a resistant strain of *Staphylococcus aureus, Escherichia coli*, and *Aspergillus niger* (Anesini & Pérez 1993; Pérez & Anesini 1994). Similarly, ethanolic extracts of *X. spinosum* failed to demonstrate antimicrobial, antiviral, and virucidal activities, or cytotoxic and mutagenic effects (Alkofahi *et al.* 1990; Faral-Tello *et al.* 2012).

The biological activity of *X. spinosum* from Serbia has not been studied. Also, there are no data related to the phenolic compounds of the separate plant parts and their biological activity. Thus, the main objective of the present study was to investigate the chemical profiles of phenolics in extracts from different parts of *X. spinosum* and to evaluate their antimicrobial potential against bacterial and fungal strains, as well as their effect on biofilm formation.

MATERIALS AND METHODS

Plant material. Whole plants of *Xanthium spinosum* were collected during the fruiting season from the village Kamenica (Serbia, N 43°26'57'', E 21°36'21'') in October 2020. The plant material was identified following professional literature (Gajić 1975). The voucher specimen (BEOU 17765) was deposited at the Herbarium of the University of Belgrade - Faculty of Biology.

Extraction of the phenolic compounds. The plant material (roots, leaves, and fruits) was air-dried prior to extraction. The separated plant parts, 10 g each, were ground using a laboratory mill and submerged in 150 mL of a solvent mixture of dichloromethane and methanol (1:1). The samples were sonicated for 30 minutes in an ultrasonic bath at 25°C. After ultrasonication and filtration through filter paper (Whatman No. 1), the ex-

tracts were evaporated to dryness using a rotary vacuum evaporator. The obtained crude extracts were then stored at 4°C prior to analysis. The extraction yield of the obtained extracts was determined according to the equation previously used (Janaćković *et al.* 2019).

Chemical profiling. The phenolic profile of the extracts was determined by UHPLC-DAD-ESI/MS^2 (Dionex Ultimate 3000 UHPLC, Thermo Scientific, San Jose, CA, USA). The compounds were separated and identified as previously described (Gašić *et al.* 2015). The MS detection was performed in negative mode using a triple quadrupole (QqQ) mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with a heated electrospray ionisation (H-ESI) source. The phenolic compounds were identified based on their chromatographic behaviour and mass spectra by comparison with standard compounds. Data acquisition was carried out with a Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA). For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the MS/MS spectra. The results were expressed as mg/g of the extract.

Microorganisms. The *Candida* species used were clinical isolates *C. albicans* (475/15), *C. krusei* (H1/16), and *C. glabrata* (4/6/15). The reference yeast strains used were *C. albicans* (ATCC 10231), *C. tropicalis* (ATCC 750), and *C. parapsilosis* (ATCC 22019). The fungal species used were clinical isolate *Aspergillus fumigatus,* and food isolate *Penicillium verrucosum* var. c*yclopium.* The reference fungal strains used were *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), and *Trichoderma viride* (IAM). The clinical bacteria used were *Bacillus cereus* and *Proteus vulgaris* (IBR P004). The resistant strains used were *Pseudomonas aeruginosa* (IBRS P001), methicillin-resistant *Staphylococcus aureus* (IBRS MRSA 011), and *Escherichia coli* (IBRS E003). The reference bacterial strains used were *Listeria monocytogenes* (NCTC 7973), *Yersinia enterocolitica* (ATCC 23715), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 35210), *Salmonella* Typhimurium (ATCC 13311), *Staphylococcus aureus* (ATCC 6538), and *Enterobacter cloacae* (ATCC 35030). The tested microorganisms were deposited at the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" - National Institute of Republic of Serbia, University of Belgrade.

Antifungal activity. The minimal inhibitory and minimal fungicidal concentrations (MIC/MFC) were determined according to the modified EUCAST (2003) procedure. Briefly, fresh overnight yeast cultures were adjusted to a concentration 1.0×10^5 CFU/well with the use of sterile saline. The microplates containing serially diluted plant extracts were incubated at 37°C for 24 h (*Candida* spp.), and at 28°C for 72 h (other fungal strains), following which the MIC and MFC were determined. The MIC values were considered as the lowest concentrations with no observed growth. Following serial subcultivations of 10 µL into microtiter plates containing 100 µL of broth per well, as well as subsequent incubation at 37°C for 24 h (*Candida* spp.), and at 28°C for 72 h (other fungal strains), the lowest concentrations with no visible growth were defined as the MFC values, indicating 99.5% killing of the original inoculum. Ketoconazole was used as the positive control (Sigma-Aldrich, Germany).

Antibacterial activity. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by serial microdilution of *X. spinosum* extracts in 96-well microtiter plates following the modified CLSI (2009) protocol. Streptomycin was used as the positive control (Sigma-Aldrich, Germany).

Inhibition of biofilm formation. The strains used for the inhibition of biofilm formation assay were *Candida albicans* (ATCC 10231), *C. parapsilosis* (ATCC 22019), and *Klebsiella pneumoniae* (ATCC 13883) according to the procedure previously described (Smiljković *et al.* 2018). The percentage of inhibition of biofilm formation was calculated and the results are presented as mean ± SD of three replicates. Microsoft Excel was used for the graph construction.

RESULTS

Extraction yield. The extraction yield of the three investigated extracts ranged from 3.85% to 11.41%. The highest extraction yield was observed in the leaf extract – LXsp (11.41%), followed by the fruit extract - FXsp (10.42%), and the root extract - RXsp (3.85%).

Chemical profile. A total of 10 compounds were identified in the studied extracts (Table 1). Six compounds (quinic acid, aesculin, chlorogenic acid, caffeic acid, rutin, and isoquercetin) were detected in all the samples, with chlorogenic acid being the most abundant (4.262 mg/g in FXsp, 0.820 mg/g in LXsp, and 0.540 mg/g in RXsp). In FXsp the second dominant compound was isoquercetin (1.110 mg/g), in RXsp quinic acid (0.054 mg/g), while in LXsp it was aesculin (0.638 mg/g). Astragalin was not detected in RXsp, while apigetrin and naringenin were not detected in LXsp. Quercetin was detected only in the FXsp extract.

Antifungal activity. All the investigated extracts showed moderate antifungal activity (Table 2). The RXsp extract was the most efficient against *Candida parapsilosis* (ATCC 22019) and *Aspergillus fumigatus* with MIC val-

Table 1. The compounds identified in the *Xanthium spinosum* extracts.

Compounds	Retention time (t_R), min	Molecular ion, *m/z*	MS/MS fragments, *m/z*	RXsp[1]	LXsp	FXsp
Quinic acid	0.92	191.000	171.00	0.054[2]	0.233	0.269
Aesculin	4.78	339.080	133.09; 177.06	0.026	0.638	0.078
Chlorogenic acid	5.14	353.103	191.28	0.540	0.820	4.262
Caffeic acid	5.57	179.004	134.00; 135.00	0.046	0.022	0.143
Rutin	6.19	609.197	299.98; 301.20	0.034	0.036	0.039
Isoquercetin	6.36	463.002	300.02; 271.01	0.009	0.018	1.110
Astragalin	6.73	447.008	284.03; 255.03	nd	0.021	0.065
Apigetrin	6.91	431.004	268.03; 239.11	0.015	nd	0.015
Quercetin	8.16	301.026	151.01; 179.00	nd	nd	0.147
Naringenin	8.82	271.036	151.01; 107.07	0.038	nd	0.052

[1] RXsp – Extract of *X. spinosum* roots. LXsp – Extract of *X. spinosum* leaves. FXsp – Extract of *X. spinosum* fruits; [2] The contents of the compounds are expressed in mg/g; "nd" – not detected

Table 2. The antifungal activity of the *Xanthium spinosum* extracts.

	Microorganisms	RXsp[1]		LXsp		FXsp		Ketoconazole	
		MIC[2,3]	MFC	MIC	MFC	MIC	MFC	MIC	MFC
1	*Candida albicans* (ATCC 10231)	0.5	1	0.5	1	0.5	1	0.002	0.006
2	*Candida albicans* (475/15)	0.5	1	0.25	0.5	0.5	1	0.003	0.006
3	*Candida parapsilosis* (ATCC 22019)	0.25	0.5	0.5	1	0.5	1	0.003	0.006
4	*Candida tropicalis* (ATCC 750)	0.5	1	0.5	1	0.5	1	0.002	0.006
5	*Candida krusei* (H1/16)	0.5	1	0.5	1	1	2	0.002	0.003
6	*Candida glabrata* (4/6/15)	0.5	1	0.5	1	1	2	0.002	0.006
7	*Aspergillus fumigatus*	0.25	0.5	0.5	1	1	2	0.2	0.5
8	*Aspergillus niger* (ATCC 6275)	0.5	1	0.5	1	1	2	0.2	0.5
9	*Aspergillus versicolor* (ATCC 11730)	0.5	1	0.5	1	1	2	0.2	0.5
10	*Penicillium funiculosum* (ATCC 36839)	0.5	1	0.125	0.25	0.5	1	0.2	0.5
11	*Penicullium verrucosum* var. *cyclopium*	0.5	1	0.25	0.5	1	2	1	1.5
12	*Trichoderma viride* (IAM)	0.5	1	0.25	0.5	0.5	1	0.2	0.3

[1] RXsp – Extract of *X. spinosum* roots. LXsp – Extract of *X. spinosum* leaves. FXsp – Extract of *X. spinosum* fruits; [2] MIC - minimal inhibitory concentration. MFC - minimal fungicidal concentration; [3] The MIC/MFC values are expressed in mg/mL

ues of 0.25 mg/mL for both strains. *Penicillium funiculosum* (ATCC 36839) and *Candida albicans* (475/15) were the most susceptible strains to LXsp with MIC 0.125 and 0.25 mg/mL, respectively. In the case of *Candida albicans*, LXsp showed better activity against clinical isolate *C. albicans* (475/15), with MIC 0.25 mg/mL compared to the *C. albicans* (ATCC 10231) reference strain (MIC 0.5 mg/mL). No significant differences in growth inhibition were observed in the case of FXsp against the tested yeast and filamentous fungal strains, and the MIC values were mostly uniform ranging from 0.5 to 1 mg/mL. Different *X. spinosum* extracts were able to inhibit the growth of *Candida* spp. and it was noted that the growth of the reference strains was more inhibited than that of the clinical isolates. This occurrence is particularly apparent in LXsp and FXsp, where these extracts had the highest MIC values against *C. krusei* (H1/16) and *C. glabrata* (4/6/15) (MIC 1 mg/mL). The MIC and MFC values for the three tested *Aspergillus* strains were equal, with the exception of *A. fumigatus* treated with RXsp (MIC 0.25 mg/mL). Greater growth inhibition was determined when *Penicillium verrucosum* var. *cyclopium* was treated with RXsp (MIC 0.25 mg/mL) than with ketoconazole (MIC 1 mg/mL). The growth of *P. funiculosum* (ATCC 36839) was also more inhibited when treated with RXsp (0.125 mg/mL), than with the positive control ketoconazole (0.2 mg/mL).

Table 3. The antibacterial activity of the *Xanthium spinosum* extracts.

	Microorganisms	RXsp[1]		LXsp		FXsp		Streptomycin	
		MIC[2,3]	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	*Staphylococcus aureus* (ATCC 6538)	0.5	1	1	2	2	4	0.05	0.1
2	*Bacillus cereus*	0.5	1	0.25	0.5	2	4	0.025	0.05
3	*Listeria monocytogenes* (NCTC 7973)	1	2	1	2	2	4	0.15	0.3
4	*Escherichia coli* (ATCC 35210)	0.5	1	0.5	1	1	2	0.05	0.1
5	*Salmonella* Typhimurium (ATCC 13311)	2	4	1	2	4	8	0.1	0.2
6	*Enterobacter cloacae* (ATCC 35030)	1	2	1	2	2	4	0.025	0.05
7	*Pseudomonas aeruginosa* (IBRS P001)	0.5	1	1	2	0.5	1	0.05	0.1
8	*Proteus vulgaris* (IBR P004)	1	2	2	4	1	2	0.1	0.2
9	*Staphylococcus aureus* (IBRS MRSA 011)	1	2	2	4	2	4	0.1	/
10	*Escherichia coli* (IBRS E003)	2	4	2	4	4	8	0.1	0.2
11	*Yersinia enterocolitica* (ATCC 23715)	1	2	1	2	1	2	0.01	0.02
12	*Klebsiella pneumoniae* (ATCC 13883)	1	2	1	2	1	2	0.005	0.01

[1] RXsp – Extract of *X. spinosum* roots. LXsp – Extract of *X. spinosum* leaves. FXsp – Extract of *X. spinosum* fruits; [2] MIC - minimal inhibitory concentration. MBC – minimal bactericidal concentration; [3] The MIC/MBC values are expressed in mg/mL

Antibacterial activity. The studied *X. spinosum* extracts exhibited moderate to low antibacterial activity (Table 3), which indicates that the tested bacteria were generally less susceptible to the extracts compared to the examined yeast and fungal strains. *Bacillus cereus* was the most sensitive bacterium with the lowest MIC 0.25 mg/mL for LXsp. The greatest growth inhibition when treated with LXsp was observed in the aforementioned *B. cereus* and *Escherichia coli* (ATCC 35210) (MIC 0.5 mg/mL). The FXsp extract demonstrated the greatest ability to reduce growth in ampicillin-resistant *Pseudomonas aeruginosa* (IBRS P001) with MIC 0.5 mg/mL. However, the highest MIC values were noted in treatment with FXsp, 4 mg/mL for *Salmonella* Typhimurium (ATCC 13311) and the *Escherichia coli* (IBRS 003) resistant strain. RXsp did not show marked growth inhibition in the tested bacteria (the MIC values ranged from 0.5 to 2 mg/mL). *Staphylococcus aureus* (ATCC 6538) was more susceptible than methicillin-resistant *Staphylococcus aureus* (IBRS MRSA 011), particularly when treated with RXsp and LXsp. FXsp was equally effective in the growth reduction of both *S. aureus* strains (MIC 2 mg/mL). A similar occurrence, but more pronounced, was noted in the case of the *E. coli* reference and resistant strains. The MIC values for *E. coli* (ATCC 35210) when treated with RXsp, LXsp, and FXsp were 0.5, 0.5, and 1 mg/mL, respectively, while the MICs for the resistant strain *E. coli* (IBRS E003) in treatment with RXsp and LXsp were 2 mg/mL, and 4 mg/mL in the case of FXsp. The most sensitive of the antibiotic resistant bacteria was *P. aeruginosa* (IBRS P001), while the least susceptible was *E. coli* (IBRS E003). Methicillin-resistant *S. aureus* (IBRS MRSA 011) and *E. coli* (IBRS E003) were equally inhibited when treated with LXsp (MIC 2 mg/mL).

Inhibition of biofilm formation. The extracts of *X. spinosum* demonstrated moderate potential in the reduction of microbial ability to establish biofilms (Fig. 1). Out of all the extracts, FXsp showed the highest percentage of inhibition (> 50%) at the MIC concentration when tested on *C. parapsilosis* (ATCC 22019) (Fig. 1B). The application of RXsp at the MIC concentration also reduced *C. parapsilosis* biofilm formation ability by 50% (Fig. 1B). The extract with the lowest percentage of inhibition of *C. parapsilosis* biofilm formation was LXsp with less than 30% reduction with the highest applied concentration (MIC) (Fig. 1B). The application of LXsp at 0.5 and 0.25 MIC concentrations did not influence biofilm formation at all (Fig. 1B).

The RXsp extract was able to reduce the ability of *C. albicans* (ATCC 10231) to develop biofilm with 45.94% of inhibition at the MIC concentration, while at the highest applied concentration (MIC) LXsp and FXsp reduced biofilm formation by less than 30% (Fig. 1A). LXsp was not able to affect biofilm formation even at the 0.5 MIC concentration (Fig. 1A). The least promising was the ability of the extracts to effect *K. pneumoniae* (ATCC 13883) biofilm, where FXsp was able to inhibit biofilm formation by less than 50%, while RXsp and LXsp caused a lower than 30% reduction with the highest applied concentration (MIC) for all the extracts (Fig. 1C). It was also noticed that FXsp and RXsp reduced biofilm formation more than streptomycin at the

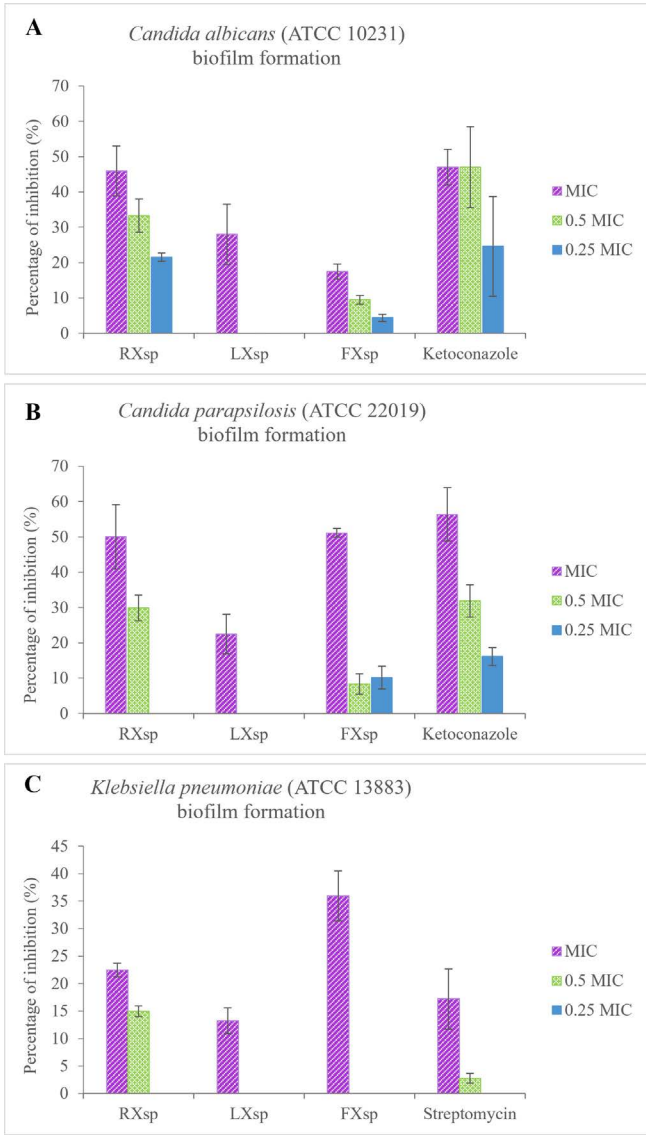

Figure 1. The percentage of inhibition of fungal and bacterial biofilm formation after treatment with the *X. spinosum* extracts (RXsp – extract of *X. spinosum* roots. LXsp – extract of *X. spinosum* leaves. FXsp – extract of *X. spinosum* fruits). The inhibition of biofilm formation of *Candida albicans* (ATCC 10231) **(A)**. *C. parapsilosis* (ATCC 22019) **(B)**. and *Klebsiella pneumoniae* (ATCC 13883) **(C)**. Error bars indicate standard deviations. The data are presented as mean ± SD (n = 3).

MIC concentrations (Fig. 1C). This assay also showed that the yeast strains were more susceptible to *X. spinosum* extracts than the tested bacterium.

DISCUSSION

Previous studies of the phytochemical profile of *X. spinosum* extracts, with an emphasis on the phenolic constituents, resulted in the identification of four compounds: axillarin, isoquercitroside, hyperoside, and isochlorogenic acid (Sanz *et al.* 1991). None of these compounds were found in the present study. Also, 38 compounds were previously identified in *X. spinosum* extracts with the majority being phenolics, and others belonging to sesquiterpenes and diterpenes (Varga *et al.* 2020). The majority of phenolic compounds belong to hydroxycinnamic derivates, followed by hydroxybenzoic derivates and flavonoids, and one benzyl alcohol was also detected (Varga *et al.* 2020). Only caffeic acid and flavonoid rutin were found in our extracts and those from the literature (Varga *et al.* 2020). The phenolics of *X. strumarium* L. have been investigated to a slightly greater extent (Ma *et al.* 1998; Lin *et al.* 2014; van Kiem *et al.* 2020; Poljuha *et al.* 2022). Caffeic acid, as well as other hydroxycinnamic acid derivatives were found in the extracts of both species. Caffeic acid, chlorogenic acid, and flavone apigenin are common constituents of *X. spinosum* extracts and those of *X. strumarium* (Ma *et al.* 1998; Lin *et al.* 2014; van Kiem *et al.* 2020; Poljuha *et al.* 2022).

Hashem and collaborators showed that different extracts of *X. spinosum* exhibit activity against *Candida albicans* and *Geotrichum candidum*, but the chloroform extract inhibited the growth of these pathogens the most, with MIC values of 51.0 µg/mL for *C. albicans* and 38.0 µg/mL for *G. candidum* (Hashem *et al.* 2019). The obtained MIC value for *C. albicans* (Hashem *et al.* 2019) differs greatly from the results of the present study where the MICs were almost 10 times higher, 0.25 and 0.5 mg/mL for the *C. albicans* clinical isolate and reference strain. It was shown that xanthatin isolated from the active fraction of the dichloromethane extract was active in concentrations of 100-10 µg/disk against two microfungi *Colletotrichum gloesporoides* and *Trichothecium roseum* (inhibition zones – IZ 25-85 mm, 20-75 mm, respectively), *Bacillus cereus* (IZ 18-12 mm), and *Staphylococcus aureus* (IZ 18-12 mm) (Ginesta-Peris *et al.* 1994). The susceptibility of these two bacteria was investigated in the present study, and different *X. spinosum* extracts exhibited moderate antibacterial activity with MICs ranging from 0.25 to 2 mg/mL.

The antimicrobial activity of *X. strumarium* is more investigated than *X. spinosum* (Scherer *et al.* 2009; Sharifi-Rad *et al.* 2014; Kumar *et al.* 2016). Strong antimicrobial activity against reference strains *S. aureus*, *P. aeruginosa*, *S. typhimurium*, and *C. perfringens*, and *E. coli* isolated from pigs was shown by extracts of *X. strumarium* obtained using different extraction methods (maceration, dynamic maceration, and Soxhlet extraction) with four different solvents [80% ethanol, 80% methanol, ethyl acetate, and dichloromethane/chloroform (1:1)] (Scherer *et al.* 2009). It was also shown that there were no differences between the antimicrobial potential of the *X. strumarium* extracts, with the exception of *S. aureus* and *C. perfringens*, which were affected by the solvent, and not by the extraction method, whereby the

ethyl acetate and dichloromethane/chloroform extracts (1:1) exhibited better antimicrobial activity (Scherer *et al.* 2009). In another study, the antibacterial activity of *X. strumarium* methanolic leaf extract was examined on methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *S. aureus* and showed that the plant extracts were effective against both strains, although their antibacterial activity was higher against MSSA, while in our study, the *X. spinosum* extracts showed weak antibacterial activity against MRSA (Sharifi-Rad *et al.* 2013). It was also shown that silver nanoparticles biosynthesised from the aqueous leaf extract of *X. strumarium* inhibited the growth of *E. coli* and *S. aureus*, more efficiently than the extract itself (Kumar *et al.* 2016). The aforementioned studies highlight that extracts of *X. spinosum* and *X. strumarium*, both species growing in Serbia, possess antimicrobial potential against numerous opportunistic pathogens.

There are several studies concerning the antimicrobial potential of chlorogenic acid. Pure chlorogenic acid was active against several clinical isolates of *Candida*, including *C. albicans* (MIC 128–512 μg/mL), *C. bovina* (MIC 256 μg/mL), *C. parapsilosis* (MIC 128 μg/mL), and *C. krusei* (MIC 128 μg/mL) (Rhimi *et al.* 2020). In the present study, chlorogenic acid was the most abundant in FXsp, but the MICs obtained for *Candida* spp. when treated with this extract ranged from 0.5 to 1 mg/mL, which is higher when compared to the RXsp and LXsp extracts (MIC 0.25–0.5 mg/mL) although chlorogenic acid was less abundant. Also, the MICs for the clinical isolates from the current study treated with the extracts were 0.25–0.5 mg/mL for *C. albicans* (475/15), which fits into the MIC range for chlorogenic acid from the aforementioned study, and 0.5–1 mg/mL for *C. krusei* (H1/16) and *C. glabrata* (4/6/15). The MIC values for the *C. krusei* clinical isolates treated with the *X. spinosum* extracts are higher when compared to the MIC obtained for chlorogenic acid. Chlorogenic acid was also shown to have an anti-arthritic effect on septic arthritis caused by *C. albicans* (Lee *et al.* 2008). In addition, the greater susceptibility of bacterial strains to pure chlorogenic acid was shown when compared to fungal strains (Lou *et al.* 2011). The potential of chlorogenic acid in the reduction of *K. pneumoniae* biofilm formation was also demonstrated (Rajasekharan *et al.* 2017). A comparison with the present study shows that *K. pneumoniae* biofilm formation was inhibited the most with FXsp (35.96 ± 4.56%). Besides chlorogenic acid, other compounds and/or their interactions might also have an impact on the extracts' bioactivities.

CONCLUSION

Xanthium spinosum is a traditionally used widespread annual herb scarcely investigated from the aspect of phytochemistry and biological activity. For the first time we determined the phenolic compounds in the extracts of different parts of *X. spinosum* and evaluated their antimicrobial and antibiofilm activity. Using liquid chromatography mass spectrometry (LC-MS) we identified a total of ten compounds in dichloromethane:methanol (1:1) extracts of the roots, leaves, and fruits. Six constituents were common to all the extracts, with chlorogenic acid as the dominant compound. The studied extracts showed moderate antimicrobial activity against 12 microfungi and 12 bacterial strains. The extracts also showed moderate antibiofilm activity against the *Candida albicans*, *C. parapsilosis*, and *Klebsiella pneumoniae* reference strains. The obtained results enhance the knowledge about the phenolics and biological activity of *X. spinosum*. Further investigation of these or different extracts is recommended in order to develop more efficient antimicrobial agents.

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REZIME

Botanica SERBICA

Sastav fenolnih jedinjenja i antimikrobna aktivnost ekstrakata *Xanthium spinosum* (Asteraceae)

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Xanthium spinosum je jednogodišnja kosmopolitska biljka koja se upotrebljava u tradicionalnoj medicini širom sveta. Iako je poznato da se tradicionalno upotrebljava, ova vrsta je nedovoljno istražena sa fitohemijskog aspekta i aspekta biološke aktivnosti. U ovom radu je istraživan sastav fenolnih jedinjenja i ispitivana je biološka aktivnost ekstrakata različitih delova vrste *X. spinosum*. Specijalizovani metaboliti, fenolna jedinjenja, su ekstrahovani iz korena, listova i plodova pomoću smeše dihlormetan:metanol (1:1) i analizirani pomoću tečne hromatografije sa masenom spektrometrijom (LC-MS). Ukupno je identifikovano i kvantifikovano 10 komponenti. Šest komponenti je identifikovano u svim biljnim delovima. Hlorogena kiselina je dominantna u svim ekstraktima (4,262 mg/g u ekstraktu plodova. 0,820 mg/g u ekstraktu listova i 0,540 mg/g u ekstraktu korena). Biološka aktivnost (antimikrobna i antibiofilm) ekstrakata je testirana na 12 mikrogljiva i 12 bakterijskih sojeva pomoću mikrodilucione metode. Svi ekstrakti su pokazali umerenu antimikrobnu i antibiofilm aktivnost i inhibirali rast većine istraživanih mikroorganizama. Dobijeni rezultati ukazuju na potencijalnu primenu testiranih ekstrakata u medicini i farmaciji.

Ključne reči: fenolne kiseline, flavonoidi, antifungalna aktivnost, antibakterijska aktivnost, antibiofilm aktivnost