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ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM WHEAT, SUNFLOWER AND MAIZE CROP RESIDUES

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Abstract

Large quantities of agricultural residues are generated every year. Most of the crop-based residues are underutilized, mainly left to decay on the land or to be burnt, which can lead to an increase in a load of environmental pollution. Considering this, different strategies have been developed to use these renewable resources as raw materials for the production of bioactive compounds, their isolation and characterization, and potential application in a wide range of fields, particularly in the food industry as natural preservatives. In this study, the antibacterial efficacy of wheat, sunflower, and maize crop residue ethanolic extracts against six bacterial strains (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica*) was evaluated by the broth microdilution method. Used extracts inhibited the growth of selected microorganisms with a minimal inhibitory concentration (MIC) of 320 µg/mL for most of the tested bacteria. *L. monocytogenes* showed a MIC value of 640 µg/mL for wheat ethanolic extract, and the MIC value

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of sunflower ethanolic extract for *S. Typhimurium* was 160 µg/mL. There were no minimum bactericidal concentration (MBC) values for any of the bacteria within the extract's concentration ranges tested (≤ 2560 µg/mL). The results of the present study indicate that crop residue ethanolic extracts could exhibit bacteriostatic effect and therefore have the potential as natural additives in food preservation.

Key words: agricultural waste, MIC and MBC, maize and sunflower stalks, wheat straw, foodborne pathogens

ANTIBAKTERIJSKA AKTIVNOST ETANOLNIH EKSTRAKATA ŽETVENIH OSTATAKA PŠENICE, SUNCOKRETA I KUKURUZA

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Kratak sadržaj

Velika količina poljoprivrednih ostataka se proizvodi svake godine. Većina žetvenih ostataka je nedovoljno iskorišćena, uglavnom se ostavlja da propadne na njivama ili se spaljuje, što dovodi do povećanog zagađenja životne sredine. Imajući ovo u vidu, razvijene su različite strategije za iskorišćavanje navedenih obnovljivih resursa kao sirovina u proizvodnji bioaktivnih jedinjenja, njihovu izolaciju i karakterizaciju i potencijalnu primenu u različitim oblastima, naročito u industriji hrane kao prirodni konzervansi. U ovoj studiji ispitivana je antibakterijska efikasnost etanolnih ekstrakata žetvenih ostataka pšenice, suncokreta i kukuruza na šest bakteri-

jskih sojeva (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* i *Yersinia enterocolitica*) primenom mikrodilucione metode u bujonu. Ekstrakti žetvenih ostataka su inhibirali rast odabranih mikroorganizama u minimalnoj inhibitornoj koncentraciji (MIC) od 320 µg/mL za većinu ispitivanih bakterija. Za *L. monocytogenes* MIC vrednost etanolnog ekstrakta pšenice bila je 640 µg/mL, a 160 µg/mL je bila MIC vrednost etanolnog ekstrakta suncokreta za *S. Typhimurium*. Minimalna baktericidna koncentracija (MBC) prema bakterijama nije postignuta u ispitivanim koncentracijama etanolnih ekstrakata (≤ 2560 µg/mL). Rezultati ukazuju da etanolni ekstrakti žetvenih rezidua bi mogli da ostvare antibakterijski efekat i stoga imaju potencijal kao prirodni aditivi u konzervisanju hrane.

Ključne reči: poljoprivredni otpad, MIC i MBC, stabljike kukuruza i suncokreta, pšenična slama, patogeni koji se prenose hranom.

INTRODUCTION

With the growth of the world's population, which in November 2022 reached 8 billion inhabitants, there is a great necessity for the production of a large amount of food (UN, 2022). Besides China and India, which are the two largest agricultural-producing countries, 157 million hectares of land were used for agricultural production in the European Union (EU) in 2020 (Eurostat, 2022). Therefore, modern agriculture produces a considerable amount of residues every year, and its vast majority is currently dumped and accumulated in landfills or burned (Sadh et al., 2018). In Serbia, the total production of three of the most represented crops in 2021 was as follows: maize 6027131 tons, wheat 3442308 tons, and sunflower 607574 tons (Stat.YearB.Serb, 2022). Even though Serbia has a relatively developed agricultural sector (Zekić et al., 2010), agricultural waste is still an underutilized resource (Maksimović, 2022).

There are two types of agricultural residues: field (crop) and process residues. Field residues remain in the field after crop harvesting and consist of leaves, stalks, straws, seed pods, stems, hulls, cobs, and weeds. Process residues are residues present even after the crop is processed into a valuable alternate resource, and these include husks, seeds, roots, bagasse, and molasses (Sadh et al., 2018). The limited and inadequate management of this agricultural waste causing environmental pollution is a global issue that emerged an urgent need to develop strategies based on new sustainable and circular models for waste timely utilization and valorization (Carpena et al., 2022).

Numerous studies have demonstrated that agro-industrial residues are essential sources of various complex and structurally diverse bioactive compounds, including flavonoids, hydroxycinnamic acid derivatives, phenolic acids, tannins ascorbates, lignans, carotenoids, tocopherols, phytosterols and arabinoxylans (Babbar and Oberoi, 2014; Sadh et al., 2018). Hence, mainly process residues are shown to be raw materials with good prospects for extracting and identifying new compounds with antimicrobial and antioxidant potential (Sihem et al., 2015; Sheng et al., 2022). On the other hand, field residues are abundant lignocellulosic biomass that varies slightly in composition with cellulose, hemicellulose and lignin as the major constituents, and the knowledge of their potential as a raw material for the extraction of different bioactive phenolics is limited (Singh nee' Nigam et al., 2009). Only few data are found in the literature regarding phenolic compounds content in crop field residues, such as wheat, maize and sunflower (Kumar and Goh, 2003; Vijayalaxmi et al., 2015; Alexandrino et al., 2021).

During the last two decades, extensive research has been devoted to discovering new antimicrobial agents, mainly from plants and other natural sources that could be applied in pharmaceutical and cosmetic products, as well as in the food industry (Nazzaro et al., 2013). With the development of the all-natural and green-label trend and consumer awareness of food safety and quality, meat and meat products that are highly susceptible to the growth of spoilage microorganisms and foodborne pathogens are of particular interest in regard to finding natural preservatives that could be used as safe antimicrobials and antioxidants in meat matrix and packaging (Ji et al., 2021).

Various basic standard methods and complex bioassays have been developed for *in vitro* antimicrobial susceptibility testing; the well-known and most commonly used methods include disk diffusion, well diffusion and broth or agar dilution (Balouiri et al., 2016). The microdilution method is considered a valuable tool for detecting the resistance to antimicrobials and comparing different susceptibility since it is a fast method that does not require many resources and provides information on the lowest concentration that inhibits bacterial or fungal growth (Kolarević et al., 2016).

Considering all mentioned above, the present work is designed to investigate the antimicrobial effect of ethanolic extracts from three different agricultural residues - wheat, sunflower, and maize against several microbial strains (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica*) which are the pathogens identified as the most common causes of foodborne diseases.

MATERIAL AND METHODS

Plant materials

Wheat, maize and sunflower harvest residues (maize and sunflower stalks, as well as wheat straw) originating from the territory of the Autonomous Province of Vojvodina (Serbia) were collected after the harvest time, between July and October 2021 and dried naturally in a shaded and well-ventilated place. A 3 kg quantity of each material was first reduced to smaller particles using a grinder; then extracted with a six-fold weight of hexane for 1 h at 40 °C in industrial stainless steel 60 L extractor. Each of obtained hexane extracts was vacuum filtered through 87 g/m² filter paper to remove the hard residues and concentrated using a DLAB RE 200 Pro industrial rotary evaporator (60 °C, 60 rpm, 216-200 mbar, 150 min). After extraction with hexane, plant material was left aside for 24 h in the open air, protected from direct sunlight, in order to remove the traces of residual solvent, and extracted again, for 1 h at 45 °C using a six-fold weight of 96% ethanol, followed by filtration and evaporation under the same working conditions.

Ethanol extracts were used for further investigations. An aliquot of each extract was taken and diluted with DMSO before the analyses, as described further in the text.

Test organisms

Six reference bacterial strains were investigated: *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19111 and *Yersinia enterocolitica* ATCC 9610.

Minimum inhibitory concentration (MIC)

The susceptibility of the selected isolates to active compounds was investigated by the broth microdilution method (CLSI, 1999; 2020; ISO, 2019). Each crop residue extract was tested in triplicates. The inoculum was prepared by the colony suspension method. The stock control culture of each of the six reference strains was sub-cultured on non-selective nutrient agar (NA) (Oxoid[®], UK) at 37 °C for 18 h to 20 h. Three to five pure colonies of each microorganism were touched with a loop and suspended in 5 mL sterile saline. The suspension was adjusted to give a turbidity equivalent to a 0.5 McFarland standard using

a spectrophotometer Cecil 2021 UV/VIS (Select Science, Bath, UK) where at 625 nm wavelength and a 1 cm path cuvette, the absorbance was in the range of 0.08 – 0.13. The prepared adjusted inoculum (approximately 1×10^8 CFU/mL) was diluted by transferring 0.1 mL of standardized isolate suspension to a tube containing 9.9 mL of Cation Adjusted Mueller-Hinton broth (CAMHB) (BBL™ Mueller Hinton II Broth, Becton, Dickinson and Company, Sparks, USA) (1:100 dilution) to obtain suspension of 1×10^6 CFU/mL, so when 50 μ l is added to an equal volume (50 μ l) of the examined solution, resulted in a final inoculum of 5×10^5 CFU/mL. For *L. monocytogenes* modified susceptibility medium, CAMHB without adding 5% lysed horse blood and 20 mg/mL β -NAD was used (Takahashi et al., 2013). Wheat, sunflower and maize crop residues extracts were diluted in DMSO (Fisher Scientific™, UK) and added to CAMHB at levels from 2560 μ g/mL to 1.25 μ g/mL by two-fold dilution in U-bottom 96-well microtiter plates (Kartell S.p.A., Italy). After inoculation, plates were incubated at 37 °C for 18-20 h. MIC was determined as the lowest concentration of an active compound that prevented the visible growth of bacteria in the broth dilution susceptibility test (CLSI, 2012). Tetracycline (Fisher Scientific™, UK) was used as a control in the range of 64 to 0.03 μ g/mL. The plates also included a negative control (media only) and a bacteria growth control (media and bacteria).

Minimum bactericidal concentration (MBC)

Following MIC determination of the crop residue extracts and antibiotic yielding a negative microbial growth after incubation, a well's content (10 μ L) was sub-cultured on the surface of NA plates to determine the number of surviving cells (CFU/mL). The plates were then incubated overnight at 37 °C. The minimum bactericidal concentration (MBC) endpoint was defined as the lowest concentration of extract that kills > 99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the NA plates (CLSI, 1999). The tests were carried out in triplicate.

RESULTS

Using described methodology, the following yields of ethanol extracts were obtained: sunflower 2.55%, wheat 1.35%, and maize 1.32%.

The *in vitro* antimicrobial activity of ethanolic wheat, sunflower and maize crop extracts and the commercial antimicrobial agent is demonstrated in Table 1. For most of the tested bacteria, the MIC value at the examination of

all three crop residue extracts was 320 µg/mL. The exception was *L. monocytogenes* which revealed MIC value of 640 µg/mL MIC for wheat ethanolic extract, while the MIC of sunflower ethanolic extract for *S. Typhimurium* was 160 µg/mL. There was no MBC value for any of the microorganisms at the extract's concentrations used (> 2560 µg/mL).

Table 1. Minimal inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) breakpoints of examined crop residues extracts for six bacterial isolates

Organism	Wheat		Sunflower		Maize		Tetracycline	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
<i>S. Typhimurium</i>	320.00	>2560.00	160.00	>2560.00	320.00	>2560.00	0.125	64.00
<i>S. Enteritidis</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	1.00	> 64.00
<i>S. aureus</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	1.00	> 64.00
<i>E. coli</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	1.00	> 64.00
<i>L. monocytogenes</i>	640.00	>2560.00	320.00	>2560.00	320.00	>2560.00	2.00	> 64.00
<i>Y. enterocolitica</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	2.00	> 64.00

DISCUSSION

The susceptibility of the bacterial strains toward the commercial antibiotic tetracycline used in the present study was in accordance with the data reported in the literature (Musumeci et al., 2003; Purushotham et al., 2010).

The chemical characterization of the main constituents of plant parts that are considered crop residues, including those examined in the present study was summarized by Sadh et al. (2018). Slight differences in the chemical composition among these three crop residues were detected; the highest content of cellulose (61.2%) and the lowest content of lignin (6.9%) were determined in maize stalk residues, sunflower stalks had the highest content of hemicellulose (29.7%), lignin (13.4%) and ash (11.17%), while wheat straw had the lowest content of cellulose (32.9%) and ash (6.7%) (Singh nee' Nigam et al., 2009; Martin et al., 2012; Sadh et al., 2018). Furthermore, previous research

investigated the antioxidant properties of different agro-industrial wastes (Cámara et al., 2020; Carpena et al., 2022); however, the antimicrobial activity has been less studied (Martin et al., 2012; Martillanes et al., 2020; Alexandrino et al., 2021). To our knowledge, there is no data on the antimicrobial effect of ethanolic maize and sunflower stalks, and wheat straw extracts. As can be observed, all three extracts inhibited the growth of gram-negative and gram-positive microorganisms in a similar way, except for the lower sensitivity of *L. monocytogenes* and the highest sensitivity of *S. Typhimurium* to wheat and sunflower extracts, respectively. The determination of the chemical composition of various plant residues revealed the presence of different classes of secondary metabolites with the most abundant phenolic compounds that have been shown to inhibit the growth of foodborne pathogens and spoilage bacteria (Vijayalaxmi et al., 2015; Gomes-Araújo et al., 2021). These phenolic compounds in the wheat, sunflower and maize crop extracts could give a preliminary explanation of the antimicrobial activities observed in the present study. The slight differences in the exhibited activity could be due to the differences in chemical composition, the concentration of the main bioactive constituents, their mechanism of action, and possible interaction with other components in the final extract (Dzotam et al., 2015; Hemeg et al., 2020). Corroborating our results, Alexandrino et al. (2021) demonstrated that the ethanolic extract of defatted sunflower seed flour exhibited antimicrobial activity against *S. aureus*, *Bacillus subtilis*, *E. coli*, and *Pseudomonas aeruginosa* with the MIC values in the range from 11.6 to 33.2 mg chlorogenic acid (CGA) eq/mL. The highest susceptibility to sunflower seed extract showed *E. coli* (Alexandrino et al., 2021), while in our study *S. Typhimurium* was the most sensitive bacteria. The total phenolic content of this sunflower seed flour was 4.00 g CGA eq/100 g on a dry basis. After concentration, the ethanolic extract had a total phenolic value of 15.44 g CGA eq/100 g, with 62% of chlorogenic acid as the predominant phenolic compound (Alexandrino et al., 2021). According to previous reports, among the analyzed phenolic compounds, chlorogenic acid was predominant in defatted sunflower kernels and shells (Weisz et al., 2009). The antibacterial effect of sunflower extracts was mainly attributed to chlorogenic acid as it can bind to the outer bacteria membrane, increase the permeability of the outer and plasma membrane and lead to its damage with the leakage of intracellular components, finally resulting in cell death (Lou et al., 2011). The efficiency of sunflower-based extracts depends on the concentration of chlorogenic acid, that is, on the purity of the obtained extracts after the extraction process and the presence of other compounds in addition to phenolic components, such as soluble sugars or proteins (Alexandrino et al., 2021). Namely, the inhibition of

the growth of various bacterial strains, including *S. aureus*, *Streptococcus pneumoniae*, *B. subtilis*, *E. coli*, *Shigella dysenteriae*, and *P. aeruginosa* is achieved at concentrations from 10 to 30 times lower using chlorogenic acid with $\geq 98\%$ purity (Lou et al., 2011; Fu et al., 2017).

Martillanes et al. (2020) found that in both aqueous and ethanolic rice bran extract, with trans-ferulic acid, *p*-coumaric acid, and γ -oryzanol as the main components, the growth of *E. coli* and *L. innocua* was inhibited. However, the percentage of inhibition was notably higher in an ethanolic extract with high γ -oryzanol and low phenolic compounds concentration. Contrarily, by examination of methanolic and ethanolic extracts from 20 different agro-industrial wastes, the positive correlation between total phenolic content and antimicrobial activity was confirmed (Martin et al., 2012). Martin et al. (2012) found that, besides the absence of an inhibitory effect against gram-negative bacteria (*S. Enteritidis* and *E. coli*), the best antimicrobial activity against *S. aureus* showed ethanol extract of peanut peel with a MIC value of 0.78 mg/mL and total phenolic value of 374.5 gallic acid equivalent (GAE)/kg, while *L. monocytogenes* growth was inhibited by guava bagasse ethanol extract (1.56 mg/mL) with a total phenolic value of 43.1 GAE/kg. Compared with the results of our study, ethanolic wheat, sunflower and corn crop residues showed higher antimicrobial potency and inhibited both gram-positive and gram-negative bacteria in concentrations twice lower than those obtained by Martin et al. (2012). Among the compounds with antibacterial activity in agro-industrial waste extracts, Martin et al. (2012) confirmed the predominant presence of dicarboxylic acids: azelaic and succinic acids, then caffeic, *p*-coumaric, syringic, gallic, ferulic acids and flavonoids: epicatechin, myricetin, and quercetin. Earlier studies reported 0.9% of polyphenols in wheat crop residues (Kumar and Goh, 2003) and the following organic acids in the wheat straw water extract: *o*-dihydroxybenzene, *p*-hydroxybenzoic acid, ferulic acid, and catechinic acid (Hongzhang and Liying, 2007).

In the present study, 96% ethanol was used for the extraction of polyphenol-rich paste from crop residues, whereas Vijayalaxmi et al. (2015) demonstrated that by using 100% ethanol, the extraction yield was better for wheat bran (3.5%) compared to corn husk (4%) and that the total polyphenols, total tannins and total flavonoids contents in corn husk extract were 35.80 g GAE/100 g extract, 29.33 g tannic acid equivalents (TAE)/100 g extract and 7.35 g quercetin equivalents (QE)/100 g extract, respectively, while in wheat bran these contents were 40.12 g GAE/100 g extract, 33.35 g TAE/100 g and 5.86 g QE/100 g extract, respectively. In that study, HPLC analysis of corn husk and wheat bran extracts detected two major peaks corresponding to gallic acid

and ferulic acid and three minor peaks identified as epicatechin, quercetin and kaempferol.

The observed antibacterial activity of our crop residue extracts could be due to the flavonoids; even though they could be found in small amounts, they exhibit membrane-disrupting activities. The mechanism of interaction involves the specific binding of flavonoids with the polar head groups of membrane lipids and non-polar compounds inside the membrane, as well as non-specific interactions of flavonoids and phospholipids that change the thickness and fluctuations of the membrane and, therefore, indirectly modulate the distribution and/or function of membrane proteins. In this way, binding to the lipid bilayer and inactivation and inhibition of intracellular and extracellular enzymes synthesis results in bacterial cell membrane damage and increased permeability (Górniak et al., 2019). In addition, tannins found in crop extracts can exhibit an antibacterial effect due to interactions with proteins in the bacterial cell wall, formation of stable water-insoluble protein components, and interfering with protein synthesis (Si et al., 2012). Si et al. (2012) found that tannins obtained by ethanolic extraction from agricultural by-products inhibited the growth of several pathogenic bacteria, including *L. monocytogenes*, *E. coli*, and Methicillin- and Vancomycin Resistant *S. aureus*.

In accordance with the present results, Alexandrino et al. (2021) did not report the bactericidal effect of the sunflower seed flour ethanolic extract against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* in the maximum concentration used (39.8 mg eq CGA/mL). On the other hand, Martin et al. (2012) showed that out of seven ethanolic extracts of different agro-industrial wastes, six had a bactericidal effect against *E. coli* and five against *L. monocytogenes*, with the observation that the bactericidal potential was lower than the inhibitory one so that, e.g., the MBC value of the most effective extract (guava bagasse) against *L. monocytogenes* was eight times higher than the MIC value.

CONCLUSION

The results of our study indicate that ethanolic extract of wheat, sunflower, and maize crop residues possess bacteriostatic activity against some of the most common foodborne pathogens. This preliminary investigation suggests that non-edible plant parts biomass, mainly consisting of lignocellulose, has the potential as a low-value renewable source for the extraction of bioactive compounds. However, for a better understanding of the mechanism of action of the examined crop residue extracts, further research is required to precisely determine the chemical composition and identify those phenolic compounds

to which antimicrobial activity can be attributed. For the appropriate exploitation of such residues in the production of added value compounds, optimizing the extraction process and applying methods that would allow the subsection of a large amount of agricultural residues to treatment with minimal environmental impact and therefore to obtain more extract in a cost-effective way is necessary. In the context of crop residue extracts as potential natural antioxidants and antimicrobial agents that could be alternatives to synthetic additives in foods, especially in meat products, research should be directed towards consumer protection in terms of determining whether such extracts are safe for consumption, and later to develop technological processes for meat product reformulation by using such new additives with a deviation in sensory characteristics of the products that would be acceptable to consumers.

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Author's Contribution:

MG and MBC - made equal contributions to conceptualization, data curation, formal analysis, investigation and methodology regarding MIC and MBC analysis and wrote an original draft. NČ carried out validation, visualization, and revised the manuscript. MŽB was involved in supervision, funding acquisition and revised the manuscript critically. JV performed microbiology analysis, revised and edited the draft. SS carried out plant material collection, the extracts preparation, reviewed and edited the manuscript. ZM made contributions to conceptualization, project administration, funding acquisition, supervision, review and editing of the draft.

Competing interest

The authors declare no conflicts of interest.

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