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Antimicrobial Assessment of *Prunus spinosa* L. Fruit Extracts

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Abstract

Prunus spinosa L., commonly known as blackthorn or sloe, is a member of the *Rosaceae* family that typically grows as a shrub on the slopes of uncultivated, wild terrain. In late autumn, fresh fruits were collected from three distinct habitats in Bosnia and Herzegovina: Borije, Vareš, and Trnovo. This study investigated the antibacterial activity of fruit extracts prepared using the Soxhlet method, employing *n*-hexane, ethanol, diethyl ether, and dichloromethane. Antibacterial activity was assessed *in vitro* using the agar well diffusion method against standard bacterial strains: Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212), Gram-negative (*Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 31194, *Escherichia coli* ATCC 25922), and fungus (*Candida albicans* ATCC 1023). Four solvents served as negative controls, while the antibiotic streptomycin and the antimycotic nystatin served as positive controls. All the blackthorn extracts demonstrated varying degrees of antimicrobial activity against the bacterial strains tested. Ethanol fruit extracts from Borije, Trnovo, and Vareš exhibited the strongest antibacterial activity against the Gram-positive bacterial strain *S. aureus,* with inhibition zones measuring 21.00, 23.50, and 25.00 mm, respectively. Compared to the control sample, the extracts from Trnovo and Vareš displayed greater potency against this bacterial strain. The diethyl ether extract from Vareš was more effective than streptomycin against *E. faecalis*, exhibiting a highly sensitive inhibition zone of 22.00 mm. These results suggest that the blackthorn fruit extracts studied possess strong antibacterial activity.

Keywords

Prunus spinosa L., antimicrobial activity, Soxhlet extraction, fruit extracts

1 Introduction

Fruits from wild shrubs have been used for centuries in traditional medicine as well as in producing foods such as teas, jams, and juices. Consumers prioritise food quality, and opt for products that not only taste good but also offer potential health benefits. In Bosnia and Herzegovina, blackthorn (*Prunus spinosa* L.), also known as sloe, is widely used in traditional medicine due to its notable phytotherapeutic properties.[1,2] Blackthorn, a perennial plant from the *Rosaceae* family, grows as a shrub in the northern hemisphere's temperate continental environment, typically on the slopes of vast, uncultivated areas. It blooms with five-petalled white flowers in March and April, followed by deep blue-purple fruits that ripen in October. The plant's flowers, bark, roots, and fruits are recognised for their medicinal properties. They are used to treat various ailments due to their cytotoxic, diuretic, spasmolytic, antibacterial, and antioxidant properties, particularly with regard to human prostate cancer cell lines.[3–5] The *in vitro* antiproliferative activity of blackthorn flower, leaf, and fruit ethanolic extracts from Bosnia and Herzegovina was investigated against human prostate cancer cell lines PC-3 and DU145. All extracts, especially leaf extracts, possess good antiproliferative activity against these cell lines.[3–5] Blackthorn extracts have been found to contain polyphenolic compounds such as kaempferol, quercetin, phenolic acids (caffeine and neochlorogenic derivatives), coumarin derivatives (umbelliferone, scopoletin, and esculetin), and anthocyanins, which are the most potent natural antioxidants and antibacterial agents.[1,6–8] Polyphenols found in fresh fruit extracts can greatly reduce the negative effects of free radicals and inhibit the growth of pathogens in the body. Extracts from blackthorn flowers are also used to treat inflammation, urinary tract disorders, and cardiovascular diseases.[3] They are crucial in preventing cancer, cardiovascular conditions, and neurodegenerative illnesses.[9] The mineral profile as well as the antioxidant and antimicrobial activity of blackthorn fruit samples were determined by *Dedić et. al.*[1] Potassium, calcium, magnesium, sodium, and iron were established as major minerals of the fruit. All plant extracts (flower, leaf, and fruit) exhibited varying degrees of antimicrobial activity against six bacterial strains tested and possessed high phenolic, flavonoid, and anthocyanin contents, and high antioxidant activity. The potent antimicrobial and antioxidative activity might result from its high content of polyphenolic compounds.[1] The growing interest in wild fruits for their antioxidant, antimicrobial, and antiproliferative properties emphasises their potential for scientific research and industrial application (dietary, pharmaceutical, and cosmetic).[10] As bacterial resistance to synthetic antibiotics, especially among Gram-negative bacteria, continues to rise, the use of plant-based preparations for bacterial control and suppression has gained importance due to the ability of plants to synthesise biologically active substances, which have an effect similar to

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antibiotics.[11,12] Studies have shown that plant antimicrobial activity can be primarily attributed to the natural phenolic antioxidants extracted from specific plant parts. Phenolic and flavonoid compounds extracted from plant material are among the main contributors to antioxidant and antimicrobial effects.[13] This study aimed to investigate the potential biological activity of wild blackthorn fruits collected from three regions in Bosnia and Herzegovina. Considering blackthorn extracts as a potential natural source of phenolic compounds, the focus of this work was placed on assessing their *in vitro* antimicrobial activity.

2 Experimental

2.1 Plant material

Fresh blackthorn (*Prunus spinosa* L.) fruits were gathered from three distinct locations in Bosnia and Herzegovina: Vareš (altitude 739 m; 44° 04′ 40.82″ N, 18° 14′ 46.41″ E), Trnovo (altitude 935 m; 43° 41′ 19.37″ N, 18° 22′ 34.39″ E), and Borije (altitude 892 m; 43° 51′ 16.60″ N, 18° 28′ 55.33″ E). The taxonomical analysis of the plant samples was conducted by the research group of Prof. Senka Barudanović at the University of Sarajevo's Faculty of Science, Department of Biology. Vouchers for the specimens were deposited at the University of Sarajevo's Faculty of Pharmacy Herbarium (voucher no. FFOH/1001-09). Fig. 1 shows the fresh blackthorn fruits collected in Trnovo. Fresh blackthorn fruits were used for the preparation of the extracts.

Fig. 1 – Blackthorn fruits

2.2 Chemicals and reagents

All the solvents, reagents, and standards used were of analytical grade. Ethanol (96 %) was obtained from Merck, Darmstadt, Germany. Diethyl ether, dichloromethane, and *n*-hexane were purchased from Sigma Aldrich, Chemie GmbH, Germany, while Mueller Hinton Agar was obtained from HI Media laboratories, India.

2.3 Sample preparation

A total of 100 g of fresh fruits were thoroughly washed with tap water followed by distilled water, and tapped dry using a paper towel. Twelve extractions were carried out using *n*-hexane, ethanol, diethyl ether, and dichloromethane in a Soxhlet apparatus for six hours. The extracted volumes were reduced to about 5–7 ml using a rotary evaporator. The resulting extracts were stored in a refrigerator at 4 °C until further analysis and investigations.

2.4 Antimicrobial activity (bacterial strains)

The bacterial strains were supplied from the American Type Culture Collection (ATCC). This study used standard bacterial strains, including Gram-positive (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212), Gram-negative (*Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 31194, *Escherichia coli* ATCC 25922), and fungal (*Candida albicans* ATCC 1023) strains (Fig. 2).

The test bacteria strains were cultivated on Petri dishes containing Mueller Hinton Agar (MHA) and incubated for 18–24 h.

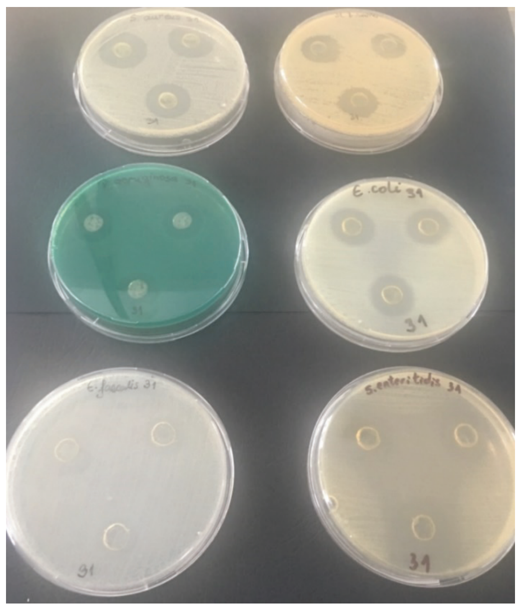

Fig. 2 – Inhibition zones for ethanol extracts from Trnovo (all six analysed bacterial strains)

2.5 Antimicrobial activity detection by the agar well diffusion method

The agar well diffusion method was used to evaluate the *in vitro* antibacterial and antifungal activities of *n*-hexane, ethanol, diethyl ether, and dichloromethane extracts against gram-positive and gram-negative bacterial strains, as well as fungi. Solvents served as negative control, while the antibiotic streptomycin (10 μg, Oxoid) and the antimycotic nystatin (10 μg, Oxoid) served as positive controls.

Wells were created in the MHA plates using a sterile Pasteur pipette. A freshly prepared bacterial suspension or spore solution, adjusted to $1.5 \cdot 10^8$ CFU/ml, was inoculated onto the agar plate surfaces using a sterile swab. One hundred microlitres of each extract were dispensed into the wells. The plates were stored at +4 °C for 2 h to allow the extracts to diffuse into the agar, and were then incubated at 37 °C for 18–24 h.[14] Each experiment was performed in triplicate. The diameters of the inhibition zones surrounding the wells were measured (Fig. 2).

3 Results and discussion

The sizes of the inhibition zones were used to assess the antibacterial activity of *n*-hexane, ethanol, diethyl ether, and dichloromethane extracts from fresh blackthorn fruits against seven pathogens, including six bacteria and one fungus. Both Gram-positive and Gram-negative bacterial strains are commonly associated with infectious diseases.

The antibacterial activity was categorised based on the sizes of the inhibition zones: Sensitive (> 18 mm), intermediate (13–17 mm), and resistant (< 13 mm).[15] The results were compared to the controls, with nystatin serving as the control for the fungus and streptomycin for bacteria.

The inhibition zones for the investigated extracts are presented in Tables 1–3. It should be noted that the antibacterial activity of each plant extract varied across the bacterial strains tested.

The ethanol fruit extracts from Borije, Trnovo, and Vareš exhibited the strongest antibacterial activity against the Gram-positive bacterial strain *S. aureus,* with inhibition

Table 1 – Antimicrobial activity of fresh blackthorn fruit extracts from Borije obtained by the Soxhlet extraction method*

Strain	Inhibition zone / mm				Control / mm
	Extract (Borije)				
	n-Hexane	Ethanol	Diethyl ether	Dichloromethane	Streptomycin
Gram-positive strains					
Bacillus subtilis	–	16.67 ± 0.58^{b}	11.00 ± 0.00^{c}	11.67 ± 1.15^{c}	28.00 ± 0.58^{a}
Staphylococcus aureus	–	21.00 ± 1.73^{b}	12.00 ± 1.00^{c}	21.33 ± 2.52^{b}	23.00 ± 0.00^{a}
Enterococcus faecalis	–	17.67 ± 0.58^{a}	–	–	12.00 ± 0.58^{b}
Gram-negative strains					
Pseudomonas aeruginosa	–	19.67 ± 0.58^{b}	11.00 ± 0.00^{c}	21.67 ± 2.08^{a}	21.00 ± 0.00^{a}
Salmonella enterica	–	14.00 ± 1.00^{b}	–	13.00 ± 0.00^{c}	22.00 ± 0.00^{a}
Escherichia coli	10.83 ± 0.29^{d}	12.00 ± 0.00^{c}	–	14.00 ± 1.00^{b}	20.00 ± 0.00^{a}
Fungi					Nystatin
Candida albicans	–	11.50 ± 0.00^{c}	–	17.00 ± 1.73^{b}	21.00 ± 0.00^{a}

* Values in a column that do not share the same letter are statistically different at the 5 % significance level

Table 2 – Antimicrobial activity of fresh blackthorn fruit extracts from Trnovo obtained by the Soxhlet extraction method*

Strain	Inhibition zone / mm				Control / mm
	Extract (Trnovo)				
	n-Hexane	Ethanol	Diethyl ether	Dichloromethane	Streptomycin
Gram-positive strains					
Bacillus subtilis	–	19.67 ± 0.51^{b}	16.67 ± 0.58^{c}	12.67 ± 0.58^{d}	28.00 ± 0.58^{a}
Staphylococcus aureus	–	23.50 ± 1.32^{a}	19.33 ± 0.58^{b}	–	23.00 ± 0.00^{a}
Enterococcus faecalis	–	16.67 ± 0.84^{a}	17.67 ± 0.58^{a}	–	12.00 ± 0.58^{b}
Gram–negative strains					
Escherichia coli	11.33 ± 0.58^{c}	19.83 ± 0.75^{a}	12.00 ± 0.00^{c}	17.67 ± 0.58^{b}	20.00 ± 0.00^{a}
Salmonella enterica	11.33 ± 0.58^{d}	20.33 ± 0.51^{b}	14.00 ± 1.00^{c}	11.00 ± 0.00^{c}	22.00 ± 0.00^{a}
Pseudomonas aeruginosa	–	19.17 ± 0.25^{b}	18.33 ± 1.15^{bc}	16.67 ± 1.53^{c}	21.00 ± 0.00^{a}
Fungi					Nystatin
Candida albicans	–	11.00 ± 0.00^{b}	–	–	21.00 ± 0.00^{a}

* Values in a column that do not share the same letter are statistically different at the 5 % significance level

Table 3 – Antimicrobial activity of fresh blackthorn fruit extracts from Vareš obtained by the Soxhlet extraction method*

Strain	Inhibition zone / mm				Control / mm
	Extract (Vareš)				
	n-Hexane	Ethanol	Diethyl ether	Dichloromethane	Streptomycin
Gram-positive strains					
Bacillus subtilis	–	15.67 ± 1.53[b]	–	–	28 ± 0.58[a]
Staphylococcus aureus	–	25.00 ± 0.00[a]	–	–	23 ± 0.00[b]
Enterococcus faecalis	–	–	22.00 ± 1.00[a]	–	12 ± 0.58[b]
Gram-negative strains					
Pseudomonas aeruginosa	–	19.00 ± 0.00[b]	10.50 ± 0.00[c]	19.00 ± 1.73[b]	21 ± 0.00[a]
Salmonella enterica	–	15.00 ± 1.00[b]	–	15.33 ± 0.58[b]	22 ± 0.00[a]
Escherichia coli	10.67 ± 0.58[c]	13.00 ± 1.00[b]	–	11.00 ± 0.00[c]	20 ± 0.00[a]
Fungi					Nystatin
Candida albicans	–	–	–	–	21 ± 0.00[a]

* Values in a column that do not share the same letter are statistically different at the 5 % significance level

zones measuring 21.00, 23.50, and 25.00 mm, respectively. For the same bacterial strain, these results were significantly different from those of the other extracts ($p < 0.05$). The extracts from Trnovo and Vareš (Fig. 3) demonstrated greater effectiveness against this bacterial strain compared to the control sample, streptomycin.

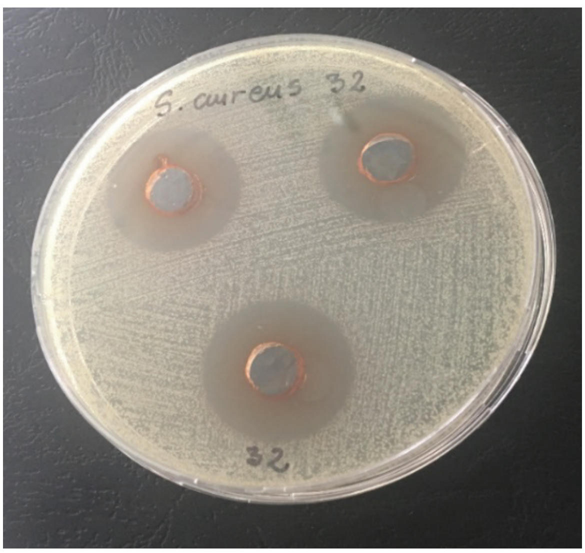

Fig. 3 – Inhibition zone of the ethanol extract from Vareš (bacterial strain *S. aureus*)

The diethyl ether extract from Vareš demonstrated stronger antibacterial activity than streptomycin against *E. faecalis*, producing a sensitive inhibition zone (22.00 mm). Data from Tables 1–3 indicate that only three extracts from the three study areas exhibited antifungal activity against *Candida albicans*. The most notable result was recorded for the dichloromethane extract from Borije, with an inhibition zone diameter of 17.00 mm. All three ethanol samples (from Borije, Trnovo, and Vareš) showed similar results against the bacterial strain *P. aeruginosa*. Similar studies reported in the literature support these findings.[1,4,16]

The phytochemical profiles of the blackthorn fruit Soxhlet extracts in *n*-hexane, ethanol, diethyl ether, and dichloromethane were determined using chemical tests, and the results are presented in Table 4. Phytosterols were identified using the Liebermann Burchard and Salkowski tests, phenols through ferric chloride and lead acetate tests, flavonoids using the lead acetate and magnesium tests, glycosides with the Killer-Kallani test, alkaloids using the Dragendorff test, and tannins through a specific test with ferric (III) chloride.

The antibacterial activity of ethanol extracts from blackthorn flowers, leaves, and fruits was tested against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. enterica*, and *E. faecalis*, as well as their antifungal activity against *C. albicans*. Fruit extracts from three distinct regions in Bosnia and Herzegovina demonstrated the highest antioxidant activity, whereas leaf extracts showed the strongest antimicrobial activity against both Gram-positive and Gram-negative bacterial strains.[1] These findings align with the study by *Veličković et al.*[4] In their study, phenolic acids (neochlorogenic and caffeic acids), flavonoids (quercetin and myricetin), and anthocyanins (cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, and peonidin-3-O-glucoside) were identified in ethanol blackthorn extracts through HPLC analysis, supporting the extracts' potent antioxidative and antimicrobial properties.[4]

All three extracts from Bosnia and Herzegovina showed sensitive and intermediate inhibition zones against *B. subtilis*. *Gegiu et al.*[16] investigated the antimicrobial activity of dried fruit pulp from *P. spinosa* L. against the reference strains *Streptococcus* ATCC 19615, *Staphylococcus* ATCC

Table 4 – Phytochemical profiles of blackthorn fruit Soxhlet extracts in different solvents

Extract	Solvent	Phytosterols	Phenols	Flavonoides	Cardio-glycozides	Alkaloids	Tanins
Fruit	*n*-Hexane	+	–	–	–	–	–
	Ethanol	+	+	+	+	–	+
	Diethyl ether	+	+	–	+	+	–
	Dichloromethane	+	–	–	+	–	–

25923, *Pseudomonas* ATCC 27853, *Enterococcus* ATCC 19433, *E. coli* ATCC 25922, and *C. albicans* ATCC 10231. Their findings, based on the disc diffusion method, revealed that while the tested solutions exhibited no antifungal activity, they showed varying levels of antibacterial activity depending on the sample concentration. It was evident that the *C. albicans* strains were completely resistant to both solution concentrations prepared from plants collected in two Romanian regions, Argeş and Tulcea.

4 Conclusion

In this study, fresh blackthorn fruit extracts prepared via Soxhlet extraction exhibited antibacterial activity against six bacterial strains and one fungus. The examined extracts exhibited strong antibacterial activity, with ethanol extracts demonstrating the most potent effects, whereas *n*-hexane extracts were the least effective. This was to be expected, since ethanol is a more polar solvent than the others tested in this study. Polyphenols, which are well-documented as effective antibacterial agents, were separated as polar components using ethanol. The diethyl ether extracts from Vareš and Trnovo exhibited significantly better antibacterial activity against *E. faecalis* bacterial strains compared to the positive control, streptomycin. Similarly, the ethanol extracts from Trnovo and Vareš were more effective than the control sample against the bacterial strain *S. aureus*. Based on these results, it can be concluded that the investigated blackthorn fruit extracts possess significant biological potential as antimicrobial agents. It is particularly important to note that the tested extracts displayed sensitive, intermediate, and resistant inhibition zones against Gram-negative bacterial strains. This aligns with the widely recognised challenge of increasing resistance among Gram-negative bacteria to certain antibiotics, such as penicillin. The results of this study suggest that blackthorn fruit extracts could serve as a complementary treatment or be incorporated into pharmaceutical formulations to enhance antibacterial efficacy.

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List of abbreviations

ATCC – American Type Culture Collection
MHA – Mueller Hinton Agar
CFU – Colony-forming Unit

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SAŽETAK

Antimikrobna procjena ekstrakta voća *Prunus spinosa* L.

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Prunus spinosa L., biljka iz porodice *Rosaceae*, poznata i kao trnjina ili crni trn, raste kao žbunje na padinama divljih, neobrađenih terena. Svježi plodovi sakupljani su u kasnu jesen s tri različita staništa: Trnovo, Vareš i Borije u Bosni i Hercegovini. Predmet istraživanja bila je antimikrobna aktivnost *n*-heksanskih, etanolnih, dietil eterskih i dihlormetanskih ekstrakata plodova trnjine dobivenih Soxhlet ekstrakcijom. U tu svrhu provedena je analiza *in vitro* metodom difuzije prema standardnim bakterijskim sojevima: gram-pozitivnim (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212), gram-negativnim (*Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 31194, *Escherichia coli* ATCC 25922) i u odnosu na gljivicu (*Candida albicans* ATCC 1023). Kao pozitivne kontrole upotrijebljeni su antibiotik streptomicin i antimikotik nistatin, a kao negativne kontrole četiri otapala. Može se primijetiti da su svi ekstrakti pokazali različit stupanj antimikrobne aktivnosti prema testiranim sojevima bakterija. Najbolju antibakterijsku efikasnost pokazali su etanolni ekstrakti plodova trnjine s područja Borija, Trnova i Vareša, sa zonama inhibicije od 21,00, 23,50 i 25,00 mm prema gram-pozitivnoj bakteriji *S. aureus*. Ekstrakt s područja Trnova i Vareša potentniji je prema tom bakterijskom soja u odnosu na kontrolni uzorak. Dietil eterski ekstrakt iz Vareša pokazao je osjetljivu zonu inhibicije (22,00 mm) prema *E. faecalis*, a potentniji je od streptomicina. Dobiveni podatci ukazuju na visoku antibakterijsku aktivnost istraživanih ekstrakata ploda trnjine.

Ključne riječi
Prunus spinosa L., antimikrobno djelovanje, Soxhlet ekstrakcija, ekstrakti ploda

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