2025 Vol. 68 | Št. 3

# ABS

## Acta Biologica Slovenica

DBS Društvo biologov Slovenije

BF UNIVERZA V LJUBLJANI Biotehniška fakulteta

**Acta Biologica Slovenica, 2025, 68 (3)**

**Založila/Published by**
Založba Univerze v Ljubljani / University in Ljubljana Press
Društvo biologov Slovenije / Slovenian biological society

**Za založbo/For the publisher**
Gregor Majdič, rektor Univerze v Ljubljani / the Rector of the University of Ljubljana
Anita Jemec Kokalj, predsednica Društva biologov Slovenije / Chairman of Slovenian Biological Society

**Izdala/Issued by**
Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo /
University of Ljubljana, Biotehnical Faculty, Department of Biology

**Za izdajatelja/For the Issuer**
Marina Pintar, dekanja Biotehniške fakultete UL / Dean of Biotechnical Faculty

**Naslov uredništva/Editorial Office Address**
Univerza v Ljubljani, Biotehniška fakulteta, Acta Biologica Slovenica,
Večna pot 111, 1000 Ljubljana, Slovenija

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**Naslovnica/Cover page**
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Izdajanje revije sofinancira Javna agencija za znanstvenoraziskovalno in inovacijsko dejavnost Republike Slovenije (ARIS)
The journal is co-financed by Slovenian Research and Innovation Agency (ARIS)

Publication is free of charge.

ISSN 1854-3073 (spletna verzija/online version) UDK 57(497.4)
DOI: 10.14720/abs.68.3
http://journals.uni-lj.si/abs/
Acta Biologica Slovenica je indeksirana v – is indexed in: CAB Abstracts, Web of Science Clarivate

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Original Research

# Unravelling spinach growth: when microbial inoculants fall short - soil quality and microbial communities across land uses

Mahmuda Parveen [1], Sumanta Ray [2], Sujit Ghosh [3,*]

## Abstract

This study aims to evaluate the efficacy of microbial inoculants as sustainable alternatives to synthetic fertilizers in spinach cultivation. Five bacterial strains (MP1–MP5) were isolated from soil and mature spinach plants based on nitrogen-free growth and phosphate solubilization abilities. These were applied individually or in combination (1 g/kg of soil per week) to containerized topsoil, collected in triplicate from different locations within agricultural fields (AG) and college premises (CP), with a control group included for comparison.Biotic interactions were studied for unified application. Spinach growth, measured by leaf area 50 days after germination, showed minimal improvement except when three bacteria were combined, suggesting soil limitations. Soil physicochemical and biochemical analyses, along with principal component analysis (PCA), revealed key components for CP soil, including bulk density, electrical conductivity, clay content, total organic carbon, nitrogen, and pH. For AG soil, significant factors included total organic carbon, pH, sand content, urease, and β-glucosidase. The PCA calculated the soil quality index (SQI) as 0.59 for CP and 0.55 for AG on a scale of 0 to 1, indicating that both soils are fair and require improvement. Effective microbial inoculation necessitates the establishment of niches in both soil types. Amplicon sequencing of 16S rRNA showed higher species richness in AG soil, with dominant species identified in each type. Notably, combining bacteria (MP-1, MP-3, MP-5) resulted in a slight improvement in spinach growth compared to other combinations, while other bacteria struggled in both soil types. These findings highlight the need for proper establishment of applied inoculants in soil for successful application. Future research should focus on building networks and identifying keystone species in the soil microbiome, as well as determining whether these species facilitate or hinder the establishment of applied microbial inoculants.

## Keywords

Amplicon sequence analysis, Applied microorganisms, Principal Component Analysis, Soil Quality Indices, Sustainable Agriculture

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**Citation:** Parveen, M., Ray, S., Ghosh, S., (2025). Unravelling spinach growth: when microbial inoculants fall short - soil quality and microbial communities across land uses. Acta Biologica Slovenica 68 (3)

**Received:** 09.10.2024 / **Accepted:** 14.04.2025 / **Published:** 17.04.2025

**https://doi.org/10.14720/abs.68.3.19915**

## Razkrivanje rasti špinače: ko so mikrobni inokulanti pomanjkljivi - kakovost tal in mikrobne združbe v različnih vrstah rabe tal

**Izvleček**

Namen te študije je oceniti učinkovitost mikrobnih inokulantov kot trajnostne alternative sintetičnim gnojilom pri gojenju špinače. Pet bakterijskih sevov (MP1-MP5) je bilo izoliranih iz tal in odraslih rastlin špinače na podlagi rasti brez dodanega dušika in sposobnosti topljenja fosfatov. Izolirani bakterijski sevi so bili uporabljeni posamično ali v kombinaciji (1 g/kg tal na teden) v kontejnerski vrhnji plasti tal, zbrani v treh ponovitvah na lokaciji univerze (CP) ali kmetijskih površin (AG), pri čemer je bila za primerjavo vključena kontrolna skupina. Za enotno uporabo so bile preučene biotske interakcije. Rast špinače, merjena s površino listov 50 dni po kalitvi, je pokazala minimalno izboljšanje, razen pri kombinaciji treh bakterijskih sevov, kar kaže na omejitve tal. Fizikalno-kemijske in biokemijske analize tal skupaj z analizo glavnih komponent (PCA) so razkrile ključne komponente za tla CP, vključno s prostorninsko gostoto, električno prevodnostjo, vsebnostjo gline, skupnim organskim ogljikom, dušikom in pH. Za tla AG so bili pomembni dejavniki skupni organski ogljik, pH, vsebnost peska, ureaza in β-glukozidaza. PCA je izračunal indeks kakovosti tal (SQI) 0,59 za CP in 0,55 za AG na lestvici od 0 do 1, kar pomeni, da sta obe prsti primerni in ju je treba izboljšati. Za učinkovito mikrobno inokulacijo je treba vzpostaviti niše v obeh vrstah tal. Sekvenciranje amplikonov 16S rRNA je pokazalo večje bogastvo vrst v tleh AG, pri čemer so bile v vsakem tipu ugotovljene dominantne vrste. Zlasti kombinacija bakterij (MP-1, MP-3, MP-5) je povzročila rahlo izboljšanje rasti špinače v primerjavi z drugimi kombinacijami, medtem ko so imele druge bakterije težave v obeh tipih tal. Te ugotovitve poudarjajo, da je za uspešno uporabo potrebno pravilno uvajanje uporabljenih inokulantov v tleh. Prihodnje raziskave bi se morale osredotočiti na oblikovanje mrež in prepoznavanje ključnih vrst v mikrobiomu tal ter ugotavljanje, ali te vrste olajšujejo ali ovirajo uveljavitev uporabljenih mikrobnih inokulantov.

**Ključne besede**

analiza zaporedja amplikonov, uporabni mikroorganizmi, analiza glavnih komponent, indeksi kakovosti tal, trajnostno kmetijstvo

# Introduction

Sustainable agriculture is increasingly challenged by soil degradation, nutrient depletion, and the growing demand for food production. Excessive use of chemical fertilizers has contributed to environmental issues such as pollution, loss of soil biodiversity, and reduced long-term soil fertility. To address these challenges, biofertilizers are a viable option that consists of microbial inoculants containing beneficial microorganisms. They can improve soil fertility, support crop productivity, and reduce the use of chemical inputs.

In particular, microbial inoculants enhance nutrient availability through biological nitrogen fixation, phosphorus solubilization, and the mobilization of micronutrients such as potassium and zinc (Nosheen et al., 2021). They also contribute to soil health by improving structure, increasing microbial biomass carbon, and promoting beneficial microbial interactions, which in turn enhance water retention and nutrient cycling (Mahanty et al., 2017). Plant growth-promoting rhizobacteria (PGPR) and endophytic bacteria are particularly important for these functions. Typically, these microbial strains are isolated, mass-cultivated, and introduced into soils to promote plant growth and enhance soil nutrient dynamics. Molina-Romero et al., 2017 demonstrated that a microbial consortium comprising *P. putida*, *Acinetobacter* sp., *Sphingomonas* sp., and *A. brasilense* enhanced maize rhizosphere colonization and improved stress resilience during early growth. Several studies have highlighted the role of microbial inoculants in sustainable crop production (Adesemoye and Kloepper, 2009; Glick, 2012; Goswami et al., 2014; Aloo et al., 2021), while others have emphasized their contribution to food security and disease suppression (Bhardwaj et al., 2014; Oteino et al., 2015).

Despite these advantages, the effectiveness of microbial inoculants remains inconsistent, varying across soil types and environmental conditions due to differences in soil

physicochemical properties, microbial community dynamics, and land-use patterns (Bastida et al., 2009; Kour et al., 2019). Furthermore, gaps exist in understanding how these microbial inoculants function across different agroecosystems.

To address these inconsistencies, several soil evaluation tools have been developed. Among them, the Soil Quality Index (SQI) and Principal Component Analysis (PCA) offer valuable insights. The SQI provides a comprehensive measure by integrating key physical, chemical, and biological indicators into a single value (Levi et al., 2021). PCA helps simplify complex soil datasets and identify the most influential indicators to guide management decisions.

Spinach (*Spinacia oleracea* L.), a nutrient-rich leafy vegetable with short growth cycles, was chosen due to its agricultural and nutritional importance in local farming systems. The study uses soils from two distinct land-use types—college premises (CP), which represent less intensively managed soil, and agricultural fields (AG), which represent conventionally cultivated soil—to evaluate microbial inoculant performance under contrasting soil conditions.

This study aims to evaluate the potential of microbial inoculants isolated from the rhizosphere and endosphere of spinach for their role in nitrogen fixation and phosphorus solubilization. It further examines their relationship with soil physicochemical properties and native microbial communities using soils collected from two contrasting land-use types, CP and AG, under controlled container conditions.

# Materials and methods

## Study Area and Soil Sampling

The study was conducted at the Jagannath Kishore (J.K) College in Purulia District, West Bengal, India. Soil samples (topsoil: 0-15 cm depth) were collected from a man-made garden area within the College (23°32' N, 86°37' E) and an adjacent agricultural field (23.1493° N, 86.4617° E) under monoculture rice cultivation. Samples were sieved (0.2 mm), stored at -20°C, and used for microbial isolation and soil characterization.

## Isolation, Characterization, and Preservation of Soil and Endophytic Microbes

### Sterilization process

Endophytes from spinach plants were isolated using a stepwise surface sterilization process to eliminate epiphytic microbes while preserving internal microorganisms. Healthy spinach tissues (roots and leaves) were collected, washed under running tap water for 5 minutes to remove soil and debris, and then subjected to sequential sterilization. This involved immersion in 70% ethanol for 1–2 minutes, followed by 1% sodium hypochlorite for 3 minutes (leaves) and 2.5% sodium hypochlorite for 5 minutes (roots). The tissues were then rinsed three times with sterile distilled water and finally with 70% ethanol for 30–60 seconds (Schulz et al., 1993). To confirm surface sterilization, 100 µL of the final rinse water was plated on nutrient agar to check for absence of microbial growth. For bacterial isolation, sterilized tissues were cut into 10 mm segments using a sterile knife and cultured on nutrient agar medium (peptone—5 g/L, NaCl—5 g/L, yeast extract—2 g/L, beef extract—1 g/L, agar—1.5%), supplemented with nystatin (10 µg/mL) from Hl-Media (India) to inhibit fungal growth. Plates were incubated at 30 ± 2°C for 3–7 days till the colonies appear.

### Isolation

Nitrogen-fixing and phosphate-solubilizing bacteria were isolated from CP and AG soils using serial dilution and spread plating on selective N-free media, including *Beijerinckia*, Burk, and *Azospirillum* media, as well as Pikovskaya's agar supplemented with nystatin for evaluating phosphate-solubilizing capacity (Srivastava et al., 2024). Plates were incubated at 30 ± 2°C for 3–7 days till the colonies appear. For bacterial culture preparation, colonies from these N-free and phosphate-solubilizing agar plates were suspended in 0.85% sterile saline, and serially diluted up to $10^{-4}$, $10^{-5}$, and $10^{-6}$. Aliquots from these dilutions were spread again on nutrient agar plates containing nystatin and incubated at 30 ± 2°C for 3-7 days till the colonies appear.

For isolation of endophytic bacteria from spinach, the same procedure was followed after surface sterilization, using the same media and plating technique with 10 mm segments of root and leaf tissues. Once bacterial growth was established, glycerol stocks (30%) were prepared for long-term preservation and future research.

### Selection of N-fixing and P-Solubilizing Microbes

Colonies exhibiting distinct morphological features were selected for further characterization. Carbon source utilization was tested in broth cultures with various carbon sources (malic acid, glucose, sucrose, mannitol) and mineral salt media at 30 ± 2°C, 150 rpm for 72 hours. Biochem-

ical tests, including amylolytic activity on starch agar with iodine flooding (Sim and Wong, 2021), urease activity on Urea Agar Base (Christensen, 1946), and the catalase test using 3% $H_2O_2$ (Reiner, 2010), were performed to identify isolates with desirable traits. The distinct colonies were selected for further experiments and stored on nutrient agar slants at 4°C for short-term use and in 20% glycerol at -80°C for long-term preservation. Media and antibiotics were procured from HiMedia, India.

**Mass Cultivation**

Selected isolates were then cultivated in nutrient broth to achieve sufficient biomass for soil amendment applications. The microbes were grown in 1 L Nutrient broth at 30 ± 2°C for three days in 10 replicas, each one inoculated with a single colony from the same plate, and then after three days the broth was centrifuged (10000 g for 5 minutes) to separate the microbes and then collected bacteria were weighted and added to soil samples from both sites at 1 g/kg, placed in a container filled with 6 kg soil, and kept at 30 ± 2°C with maintaining 60% water holding capacity and 12-hour light/dark cycles.

## Experimental Setup for Microbial Treatment and Analysis

Microbes were grown in Nutrient broth and then centrifuged to separate the microbes and then added to soil samples from both sites at 1 g/kg, placed in a container filled with 6 kg soil, and kept at 28 ± 1°C with 60% water holding capacity and 12-hour light/dark cycles. Treatments for CP and AG soils included Control (no microbes), Microbes MP1, MP2, MP3, MP4, MP5, MP1+MP3, MP2+MP4, and MP1+MP3+MP5 at 7-day intervals. Before application, combined inoculants were tested for biotic interactions on nutrient agar plates. Spinach seeds were planted, and leaf area was measured after 50 days under different treatments and soil types. Two-way analysis of variance (ANOVA) was conducted to determine the significance of treatment using https://www.statskingdom.com/.

## Soil Quality Analysis

A total of 15 soil parameters (n=75), including physical, chemical, and biochemical properties, were used to assess the underlying soil conditions. The physical properties examined were texture (Hwang et al., 2004), bulk density (Sobek et al., 1978), porosity (Sobek et al., 1978), and water-holding capacity (Cassel and Nielsen, 1986). The chemical properties analyzed were pH (Thomas, 1996), electrical conductivity (EC) (Lund, 2008), available phosphorus (P) (Olsen et al., 1954), potassium (K) (Habib et al., 2014), total organic carbon (TOC) (Prasad et al., 2010), and total nitrogen (TN) (Bremner, 1960). Additionally, the biological properties assessed included the activities of enzymes such as urease (Tabatabai and Bremner, 1972), acid phosphatase (Eivazi and Tabatabai, 1977), and glucosidase (Wood and Bhat, 1988).

## Principal Component Analysis (PCA) and Minimum Data Set (MDS) with Soil Quality Index (SQI)

PCA reduces data dimensionality by transforming original variables into uncorrelated principal components using eigenvalues and eigenvectors from the covariance matrix, thereby revealing key soil properties influencing quality variation (Table 1). It retained components with eigenvalues above one based on the Kaiser criterion (Jolliffe, 2002) and selected highly weighted indicators for the MDS to identify key soil quality variables. The visualizations were generated using the factoextra package in R (v. 4.4.3) (Kassambara and Mundt, 2017).

To quantify soil quality across CP and AG soils, the Soil Quality Index (SQI) was calculated by integrating standardized soil indicator scores (Si) with weight factors (Wi), following the methods of Levi et al., 2021 and Das et al., 2021. The weight factors (Wi) were derived from PCA and calculated as the percentage variance of each principal component divided by the cumulative variance. The score was used to standardize the MDS values based on indicator behaviour: 'More is better' ($S_i$ = [1 + e(-b(x-a)))-1), 'Less is better' ($S_i$ = (1 + e(b(x-a)))-1), and 'Optimum' ($S_i$ = 1 x e (- (x-a)2 / b)). Where Si is the soil property score, x is the parameter value, a is the soil property average, and b is $2\sigma^2$ of the data. The final SQI was calculated using the equation SQI = $\sum (W_i \times S_i)$, and the resulting values (Fig. 1) were used to evaluate the overall soil quality class, categorized from "very good" to "very bad" (Istijono and Harianti, 2019) (Table 2).

## Isolation and Sequencing of Bacterial DNA

DNA from both soil and bacterial samples (MP1 to MP5) was isolated using the DNeasy Power Soil Kit (Qiagen), following the manufacturer's instructions. The V3–V4 region of the

**Table 1.** Soil indicator and its scoring function.

**Tabela 1.** Lastnosti tal in njegova utež pri ocenjevanju.

| Indicator | Scoring Curve | Aq.E. |
|---|---|---|
| **Sand** | less is better | Authors opinion |
| **Silt** | More is better | ICAR (https://nishantsinha51.shinyapps.io/SQICAL) |
| **Clay** | optimum is better | Istijono, B., andHarianti, M. (2019) |
| **Water Holding Capacity** | More is better | Das et al., 2021 |
| **Bulk Density** | Less is better | Askari and Holden, 2014. |
| **porosity** | Optimum | Uthappa et al., 2024 |
| **pH** | Optimum | Chaudhry et al., 2024 |
| **Electrical Conductivity** | Optimum | Andrews et al., 2002 |
| **Available phosphorus** | Optimum | Andrews et al., 2002 |
| **Available potassium** | More is better | Das et al., 2021 |
| **Total Organic Carbon** | More is better | Askari and Holden, 2014. |
| **Total nitrogen** | More is better | Askari and Holden, 2014. |
| **Beta-glucosidase** | More is better | Authors' opinion |
| **Urease** | More is better | |
| **Acid Phosphatase** | More is better | |

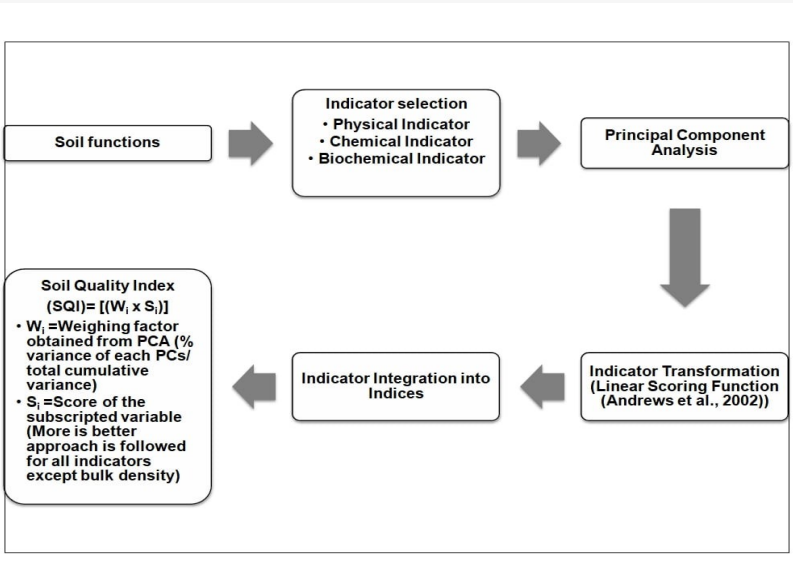


**Figure 1.** Flow diagram illustrating the process of Soil Quality Index (SQI) development using Principal Component Analysis (PCA).

**Slika 1.** Diagram poteka, ki prikazuje postopek razvoja indeksa kakovosti tal (SQI) z uporabo analize glavnih komponent (PCA).

**Table 2.** Classes of Soil Quality (As per Istijono and Harianti, 2019).

**Tabela 2.** Razredi kakovosti tal (po Istijono in Harianti, 2019).

| Soil Quality | Scale | Class |
|---|---|---|
| Very good | 0.8-1 | 1 |
| Good | 0.6-0.79 | 2 |
| Fair | 0.35-0.59 | 3 |
| Bad | 0.20-0.34 | 4 |
| Very bad | 0-0.19 | 5 |

16S rRNA gene was sequenced on the Illumina platform at QBiogen Lab, Chennai, India. Sequences were compared against the GenBank database using BLAST (NCBI), and bacterial isolates were identified based on a minimum of 97% similarity to known type strains.

### Bioinformatic Analysis

Bioinformatic analysis was conducted using QIIME 2. Raw reads were demultiplexed, quality filtered, and denoised with DADA2 to generate feature tables and corrected sequences. Taxonomy was assigned using a Naive Bayes classifier trained on the Greengenes database. Alpha and beta diversity metrics assessed community richness and dissimilarity, with rarefaction curves and PCoA visualizing sequencing depth and sample clustering. ANCOM identified differentially abundant taxa and phylogenetic trees examined evolutionary relationships. Bar plots displaying taxonomic profiles were created in R using the "ggplot2" package (Wickham and Wickham, 2016).

## Results

The study was conducted at two land use types, designated as CP and AG, with soil types characterized as sandy loam for CP and loamy sand for AG. Morphology, carbon preference, growth on selective media, and enzymatic activities of selected microbes are presented in Tables 3, 4, and 5.

**Table 3.** Morphological characteristics of bacterial isolates (MP-1 to MP-5).

**Tabela 3.** Morfološke značilnosti bakterijskih izolatov (MP-1 do MP-5).

| Characteristics | MP-1 | MP-2 | MP-3 | MP-4 | MP-5 |
|---|---|---|---|---|---|
| **Growth on Nutrient Agar** | + | + | + | + | + |
| **Growth on Burk Media** | – | – | – | – | + |
| **Growth on *Beijerinckia* Media** | – | – | – | + | + |
| **Growth on *Azospirillum* Media** | – | + | – | – | – |
| **Growth on Pikovskaya Media** | + | + | + | + | + |
| **Growth on Cetrimide agar Media** | + | – | – | – | – |
| **Growth of Tryptic Soy Agar** | + | – | – | – | + |
| **Cell Shape** | Rod | Rod | Rod | Slightly curved rod | Rod |
| **Pigment/Colony Color** | Green fluorescent | Very light yellow, slightly opalescent | Colourless, translucent | Brownish-white | Light yellow |
| **Colony Morphology** | Convex | Slightly convex | Raised with filaments | Circular, raised, mucoid (on N-free medium) | Circular, slightly convex, smooth appearance |
| **Gram Stain** | Negative | Negative | Positive | Negative | Negative |
| **Motility** | Motile | Motile | Motile | Motile | Motile |

**Table 4.** Carbohydrate utilization profile of bacterial isolates (MP-1 to MP-5).

**Tabela 4.** Profil uporabe ogljikovih hidratov bakterijskih izolatov (MP-1 do MP-5).

| Bacterial isolates | Malic Acid | Glucose | Sucrose | Mannitol | Mannose |
|---|---|---|---|---|---|
| **MP-1** | – | + | + | – | – |
| **MP-2** | ++ (fast) | + | + | – | – |
| **MP-3** | – | ++ (early) | ++ (early) | – | – |
| **MP-4** | – | + | + | + | – |
| **MP-5** | + | + | + | + | + |

**Table 5.** Growth onset and enzymatic activity of bacterial isolates (MP-1 to MP-5).

**Tabela 5.** Začetek rasti in encimska aktivnost bakterijskih izolatov (MP-1 do MP-5).

| Bacterial isolates | Growth onset in nutrient broth (28°C, pH 7) | Amylase activity | Urease activity | Catalase activity |
|---|---|---|---|---|
| **MP1** | Within 4 hours | Strong | Absent | Present |
| **MP2** | Within 12 hours | Weak | Present | Present |
| **MP3** | Within 4 hours | Strong | Present | Present |
| **MP4** | Within 14 hours | Strong | Absent | Present |
| **MP5** | Within 4 hours | Strong | Present | Present |

## Microbial Interaction Studies

The potential interactions between the different microbes (Table 6), when used as an inoculums /consortium, could enhance plant growth. This study investigates various combinations of microbial inoculants for a plant application, considering five distinct microorganisms labelled MP-1 through MP-5. These can be used alone or in different combinations. For single applications, each microorganism (MP-1, MP-2, MP-3, MP-4, and MP-5) can be applied independently. In double combinations, MP-1 (a root endophyte) and MP-3 (a leaf endophyte) are used together at 0.5 g/kg each in soil, while MP-2 and MP-4 (both soil microorganisms) are paired at 0.5 g/kg each. A triple combination involves MP-5 (a leaf endophyte) combined with MP-1 (root endophyte) and MP-3 (leaf endophyte), with each microorganism applied at 0.33 g/kg in soil. These combinations aim to enhance plant leaf growth, utilizing the potential synergistic interactions between the microorganisms.

This study identified the following microbial isolates and their interactions:

**MP-1:** A *Pseudomonas* sp. (100% similarity with Accession MT256252.1), a root endophyte, which showed synergistic interactions with MP-3 and MP-5.

**MP-2:** An *Azospirillum* sp. (97.44% similarity with Accession MK108951.1), a soil bacterium, which exhibited mixed interactions with other isolates, including a competitive relationship with MP-5.

**MP-3:** A *Bacillus* sp. leaf endophyte (98.14% similarity to *Bacillus mannanilyticus*, Accession HE663240.1), which displayed strong compatibility with MP-5 and moderate interaction with MP-1.

**MP-4:** A *Beijerinckia* sp. (100% similarity with Accession MG904942.1), a soil bacterium, which generally showed competitive interactions with MP-1, MP-3, and MP-5.

**MP-5:** A *Flavobacterium ummariense* leaf endophyte (100% similarity with Accession KF844048.1), which exhibited the most compatible interactions, particularly with MP-1 and MP-3, highlighting its potential role in enhancing the synergistic effects within the consortium.

In this interaction study, MP-1 (*Pseudomonas*, root endophyte) showed mutual growth with MP-3 (*Bacillus*, leaf endophyte) (+/+) and facilitative synergy with MP-5 (*Flavobacterium*, leaf endophyte) (+/++), suggesting cooperative endophytic colonization. MP-1 exhibited growth while inhibiting MP-2 (*Azospirillum*, soil) and MP-4 (*Beijerinckia*, soil) (+/–), indicating selective compatibility. MP-2 and MP-4 interacted synergistically (+/++), whereas MP-2 was strongly inhibited by MP-5 in a –/+++ pattern. MP-5 showed strong growth with MP-3 (+++/+) and a slightly suppressive effect on MP-2 (+/–), while MP-4 experienced inhibition in most pairings (–/+, –/++). MP-5 demonstrated the highest compatibility with other endophytes, especially MP-1 and MP-3, highlighting its potential as a keystone in consortium design. Collectively, these results reveal that endophytes (MP-1, MP-3, MP-5) engage in more synergistic interactions than soil-derived strains (MP-2, MP-4), underscoring the functional advantage of endophytic consortia in microbial formulations.

## Growth of spinach in two types of soil

The leaf area ($cm^2$) measurements for both soil types and with microbial inoculant treatments were represented (Table 7). ANOVA results, model summary and regression analysis, are represented in Supplementary Tables S1-S3.

**Table 6. Interaction between Microbes for Consortium Development:** the potential interactions between the microbes when used together as a consortium.

**Tabela 6. Interakcije med mikrobi za razvoj konzorcija:** možne interakcije med mikrobi, kadar se uporabljajo skupaj kot konzorcij.

| Microbe | Source | MP-1 (*Pseudomonas*), Root endophyte | MP-2 (*Azospirillum*, Soil) | MP-3 (*Bacillus*, Leaf Endophyte) | MP-4 (*Beijerinckia*, Soil) | MP-5 (*Flavobacterium*, Leaf Endophyte) |
|---|---|---|---|---|---|---|
| **MP-1** | Root Endophyte | 0 | +/- | +/+ | +/- | +/++ |
| **MP-2** | Soil | - /+ | 0 | -/+ | +/++ | -/+++ |
| **MP-3** | Leaf Endophyte | +/++ | +/- | 0 | +/- | +/++ |
| **MP-4** | Soil | -/+ | +/+ | - /+ | 0 | -/++ |
| **MP-5** | Leaf Endophyte | +/++ | +/- | +++/+ | +/- | 0 |

The notation "+/-" OR "-/+" indicates that the presence of one microbe can inhibit the other in a nutrient agar plate, i.e. one microbe is dominant over the other, "+/+" indicates no significant effect on each other, and increasing the number of "+" signs indicates a higher level of dominance compare to other.)

**Table 7.** Leaf area ($cm^2$ ± SD(SE) of Spinach plants grown in CP and AG soils under various microbial inoculant treatments.

**Tabela 7.** Listna površina ($cm^2$ ± SD(SE)) špinače, gojene v tleh CP in AG, pri različnih postopkih z mikrobnimi inokulanti.

| Treatment | CP Leaf Area ($cm^2$) | AG Leaf Area ($cm^2$) | p-value |
|---|---|---|---|
| **Control** | 72.97 ± 0.34 (0.11) | 67 ± 0.16 (0.05) | 0.049 |
| **MP-1** | 72.96 ± 0.3 (0.09) | 68.95 ± 0.22 (0.07) | 0.909 |
| **MP-2** | 72.76 ± 0.24 (0.07) | 67.55 ± 0.36 (0.12) | 0.094 |
| **MP-3** | 73.06 ± 0.15 (0.05) | 68.95 ±0.22 (0.07) | 0.814 |
| **MP-4** | 72.96 ± 0.32 (0.1) | 67.55 ± 0.25 (0.08) | 0.139 |
| **MP-5** | 74.45 ± 0.24 (0.07) | 68.95 ± 0.26 (0.08) | 0.000* |
| **MP-1 + MP-3** | 74.96 ± 0.29 (0.09) | 68.21 ± 0.29 (0.09) | 0.127 |
| **MP-2 + MP-4** | 73.05 ±0.36 (0.11) | 67.8 ±0.26 (0.08) | 0.258 |
| **MP-1 + MP-3 + MP-5** | 74.96 ± 0.21 (0.06) | 69.75 ± 0.37 (0.12) | 0.007* |

Note: MP = Microbial inoculant (before species identification).
*$p < 0.01$

## Selecting soil properties

After observing insignificant plant growth in both soils, with or without the addition of microbial inoculants, further analysis was carried out to characterize the soils' physiochemical and biochemical properties. This was essential to identify the underlying factors that might be limiting plant growth and to evaluate the potential effectiveness of the microbial treatments, as the initial growth data alone did not provide sufficient explanation for the observed outcomes.

Soil physical properties, such as texture, water-holding capacity, and bulk density, were analyzed to assess their influence on hydration, drainage, and root proliferation. Chemical properties, including pH, electrical conductivity, available phosphorus and potassium, total organic carbon, and total nitrogen, were evaluated to determine nutrient availability and overall soil fertility. Biochemical properties, particularly enzyme activities such as urease, acid phosphatase, and β-glucosidase, were assessed to understand the microbial activity and nutrient cycling essential for supporting plant growth.

An independent t-test was conducted to statistically validate the observed differences between the two soil types. The results revealed highly significant differences (***$p < 0.001$) across all measured parameters, highlighting the contrasting characteristics between the soils (Supplementary Table S4-6).

**Table 8.** Results of principal component analysis (PCA) of soil parameters from the different sites of CP soil (Bold values from each PC were selected as the most important soil property).

**Tabela 8.** Rezultati analize glavnih komponent (PCA) parametrov tal z različnih lokacij tal CP (krepko označene vrednosti iz vsake PC so bile izbrane kot najpomembnejše lastnosti tal).

| Principal Component | PC-1 | PC-2 | PC-3 | PC-4 | PC-5 | PC-6 | PC-7 |
|---|---|---|---|---|---|---|---|
| Eigenvalues | 2.32 | 1.84 | 1.83 | 1.33 | 1.22 | 1.01 | 1.00 |
| Variation (%) | 28.209 | 17.700 | 15.741 | 8.19 | 7.04 | 5.26 | 5.23 |
| Cumulative Variation (%) | 28.209 | 45.91 | 61.65 | 69.85 | 76.88 | 82.15 | 87.38 |
| Factor loading/eigenvectors | | | | | | | |
| Sand | -0.130 | -0.393 | -0.133 | 0.263 | -0.148 | -0.205 | 0.248 |
| Silt | -0.124 | -0.202 | 0.509 | 0.011 | 0.136 | -0.045 | 0.224 |
| Clay | 0.109 | -0.087 | **0.617** | -0.009 | -0.046 | 0.018 | -0.232 |
| pH | -0.170 | 0.150 | -0.017 | 0.139 | -0.563 | -0.024 | **0.409** |
| Water holding capacity | 0.237 | -0.378 | -0.257 | -0.154 | -0.278 | -0.196 | -0.166 |
| Bulk Density | **0.330** | 0.296 | -0.153 | 0.133 | **0.385** | -0.054 | 0.178 |
| Porosity | -0.422 | -0.070 | 0.335 | -0.122 | 0.040 | -0.129 | -0.004 |
| Total Organic Carbon | -0.067 | -0.220 | -0.048 | **0.605** | 0.165 | 0.396 | 0.244 |
| Total Nitrogen | -0.148 | 0.142 | -0.009 | -0.140 | -0.205 | **0.799** | -0.108 |
| Available Phosphorus | -0.191 | -0.215 | -0.125 | 0.273 | 0.318 | 0.038 | -0.539 |
| Available Potassium | 0.325 | -0.116 | 0.089 | 0.121 | -0.423 | 0.088 | -0.323 |
| Electrical Conductivity | -0.058 | **0.554** | 0.122 | 0.174 | -0.041 | -0.204 | -0.070 |
| Beta-Glucosidase | 0.322 | -0.304 | 0.125 | -0.284 | 0.186 | 0.172 | 0.343 |
| Urease | 0.316 | 0.048 | 0.257 | 0.514 | -0.145 | -0.075 | -0.074 |
| Acid Phosphatase | -0.453 | -0.084 | -0.122 | 0.059 | -0.055 | -0.114 | -0.100 |

**Table 9.** Results of principal component analysis (PCA) of soil parameters from the different sites of AG soil (Bold values from each PC were selected as the most important soil property).

**Tabela 9.** Rezultati analize glavnih komponent (PCA) parametrov tal z različnih lokacij tal AG (krepke vrednosti iz vsake PC so bile izbrane kot najpomembnejše lastnosti tal).

| Principal Component | PC-1 | PC-2 | PC-3 | PC-4 | PC-5 | PC-6 |
|---|---|---|---|---|---|---|
| Eigenvalues | 2.47 | 1.90 | 1.70 | 1.49 | 1.19 | 1.06 |
| Variation (%) | 30.72 | 17.32 | 13.79 | 10.09 | 7.27 | 5.41 |
| Cumulative Variation (%) | 30.72 | 48.04 | 61.83 | 71.93 | 79.2 | 84.61 |
| Factor loading/eigenvectors | | | | | | |
| Sand | -0.088 | 0.253 | **0.450** | -0.239 | -0.381 | 0.088 |
| Silt | -0.210 | -0.495 | 0.190 | -0.208 | 0.120 | 0.177 |
| Clay | 0.161 | -0.526 | -0.117 | 0.070 | 0.205 | 0.219 |
| pH | -0.297 | **0.316** | -0.388 | -0.151 | -0.0002 | 0.200 |
| Water holding capacity | -0.423 | -0.219 | 0.207 | 0.150 | 0.099 | -0.159 |
| Bulk Density | -0.430 | -0.073 | -0.258 | -0.292 | -0.106 | -0.071 |
| Porosity | -0.114 | -0.236 | 0.309 | 0.235 | -0.355 | -0.223 |
| Total Organic Carbon | **0.294** | 0.090 | 0.042 | **0.299** | -0.089 | -0.202 |
| Total Nitrogen | 0.246 | 0.137 | 0.275 | 0.110 | 0.256 | -0.143 |
| Available Phosphorus | 0.166 | -0.026 | 0.026 | -0.410 | 0.369 | -0.211 |
| Available Potassium | 0.141 | -0.368 | -0.320 | 0.095 | -0.200 | -0.205 |
| Electrical Conductivity | 0.104 | -0.111 | -0.084 | -0.434 | -0.317 | -0.603 |
| Beta-Glucosidase | 0.209 | -0.157 | 0.285 | -0.306 | -0.290 | **0.470** |
| Urease | 0.009 | 0.039 | 0.270 | -0.305 | **0.439** | -0.183 |
| Acid Phosphatase | -0.453 | 0.030 | 0.216 | 0.230 | 0.145 | -0.156 |

## Key indicators and SQI

PCA was performed on 15 soil parameters for CP and AG soils to identify key indicators for the SQI. For CP soil (Table 8), PCA revealed seven principal components (PCs) with eigenvalues > 1, explaining 87.38% of the MDS variation. Key indicators included bulk density (PC-1), electrical conductivity (PC-2), clay (PC-3), total organic carbon (PC-4), total nitrogen (PC-6), and pH (PC-7). According to Sharma et al., 2008 those having higher factor loadings were considered sensitive key indicators for the MDS but not for SQI calculation. The weighted factors for PCs 1 to 7 were 0.323, 0.202, 0.180, 0.094, 0.080, 0.060, and 0.059, respectively. Bulk density reappeared as a highly weighted variable in PC 5, leading to its omission in the SQI calculation. Thus, SQI =Σ [(bulk density score * 0.323) + (electrical conductivity score * 0.202) + (clay score *0.180) + (Total Organic Carbon score * 0.094) + (Total Nitrogen score*0.060) + (pH score*0.059].

For AG soil (Table 9), six PCs accounted for 84.61% of MDS variation, with key indicators being total organic carbon (PC-1), pH (PC-2), sand (PC-3), urease (PC-5), and β-glucosidase (PC-6). Thus, weighted factors for PC1 to PC6 were 0.363, 0.205, 0.163, 0.119, 0.086, and 0.064 respectively. In PC4, Total Organic Carbon reappeared as a highly weighted variable; therefore, it was omitted during the SQI calculation. Thus, SQI =Σ [(total organic carbon score * 0.363) + (pH score * 0.205) + (sand score *0.163) + (Urease activity*0.086) + (β-glucosidase activity score*0.064] (Supplementary Material).

## 16s rRNA Amplicon Sequence of V3-V4 region of two soil types

Metagenomic analysis based on 16S rRNA amplicon sequencing (V3–V4 region) revealed clear differences in bacterial diversity and community structure between CP and AG soils. Alpha diversity indices at 97% sequence identity showed that AG soil had significantly higher microbial richness and diversity compared to CP soil (Table 10). The number of observed species in AG soil was 1413, more than three times that in CP soil (427). Similarly, the Shannon and Simpson indices were higher in AG soil (8.292 and 0.991, respectively) than in CP (4.068 and 0.800), indicating a more even and diverse microbial population.

Predicted richness estimators Chao1 and ACE were also higher in AG (1421.873 and 1422.836) compared to CP (436.130 and 433.431), while the phylogenetic diversity (PD Whole Tree) was nearly double in AG (111.677) compared to CP (65.239), suggesting a broader evolutionary diversity. Good's coverage was 1.000 for both samples, indicating sufficient sequencing depth.

At the taxonomic level, the bacterial community composition differed markedly between the two soil types. CP soil was primarily dominated by Proteobacteria (50%) and Firmicutes (35.4%), whereas AG soil exhibited a higher relative abundance of Actinobacteria (32.77%) and Proteobacteria (16.27%) (Fig. 2 and 3). At the order level, distinct differences were also observed (Fig. 4 and 5). In CP soil, Enterobacterales (43.61%) and Bacillales (30.36%) were the most abundant, while in AG soil, Solirubrobacterales (9.6%) and Rhizobiales (8.9%) were predominant.

The most abundant bacterial species also varied notably between the two soils (Table 11). CP soil was characterized by the dominance of *Bacillus mannanilyticus* (1.62%) and *Lysinibacillus* sp. YS11 (1.59%), whereas *Bradyrhizobium elkanii* (0.937%) and *Streptomyces puniciscabiei* (0.476%) were among the most prominent species in AG soil.

**Table 10.** Diversity Metrics of Soil Microbial Communities.

**Tabela 10.** Metrike raznolikosti mikrobnih združb v tleh.

| Alpha Diversity Indices | Sample | Observed Species | Shannon | Simpson | Chao1 | ACE | Good's Coverage | PD Whole Tree |
|---|---|---|---|---|---|---|---|---|
| **97% Sequence Identity** | CP | 427 | 4.068 | 0.800 | 436.130 | 433.431 | 1.000 | 65.239 |
| | AG | 1413 | 8.292 | 0.991 | 1421.873 | 1422.836 | 1.000 | 111.677 |

Note: Shannon: Shannon Diversity Index, Simpson: Simpson Diversity Index, Chao1: Chao1 Richness Estimator, ACE: Abundance-based Coverage Estimator. Good's Coverage: Good's Coverage Estimate, PD Whole Tree: Phylogenetic Diversity Whole Tree.

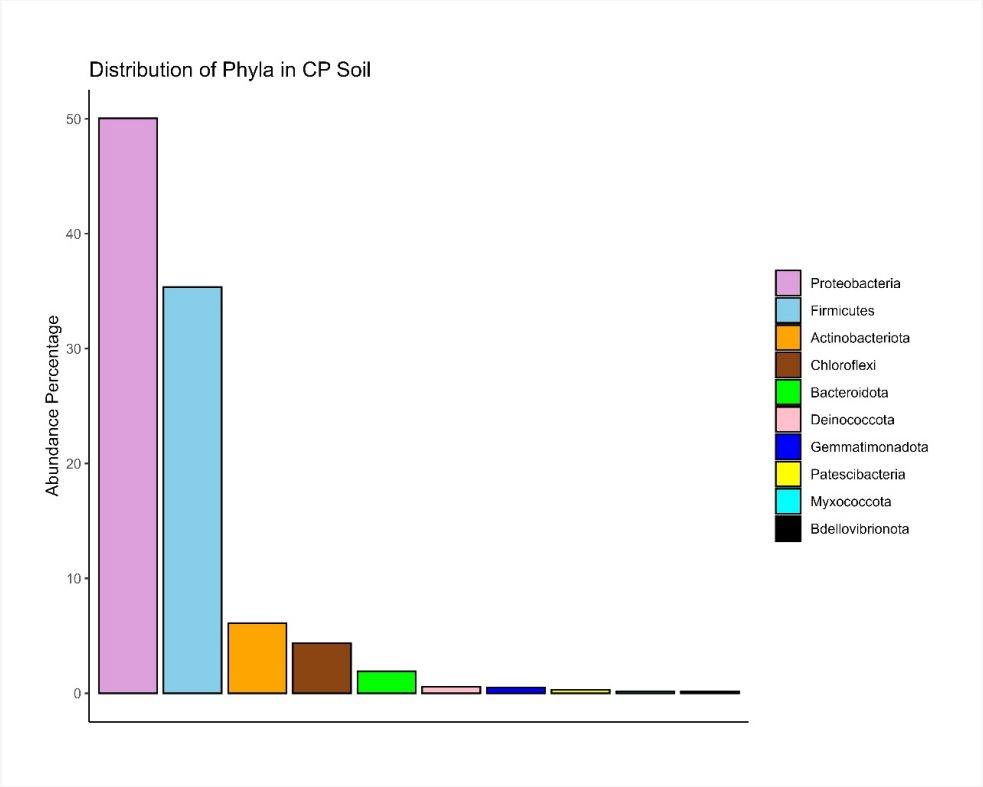


**Figure 2.**
Phylum-wise bacterial distribution is in CP (College Premises) soil.

**Slika 2.**
Razporeditev bakterij v tleh CP (College Premises) po posameznih skupinah bakterij

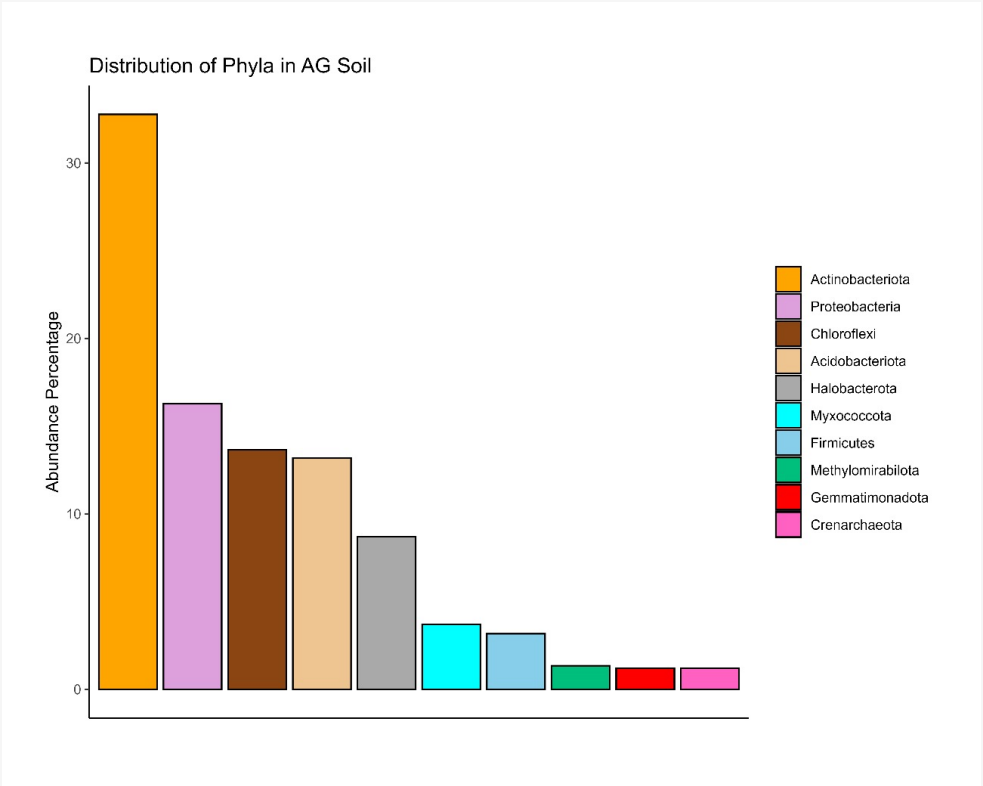


**Figure 3.**
Phylum-wise bacterial distribution in AG (Rice monoculture agricultural field) soil.

**Slika 3.**
Porazdelitev bakterij po posameznih skupinah v tleh AG (monokulturno polje z rižem).

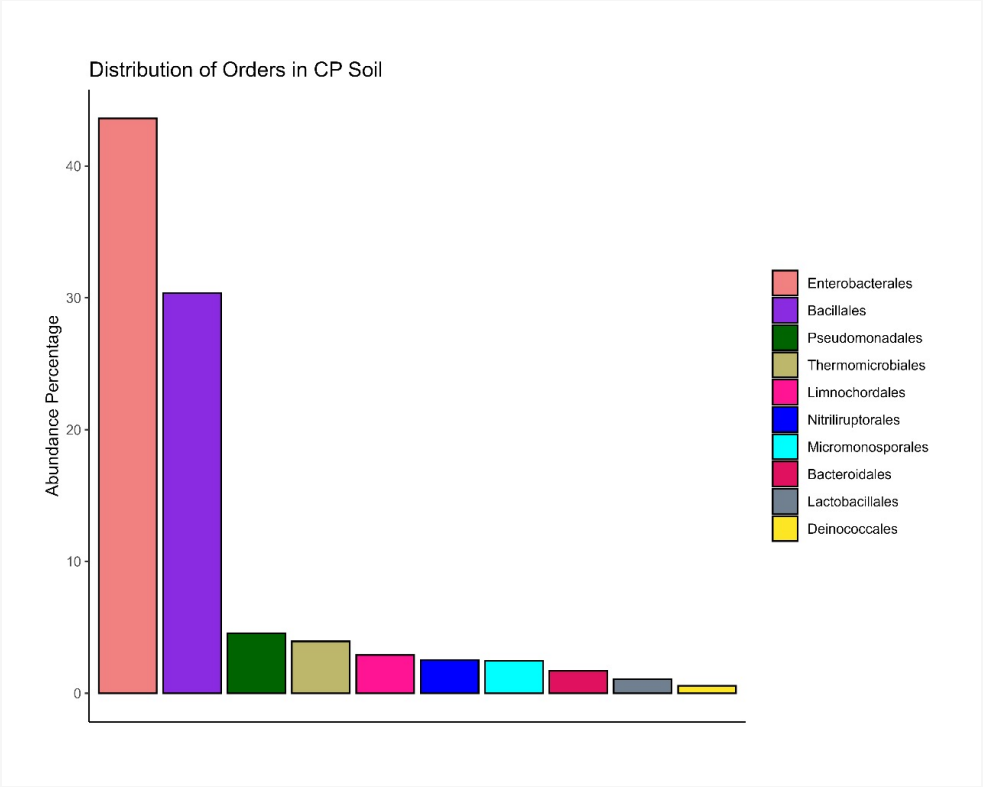


**Figure 4.**
Order-wise bacterial distribution is in CP (College Premises) soil.

**Slika 4.**
Razporeditev bakterij po vrstnem redu v tleh CP (prostori kolidža).

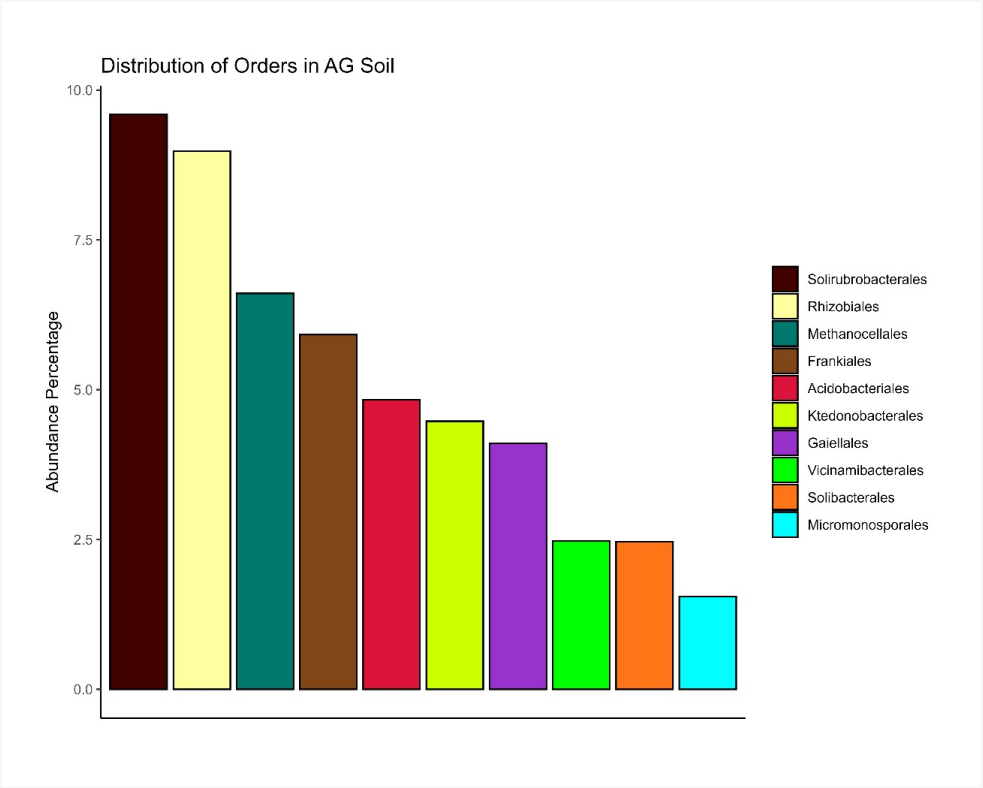


**Figure 5.**
Order-wise bacterial distribution in AG (Rice monoculture agricultural field) soil.

**Slika 5.**
Razporeditev bakterij po vrstnem redu v zemlji AG (monokulturno polje z rižem).

**Table 11.** Lists of abundant bacterial species in each soil type.

**Tabela 11.** Seznami pogostih bakterijskih vrst v vsakem tipu tal.

| CP soil | | AG soil | |
|---|---|---|---|
| **Name of the Bacterial species** | **Abundance (%)** | **Name of the Bacterial species** | **Abundance (%)** |
| *Bacillus mannanilyticus* | 1.62 | *Bradyrhizobium elkanii* | 0.937 |
| *Lysinibacillus sp. YS11* | 1.59 | *Streptomyces puniciscabiei* | 0.476 |
| *Lactobacillus kefiranofaciens* | 1.048 | *Micromonospora echinospora* | 0.465 |
| *Escherichia coli* | 0.165 | *Clostridium beijerinckii* | 0.198 |
| *Massiliprevotella massiliensis* | 0.104 | *Nocardioides sp.* | 0.086 |
| *Brachybacterium paraconglomeratum* | 0.073 | *Paenibacillus chartarius* | 0.059 |
| *Moraxella osmosis* | 0.053 | *Clostridium magnum* | 0.050 |
| *Staphylococcus nepalensis* | 0.050 | *Ruminiclostridium hungatei* | 0.031 |
| *Prevotella intermedia* | 0.042 | *Lysinibacillus sp. YS11* | 0.021 |
| *Micromonospora echinospora* | 0.029 | *Hydrogenispora ethanolic* | 0.013 |

# Discussion

This study explored microbial growth and enzymatic activity in two distinct land use types, CP and AG, characterized by sandy loam and loamy sand soils, respectively. The phenotypic characterization of bacterial isolates MP-1 to MP-5—including morphological traits (Table 3), carbohydrate utilization profiles (Table 4), and growth onset and enzymatic activities (Table 5)—reveals notable diversity, providing important clues to their taxonomic identities and ecological functions. MP-1, an endophyte, exhibited rod-shaped, Gram-negative, motile cells and formed green fluorescent, convex colonies on Cetrimide agar, suggesting a *Pseudomonas*-like identity, likely *P. aeruginosa*. This identification is further supported by its rapid growth onset within 4 hours and strong amylase and catalase activities. MP-1's limited carbohydrate utilization—positive only for glucose and sucrose—is consistent with the metabolic preference of *Pseudomonas* species, which often favour simple sugars (Udaondo et al., 2018). MP-3, the only Gram-positive endophyte, produced translucent, raised colonies with radial filaments on nutrient agar, indicative of a *Bacillus*-related lineage. Its rapid utilization of glucose and sucrose, early growth within 4 hours, and high levels of amylase, urease, and catalase activity suggest a metabolically versatile profile typical of *Bacillus* spp., which are known for their ecological adaptability (Connor et al., 2010). The co-occurrence of *Pseudomonas* and *Bacillus*-like isolates in this study supports findings by Fariska et al., 2024, who reported their association as endophytes in cassava leaves. MP-5, another endophytic isolate, displayed smooth, light-yellow colonies on both nutrient and tryptic soy agar and consisted of Gram-negative, motile rods. It showed the broadest carbohydrate utilization capacity—positive for malic acid, glucose, sucrose, mannitol, and mannose—along with rapid growth (within 4 hours) and strong amylase, urease, and catalase activity. These traits suggest a *Flavobacterium*-like identity with high metabolic plasticity, aligning with earlier observations of *Flavobacterium* as endophytes in rice seeds (Walitang et al., 2017).

In contrast, MP-2 (a soil isolate), grown on *Azospirillum* medium, exhibited Gram-negative, motile rods forming light yellow, opalescent colonies and demonstrated fast, strong utilization of malic acid in addition to glucose and sucrose. This metabolic profile, along with its slower growth onset (12 hours), weak amylase activity, and positive urease and catalase activities, supports its identification as an *Azospirillum* species—organisms well known for their nitrogen-fixing abilities and plant growth-promoting traits (Higdon et al., 2020). MP-4, another soil isolate cultured on *Beijerinckia* medium, consisted of slightly curved, Gram-negative rods forming mucoid, brownish-white colonies, characteristics consistent with *Beijerinckia* spp., a group of free-living nitrogen fixers. Its ability to utilize glucose, sucrose, and mannitol, along with a slower growth onset (14 hours) and strong amylase and catalase activities,

suggests metabolic versatility and an ecological adaptation to nutrient-limited environments—features commonly associated with diazotrophic bacteria.

The isolates demonstrated catalase activity and motility, indicating oxidative stress tolerance and flagellar-mediated adaptability to environmental conditions. Displaying catalase activity indicates aerobic or facultative anaerobic capacity to manage oxidative stress. Distinct enzymatic and carbohydrate utilization patterns revealed specialized metabolic strategies, with MP-2 efficient in malic acid metabolism and MP-5 showing broad substrate versatility. MP-1 and MP-5 appeared suited to nutrient-rich habitats, while MP-2 and MP-4 were better adapted to nitrogen-limited environments. Growth onset further supported these ecological roles, with faster growth in MP-1, MP-3, and MP-5 and slower responses in MP-2 and MP-4, suggesting niche-specific adaptations. All inoculants showed phosphate-solubilizing activity on the Pikovskaya agar medium, reinforcing their potential to enhance phosphorus availability.

Out of all the applied inoculants, either individually or in combination, only "MP-1+MP-3+MP-5" has the most substantial positive impact, and CP soil produces larger leaf areas than AG soil. The observed synergistic effects of combined treatments on leaf area further emphasize the need for integrated microbial applications in agricultural settings. For instance, earlier studies have demonstrated the importance of phosphate-solubilizing bacteria in improving phosphorus availability to plants (Goswami et al., 2014). Similarly, the role of nitrogen-fixing bacteria in enhancing soil fertility and plant growth is well-documented (Oteino et al., 2015). The observed synergistic effects of combined treatments are consistent with research by Wani et al., 2007 and Vassileva et al., 2020 indicating the potential for combined microbial applications to achieve better agricultural outcomes.

## Microbial Interaction Studies

These cooperative associations suggest that endophytic *Pseudomonas*, *Bacillus*, and *Flavobacterium* may co-colonize plant tissues, potentially enhancing nutrient uptake and promoting plant growth through mutualistic relationships. Similar findings were reported by Sher et al., 2024, who studied microbial communities on a Hawaiian lava field and revealed that non-diazotrophic strains, particularly *Sphingomonas* sp. (HT1-2), significantly enhanced nitrogenase activity in diazotrophic *Azorhizobium* spp. isolates. This synergy was critical to achieving nitrogen fixation rates that surpassed those of individual strains. The study underscored that microbial cooperation—rather than individual performance - is essential in supporting key ecosystem functions like biological nitrogen fixation in oligotrophic environments. Taken together, these observations reinforce the ecological significance of microbial consortia, where synergistic interactions between endophytes such as *Pseudomonas*, *Bacillus*, and *Flavobacterium* may collectively drive nutrient mobilization, plant growth promotion, and stress resilience under environmental constraints. Studies like Santoyo et al., 2021 have shown that endophytic microbes can form synergistic relationships, enhancing nutrient cycling and promoting plant growth when working together.

## Comparative Analysis of CP and AG Soils: Physical, Chemical, and Biological Properties

The physical, chemical, and biological properties of soils can vary significantly depending on their use and management practices (Brady and Weil, 2008). This is evident from the distinct differences observed between the CP and AG soils in the present study. The CP soil exhibits a reddish colour, sandy loam texture, higher clay content (18.14%), and higher bulk density (1.184 g/cc), indicating a finer texture and more compacted state compared to the agricultural soil has a coarser texture (Hillel, 2014).

In contrast, the AG soil appears blackish with a loamy sand texture, higher sand content (86.34%), and lower bulk density (1.0903 g/cc), suggesting a coarser texture and less compacted state. Higher bulk density in CP soil indicates greater soil compaction, which is likely caused by frequent human activity, infrastructure development, and reduced biological activity in college premises. Conversely, porosity and water-holding capacity were significantly higher in AG soils, suggesting better soil structure and moisture retention. This could be attributed to regular agricultural practices such as tilling, organic matter application, and crop rotation, all of which can improve soil aeration and water retention. Textural components also varied markedly. Clay and silt contents were higher in CP, while sand content was significantly greater in AG. This might reflect natural differences in parent material or long-term management impacts, such as erosion control in agricultural fields and surface sealing in urban soils.

Chemically, the AG soil has higher available phosphorus (55.98 mg/kg), reflecting intensive fertilizer use in cultivated fields that boosts phosphorus availability. (Havlin et al., 1999). Electrical conductivity (EC), which reflects soluble salt content, was substantially higher in AG (192.92 µS/cm) than in CP (114.36 µS/cm), suggesting increased salinity due to fertilizer residues and irrigation practices. Available potassium showed a significantly lower concentration in AG (81.61 mg/kg) than in CP (110.25 mg/kg), possibly due to crop uptake and depletion over successive growing seasons. The total nitrogen level in AG (1120.43 mg/kg) was nearly three times greater than in CP (357.69 mg/kg), indicative of regular nitrogen fertilization enhancing soil fertility. Total organic carbon (TOC) also followed this trend, with AG soil (1.25%) showing significantly more organic matter than CP soil (0.46%), likely due to the incorporation of organic residues and better soil management practices in agriculture (Doran and Zeiss, 2000). The pH of AG soil (7.10) was slightly more alkaline than CP soil (6.90), though both remained within the optimal range for most crops. This difference could stem from liming practices or the nature of parent materials.

The biological properties further differentiate the two soils, reflecting differences in microbial activity levels. The higher urea availability in CP soil—likely due to reduced plant uptake, as crop plants are not grown as abundantly as in agricultural fields—may stimulate urease-producing microbes. Additionally, the dominance of specific urease-producing taxa, such as *Bacillus mannanilyticus*, as revealed by microbial sequencing data, could contribute to the elevated urease activity observed in CP soils. In contrast, the AG soil has higher acid phosphatase (61.20pNP/g/12h) and beta-glucosidase (141.36µgpNP/g/h) activities, suggesting a more active microbial community and higher organic matter content, likely due to the inputs and management practices associated with agricultural production (Nannipieri et al., 2018; Tabatabai, 1994).

The independent t-test results showed very highly significant differences (*** $p < 0.001$) across all measured soil parameters. These differences reflect the distinct land use practices and environmental conditions influencing soil properties in the two systems.

## PCA and soil quality indexing

After analyzing two soil types, CP and AG, using PCA to understand the variation in their properties, for CP soil, PCA revealed seven main factors that explain about 87% of the variation, with bulk density, electrical conductivity, clay total organic carbon, total nitrogen and pH being the most important. Using these key indicators, a SQI of 0.59 was calculated for CP soil. For AG soil, PCA identified six main factors explaining around 84.61 % of the variation, with total organic carbon, pH, sand, urease and β-glucosidase activity of soil being the most significant. The SQI for AG soil was determined to be 0.55.

According to Istijono and Harianti (2019), soil quality can be classified on a scale from 0 to 1, with higher values indicating better quality. SQI of both soils falls into the "Fair" category, and CP soil represents slightly better quality than AG soil. This quality is likely due to favourable physical, chemical, and biological properties, such as optimal nutrient levels and good soil structure, all of which support comparatively better plant growth and ecosystem functions.

## Study of 16s rRNA Amplicon Sequence of V3-V4 region

The AG soil sample shows higher species richness compared to CP soil (1413 vs. 427), Shannon diversity index (8.292 vs. 4.068), and Simpson's diversity index (0.991 vs. 0.800), indicating a more diverse microbial community. This study aligns with the findings of Zhou and Fong (2021), who emphasized the importance of soil microbial communities as indicators of soil health influenced by agricultural management, plant selection, and soil compartment and observed higher microbial alpha diversity and species richness in soils with comparatively higher organic carbon content, consistent with this study. This higher diversity in AG soil suggests greater resilience and resistance to invasion by new microbes, whereas the lower diversity in CP soil may mean it is more susceptible to changes. However, high diversity in AG soil might be challenging for introducing microbial inoculants due to increased competition among species (Callaghan et al., 2022).

A metagenomic study of CP and AG soils revealed that the most abundant phylum (Fig. 2,3) in both is Proteobacteria, making up about 50% of the bacterial population. Firmicutes and Actinobacteriota follow, with approximately 35.4% and 6.11% abundance, respectively. The dominance of Actinobacteriota (32.77%) in AG soil aligns with the findings of Dai et al. (2018), who reported that long-term nitrogen fertilization increased the relative abundance of Actinobacteria, suggesting that elevated nitrogen availabil-

ity favours copiotroph bacterial groups in agro-ecosystems. In AG soil, there is a more diverse bacterial community, with notable presences of Acidobacteria, Chloroflexi, and Myxococcota, in addition to the dominant phyla.

In terms of bacterial orders (Figure 4,5), CP soil is predominantly composed of Enterobacterales (approximately 43.6%), followed by Bacillales (30.4%) and Pseudomonadales (4.5%). Other major orders include Thermomicrobiales (3.9%), Limnochordales (2.89%), and Micromonosporales (2.4%). In AG soil, the distribution is more diverse, with Solirubrobacterales (9.6%) and Rhizobiales (9%), Micromonosporales (1.5 %) being the most prominent orders, followed by Methanocellales, Frankiales, and Acidobacteriales. Micromonosporales present in both CP and AG, widely found in various environments, including soil, contributes to soil fertility by breaking down chitin, cellulose, lignin, and pectin, enhancing nutrient availability and supporting plant growth (Hirsch and Valdés, 2010). The Rhizobiales order under Alphaproteobacteria was found in higher abundance in AG (9%) compared to CP (0.36), contributing to essential plant-associated functions such as nitrogen fixation and nutrient uptake (Suleiman et al., 2019).

In terms of species, in CP soils, under Bacillales, *Bacillus mannanilyticus* (1.62%), *Lysinibacillus* sp. YS11 (1.59%) and under Lactobacillales, *Lactobacillus kefiranofaciens* (1.048%), first one is prominent for breaking down complex organic matter and cycling nutrients. These soils also harbour bacteria associated with humans and animals, like *Escherichia coli* and *Massiliprevotella massiliensis*, and species found on human skin and mucous membranes, such as *Moraxella osloensis* and *Staphylococcus nepalensis*. *Lysinibacillus* sp. YS11 is known for its role in calcium carbonate formation, influencing soil structure (Lee et al., 2017). In contrast, AG soil contains *Bradyrhizobium elkanii* (0.938%) under the order Rhizobiales, the plant pathogenic *Streptomyces puniciscabiei* (0.478%) under the order Streptomycetales , and the copiotrophic *Micromonospora echinospora* (0.465%) under the order Micromonosporales. *Bradyrhizobium elkanii* plays a key role in symbiotic relationships with plants and contributes to soil nutrient cycling. AG soil also contains species like Gemmatimonadetes bacterium LX87 (0.130%) and *Nocardioides* sp. (0.086%), known for their roles in biogeochemical processes.

# Conclusions

This study demonstrated that the combined application of MP-1 (*Pseudomonas* sp.), MP-3 (*Bacillus* sp.), and MP-5 (*Flavobacterium* sp.) significantly improved spinach growth, whereas individual treatments had limited effect. This reduced efficacy may be attributed to niche unavailability, suboptimal soil conditions, and the limited multifunctionality of single strains. Principal Component Analysis (PCA) helped identify key soil properties responsible for variability between CP and AG soils. In CP soil, bulk density, electrical conductivity, clay content, total organic carbon, nitrogen, and pH were significant contributors, while in AG soil, total organic carbon, pH, sand content, urease, and β-glucosidase were prominent. These findings suggest that enhancing specific soil parameters is essential for optimizing spinach growth and improving the performance of microbial inoculants. The Soil Quality Index (SQI) values for CP (0.59) and AG (0.55) classify both soils as fair, indicating considerable scope for improvement in achieving higher crop productivity. Distinct native microbial communities and the absence of applied inoculants in both soils underscore the need for improved inoculant establishment strategies. Future research should prioritize enhancing soil health, integrating inoculants into existing microbial networks, and identifying keystone species to increase the reliability and sustainability of microbial inoculant applications.

## Supplementary Materials

https://journals.uni-lj.si/abs/article/view/19915/18325

## Conflict of interest

The authors declare no conflict of interest.

## Funding

This work did not receive any funding.

## Author's contributions

Conceptualization, M.P., S.G.; Methodology, M.P., S.R., and S.G.; Investigation, M.P., S.G.; Formal analysis, M.P.; Writing – original draft, M.P., Supervision, S.G.; Writing - editing and manuscript preparation, S.G.

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Original Research

# Ethnobotanical investigation of therapeutic plants in Thiruthuraipoondi, Tamil Nadu

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## Abstract

Field surveys were conducted in 25 villages of Thiruthuraipoondi, Tiruvarur district, Tamil Nadu, India, to methodically record and enumerate the traditional knowledge (TK) that the villagers had about the use of medicinal plants in treating various human ailments. A comprehensive collection of ethnomedical data was accomplished, including botanical names, vernacular names, family information, habits, parts used, modes of application, use value (UV), Relative frequency citation (RFC) and therapeutic uses. A thorough documentation of the medicinal uses of 63 plant species from 28 families was presented out of the exploration. The study area was dominated by Fabaceae (12.7%), Malvaceae (9.5%), Asteraceae and Euphorbiaceae (7.9%), Apocynaceae, Lamiaceae and Solanaceae (6.3%) plant families. Based on habit, the majority of plants were shrubs (40%), succeeded by herbs (37%), trees (17%) and climbers (6%). Amongst the various plant components used to treat ailments, the most popular ones were the leaves (65%), trailed by whole plants (11%), fruits (8%), roots (5%), seeds (5%), flowers and bark (3% each). The various forms of drug preparations include cooked (24%), decoction (24%), paste (22%), juice (14%), raw (8%), powder (6%), and oil (2%). The most common form of administration was oral. With the highest UV of 0.13 and RFC of 0.33, *Acalypha indica* L. became the most often utilized species. This plant, in particular, attracted a lot of interest from the local population because of its reputation for treating a variety of ailments.

## Keywords

Ethnomedicine, study area, documentation, medicinal plants, informants, therapeutics.

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**Citation:** Govindaraj, S., Dhandapani G., Pappaiyan, S., (2025). Ethnobotanical investigation of therapeutic plants in Thiruthuraipoondi, Tamil Nadu. Acta Biologica Slovenica 68 (3)

**Received:** 01.01.2025 / **Accepted:** 22.04.2025 / **Published:** 25.04.2025

**https://doi.org/10.14720/abs.68.3.21545**

## Etnobotanična raziskava zdravilnih rastlin v Thiruthuraipoondi, Tamil Nadu

**Izvleček**

V 25 vaseh Thiruthuraipoondi, okrožje Tiruvarur, Tamil Nadu, Indija, so bile opravljene terenske raziskave, da bi metodično zapisali in našteli tradicionalno znanje vaščanov o uporabi zdravilnih rastlin pri zdravljenju različnih človeških bolezni. Opravljeno je bilo obsežno zbiranje etnomedicinskih podatkov, vključno z botaničnimi imeni, domačimi imeni, podatki o družini, navadah, uporabljenih delih rastlin, načinih uporabe, uporabni vrednosti (UV), relativni navedbi pogostosti (RFC) in terapevtski uporabi. Skupaj predstavljamo uporabnost 63 zdravilnih rastlinskih vrst iz 28 taksnomskih družin. Na območju raziskave so prevladovale rastlinske družine Fabaceae (12,7 %), Malvaceae (9,5 %), Asteraceae in Euphorbiaceae (7,9 %), Apocynaceae, Lamiaceae in Solanaceae (6,3 %). Glede na habitus je bila večina rastlin grmovnic (40 %), sledila so jim zelišča (37 %), drevesa (17 %) in plezalke (6 %). Med različnimi rastlinskimi sestavinami, ki se uporabljajo za zdravljenje bolezni, so bili najbolj priljubljeni listi (65 %), sledile so cele rastline (11 %), plodovi (8 %), korenine (5 %), semena (5 %), cvetovi in lubje (po 3 %). Različne oblike pripravkov vključujejo kuhane dele rastlin (24 %), poparek (24 %), pasto (22 %), sok (14 %), surovine (8 %), prah (6 %) in olje (2 %). Najpogostejša oblika jemanja je bila peroralna. *Acalypha indica* L. je bila rastlinska vrst z najvišjim UV 0,13 in RFC 0,33. Ta rastlina je zlasti zaradi svojega slovesa pri zdravljenju različnih bolezni vzbudila veliko zanimanja lokalnega prebivalstva.

**Ključne besede**

etnomedicina, študijsko območje, dokumentacija, zdravilne rastline, informatorji, terapija.

# Introduction

The traditional knowledge of medicinal flora forms a major element of primary health care in almost all societies, particularly in remote areas (Khajuria et al., 2021). Medicinal plants have been a vital aspect of primary healthcare around the globe. Due to population growth, drug shortages, high healthcare costs, side effects of many synthetic drugs, and the emergence of drug resistance, the use of plant materials in drug production has increased to address many human health problems. The World Health Organization (WHO) recently estimated that 80% of the world's population relies on medicinal plants for their basic medical needs. According to the World Health Organization (WHO), approximately 21,000 plant species have potential medicinal uses (Tefera and Kim, 2019). Other drugs are used for self-medication or under the advice of doctors and therapists. They rely primarily on medicinal plants and, to a lesser extent, on animals (Kose et al., 2015). Official medical care is typically based on commercial pharmaceuticals that must be acquired with money, whereas a traditional medical visit has a far lower cost, including the use of the medicinal plants required (Macía et al., 2005). The geographical location and the climatic conditions of the region have encouraged the development of an extremely lush and diverse plant life that has been used to cure many diseases from time immemorial (Bouasla and Bouasla, 2017). Millions of people in developing nations use medicinal plants for essential health care, income, and upgrading of their life standards (Polat and Satil, 2012). Attention paid to ethnobotany in recent years shows how people interact with plants and other natural resources in environments around the world (Dery et al., 2023). Traditional knowledge (TK) is widely used by various ethnic groups and communities in remote areas to treat a variety of ailments using a variety of plant species (Bhattacharjya et al., 2023).

In the past, traditional medical practices were not properly documented in Thiruthuraipoondi. However, scientific research using sophisticated analytical techniques and new experimental approaches is reviving interest in the use of medicinal plants. These efforts are gradually building a large body of empirical evidence supporting the effectiveness of plants in treating a variety of conditions (Bamogo et al., 2023). Throughout human history, people have used plants to promote health and overall well-being. Herbal remedies were utilized to tackle a diverse array of ailments, and a considerable segment of the population favours these natural products as their preferred method

of treatment (Brahmi et al., 2022). Traditional medicine has a major impact on public health and is closely linked to cultural celebrations and belief systems (Kiewhuo et al., 2023). This view states that medicinal plants are the source of about 25% of modern medicines. Traditional and ethnic knowledge of medicinal plant documentation is extremely limited due to the lack of research and inventory of medicinal plants used by local people (Tamang et al., 2023). Ethnobotanical research is important for the conservation and use of biological resources because it records indigenous knowledge. As a result, identifying indigenous plants and exploring their applications offers considerable potential socioeconomic advantages (Cakilcioglu and Turkoglu, 2010). About 75% of the plants used in Unani, Ayurveda and Siddha medicinal systems come from tropical forests and 25% from temperate forests. These systems use over 400 species of plants and traditional medicines (Khan et al., 2022). Anti-dysenteric, expectorant, anthelmintic, abortifacient and insecticidal properties are extended by the bioactive compounds present in medicinal fruits, seeds and bark (Thavamurugan et al., 2022).

The Eastern and Western Ghats mountain ranges surround the state of Tamil Nadu in the eastern part of the Indian Peninsula and are home to a diverse ethnic population well-versed in the medicinal plants of the region (Srinivasan et al., 2022). Through the discovery and documentation of traditional medicinal uses of native plants, ethnobotany has become an important part of our world (Özdemir and Alpinar, 2015). Many more medicinal plants and their local uses have yet to be documented (Tugume et al., 2016). It has been suggested that written literature like historical Materia Medica, popular books on plant usage, and other written sources, conditions, and oral ethnobotanical knowledge make ethnopharmacological practices more conservative (Miara et al., 2018). This study documents and provides a quantitative analysis of the traditional knowledge of Thiruthuraipoondi taluk of Tamil Nadu, India.

# Materials and methods

## Study area

The ethnobotanical survey was conducted in 25 villages of Thiruthuraipoondi Taluk, Tiruvarur district, Tamil Nadu, India. The taluk covers a total area of 563.24 km$^2$, with 538.51 km$^2$ being rural and 24.73 km$^2$ urban (Figure 1). It has a population of 214,995, with 168,869 living in rural areas and 46,126 in urban areas (Village info, 2024). The region's soil is predominantly red loam and coastal sand alluvium. The climate is marked by a southwest monsoon from June to September, a northeast monsoon from October to December, a winter season from January to February, and a hot season from March to May. Average rainfall ranges from 10 mm in March to 189 mm in October. Temperatures vary between 25°C in January and 31.5°C in May (Chekole et al., 2015).

## Data collection

Field surveys were carried out in Thiruthuraipoondi, Tiruvarur district, Tamil Nadu, India, from 2023 to 2024 to document the use of therapeutic herbs. Folk medicinal usage documentation was sought from traditional healers, traders, local vendors, and Siddha medicine practitioners in the age groups of 20-90 (Table 1). The biographical characteristics of the respondents in this study included gender, age at which they began practising traditional healing, occupation, and source of income. This study focused on the use of indigenous herbal medicines by traditional healers (Cheikhyoussef et al., 2011).

## Plant Identification

The informants frequently pointed out the location of the medicinal species collection throughout the interviews, and in a few instances, they even assisted with plant collection, demonstrating a clear acquaintance with the documented regional flora (Gamble, 1935; Matthew, 1983; Sankaranarayanan et al., 2010). The species were categorized using Angiosperm Phylogeny Group III (The Angiosperm Phylogeny Group, 2009), and the International Plant Names Index (IPNI) was used to verify the binomial. Specimens of plants were gathered and placed in the PG and Research Department of Botany's herbarium at TPGASC in Trichy, Tamil Nadu.

## Data Analysis

To analyze the collected ethnobotanical data, we used Use Value (UV) and Relative Frequency Citation (RFC). These methods helped to find out which plant species are best for treating illnesses, figure out how important they are, and check their possible uses.

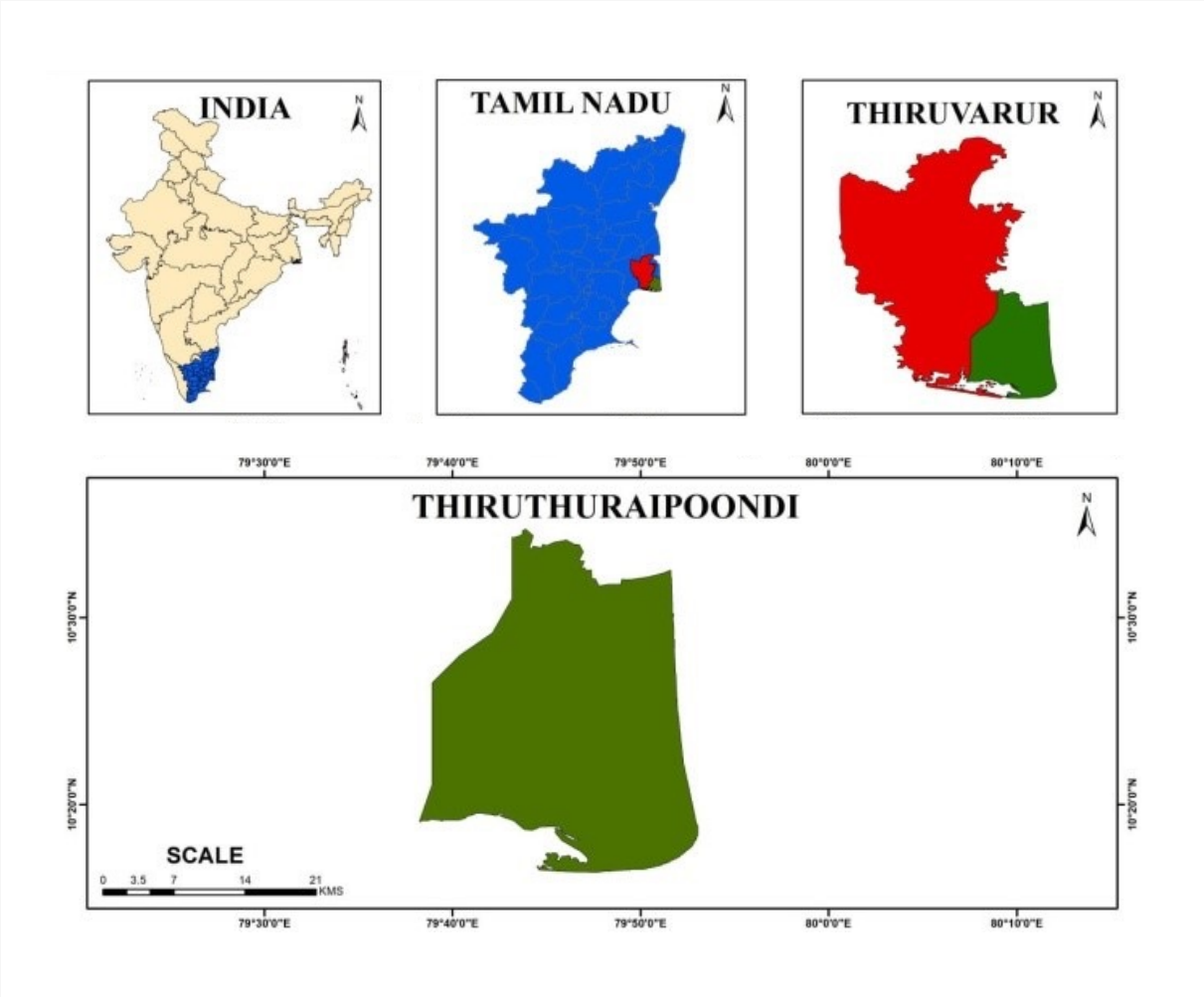


**Figure 1.** Map of Thiruthuraipoondi, Thiruvarur District, Tamil Nadu, India.

**Slika 1.** Zemljevid Thiruthuraipoondi, okrožje Thiruvarur, Tamil Nadu, Indija.

**Table 1.** Demographic profile of the informants.

**Tabela 1.** Demografski profil informatorjev.

| Age group | Male | Female | Total |
|---|---|---|---|
| 20-30 | 2 | - | 2 |
| 30-40 | 3 | 2 | 5 |
| 40-50 | 2 | 3 | 5 |
| 50-60 | 4 | 2 | 6 |
| 60-70 | 2 | 3 | 5 |
| 70-80 | 2 | 1 | 3 |
| 80-90 | 1 | - | 1 |
| **Total** | **16** | **11** | **27** |

### Relative Frequency Citation (RFC)

RFC was developed to measure how much informants agree on a particular plant's classification. The number of informants who mention using a species (also known as the frequency of citation, or FC) divided by the total number of survey respondents (N) yields this index. Each species' local significance is reflected in it (Shaheen et al., 2017).

RFC = FC / N (0 < RFC < 1)

It measures zero if no informant reported any species to be of value and scales to 1 when at least some minimum number of informants report that a particular plant species is useful.

### Use Value (UV)

The use value shows how important local plant species are by counting the number of ways each one is used. It is calculated with a special formula,

UV = U / n,

In this formula, "n" is the total number of informants who discussed a species, and "U" is the number of times each informant reported using that species (Uzun and Koca, 2020).

## Results and Discussion

### Taxonomic diversity of medicinal plants

One of the oldest known medical practices to humans on earth is the use of herbal treatments. Prior to the advent of modern medicine, many communities' long-standing traditional medical systems, which have developed over the years, are still preserved as a valuable source of traditional knowledge for herbal remedies. The purpose of the current study was to document the therapeutic herbs used by the villagers of Thiruthuraipoondi. This study recorded about 63 ethnomedicinal plants belonging to 28 families. Information on botanical names, vernacular names, family, habit, plant part used, preparation method, UV, RFC, and therapeutic uses were furnished (Table 2). The investigation site was found abundant with Fabaceae (12.7%), Malvaceae (9.5%), Asteraceae and Euphorbiaceae (7.9%), Apocynaceae, Lamiaceae and Solanaceae (6.3%), Amaranthaceae (4.7%) and further plant families (Figure 2). People who live in different rural communities have been using medicinal plants to cure a wide range of illnesses and conditions, including skin allergies, cough, ulcers, urinary tract infections, diarrhoea, antibacterial activity, diabetes, jaundice, asthma and anti-inflammatory issues. In tropical regions, legumes constitute an essential source of sustenance. Plants in the Fabaceae family, which are used medicinally in the area, are shown with pertinent information. Fabaceae was reported as the most frequently utilized plant family (Vijayakumar et al., 2016). The extensive recommendation of therapeutic species, as of present, belongs to the Fabaceae family more than to any other plant family in the world (Parthiban et al., 2016).

An essential parameter is the wide distribution of Fabaceae in various ecosystems, including tropical rainforests, to temperate grasslands (Welcome and Van Wyk, 2019). The wide geographic distribution of Fabaceae increases the possibility of human interaction and utilization. Their ability to improve both nutritional quality and soil fertility is greatly enhanced by their ability to fix nitrogen through a symbiotic relationship with rhizobia bacteria present in their root nodules (Nnanamani et al., 2017). This attribute makes Fabaceae highly relevant in agricultural settings and critical to traditional farming practices. The significance of these plants as vital nutritional resources and dietary supplements across various cultures is attributed to their substantial protein levels and essential nutrients. The regular incorporation of Fabaceae species in ethnobotanical research concerning edible flora underscores their nutritional importance. Furthermore, the diverse array of secondary metabolites extant in this family, including alkaloids, flavonoids, and terpenoids, augments its therapeutic applications (Ribeiro et al., 2017). The therapeutic properties of these substances are associated with the wide use of Fabaceae species in the context of traditional drug preparation.

### Habit-wise distribution of medicinal plants

Based on the distribution of plants, shrubs account for the largest portion at 40%, followed by 37% herbs, 17% trees and 6% climbers (Table 2). This finding defies the trend observed in most medicinal inventories, which favours herbaceous medicinal plants (Singh and Singh, 2009). In our study, we found that shrubs, with their high species richness and variety, constitute a common home for medicinal plants in the research area.

**Table 2.** List of Ethnomedicinal plants recorded in the study area.

**Tabela 2.** Seznam etnomedicinskih rastlin, zabeleženih na preučevanem območju.

| Botanical Name | Vernacular Name | Family | Habit | Parts used | Mode of Preparation | UV | RFC |
|---|---|---|---|---|---|---|---|
| *Abrus Precatorius* L. | Rosary pea | Fabaceae | Climber | Leaves | Decoction | 0.09 | 0.25 |
| *Abutilon indicum* (L.) Sweet | Indian mallow | Malvaceae | Shrub | Leaves | Decoction | 0.09 | 0.25 |
| *Acalypha indica* L. | Indian Acalypha | Euphorbiaceae | Herb | Leaves | Paste | 0.13 | 0.33 |
| *Acalypha wilkesiana* Mull. Arg. | Copper leaf | Euphorbiaceae | Shrub | Leaves | Paste | 0.02 | 0.14 |
| *Achyranthes aspera* L. | Chaff flower | Amaranthaceae | Herb | Leaves | Juice | 0.03 | 0.22 |
| *Ageratum conyzoides* L. | Billy goat weed | Asteraceae | Herb | Leaves | Paste | 0.01 | 0.18 |
| *Aloe vera* (L.) Burm. f. | Aloe | Asphodelaceae | Herb | Leaves | Raw | 0.02 | 0.18 |
| *Amaranthus viridis* L. | Amaranth | Amaranthaceae | Herb | Leaves | Cooked | 0.03 | 0.14 |
| *Anacardium occidentale* L. | Cashew nut | Anacardiaceae | Tree | Seed | Raw | 0.02 | 0.14 |
| *Azadirachta indica* A. Juss. | Neem | Meliaceae | Tree | Whole plant | Juice | 0.10 | 0.22 |
| *Bauhinia purpurea* L. | Orchid tree | Fabaceae | Tree | Leaves | Decoction | 0.04 | 0.18 |
| *Caesalpinia pulcherrima* L. | Peacock Flower | Fabaceae | Tree | Bark | Decoction | 0.06 | 0.22 |
| *Calotropis gigantea* (L.) Dryand. | Milkweed | Apocynaceae | Shrub | Leaves | Paste | 0.02 | 0.22 |
| *Carica papaya* L. | Papaya | Caricaceae | Tree | Fruit | Raw | 0.02 | 0.14 |
| *Catharanthus roseus* (L.) G.Don. | Periwinkle | Apocynaceae | Herb | Leaves | Decoction | 0.07 | 0.22 |
| *Coleus amboinicus* Lour. | Indian borage | Lamiaceae | Herb | Leaves | Decoction | 0.02 | 0.18 |
| *Cosmos caudatus* Kunth | Wild cosmos | Asteraceae | Herb | Leaves | Juice | 0.01 | 0.22 |
| *Cucurbita maxima* Duchesne | Pumpkin | Cucurbitaceae | Climber | Fruit | Cooked | 0.02 | 0.22 |
| *Cynodon dactylon* (L.) Pers. | Bermuda grass | Poaceae | Herb | Whole plant | Decoction | 0.02 | 0.14 |
| *Datura metel* L. | Thorn apple | Solanaceae | Shrub | Leaves | Paste | 0.03 | 0.22 |
| *Delonix elata* (L.) Gamble | White gul mohur | Fabaceae | Tree | Bark | Powder | 0.06 | 0.25 |
| *Dracaena trifasciata* (Prain) Mabb. | Snake Plant | Asparagaceae | Herb | Leaves | Paste | 0.02 | 0.14 |
| *Euphorbia hirta* L. | Asthma weed | Euphorbiaceae | Herb | Whole plant | Powder | 0.10 | 0.25 |
| *Euphorbia tithymaloides* L. | Redbird | Euphorbiaceae | Shrub | Leaves | Paste | 0.02 | 0.18 |
| *Gomphrena serrata* L. | Globe amaranth | Amaranthaceae | Herb | Leaves | Powder | 0.02 | 0.18 |
| *Hamelia patens* Jacq. | Firebush | Rubiaceae | Shrub | Leaves | Juice | 0.01 | 0.18 |
| *Hibiscus cannabinus* L. | Java jute | Malvaceae | Shrub | Leaves | Decoction | 0.03 | 0.18 |
| *Hibiscus rosa-sinensis* L. | China rose | Malvaceae | Shrub | Flower | Paste | 0.05 | 0.25 |
| *Hibiscus sabdariffa* L. | Indian roselle | Malvaceae | Shrub | Leaves | Cooked | 0.04 | 0.22 |
| *Ipomoea carnea* Jacq. | Bush morning glory | Convolvulaceae | Shrub | Whole plant | Powder | 0.02 | 0.11 |
| *Justicia adhatoda* L. | Malabar nut | Acanthaceae | Shrub | Whole plant | Decoction | 0.06 | 0.22 |
| *Kerria japonica* (L.) DC. | Japanese rose | Rosaceae | Shrub | Leaves | Cooked | 0.02 | 0.18 |
| *Lagenaria siceraria* (Molina) Standl. | Calabash | Cucurbitaceae | Climber | Fruit | Cooked | 0.01 | 0.18 |
| *Lantana canescens* Kunth. | Hammock shrub | Verbenaceae | Shrub | Leaves | Raw | 0.03 | 0.22 |
| *Leucas aspera* (Willd.) Link | Thumbai | Lamiaceae | Herb | Leaves | Juice | 0.04 | 0.14 |
| *Mangifera indica* L. | Mango | Anacardiaceae | Tree | Whole plant | Decoction | 0.02 | 0.18 |
| *Manihot esculenta* Crantz | Cassava | Euphorbiaceae | Shrub | Root (pulp) | Cooked | 0.01 | 0.18 |
| *Martynia annua* L. | Cat's claw | Martyniaceae | Shrub | Leaves | Decoction | 0.03 | 0.22 |
| *Millettia pinnata* (L.) Panigrahi | Pongame oil tree | Fabaceae | Tree | Seed | Oil | 0.05 | 0.22 |
| *Moringa oleifera* Lam. | Moringa | Moringaceae | Tree | Leaves | Cooked | 0.04 | 0.22 |
| *Musa acuminata* Colla | Banana | Musaceae | Tree | Root | Cooked | 0.06 | 0.22 |
| *Nelumbo nucifera* Gaertn. | Indian Lotus | Nelumbonaceae | Aquatic Herb | Root | Cooked | 0.02 | 0.22 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Nerium oleander* L. | Rosebay | Apocynaceae | Shrub | Leaves | Paste | 0.02 | 0.14 |
| *Ocimum basilicum* L. | Great basil | Lamiaceae | Shrub | Leaves | Decoction | 0.02 | 0.22 |
| *Ocimum tenuiflorum* L. | Tulasi | Lamiaceae | Shrub | Leaves | Raw | 0.03 | 0.22 |
| *Oryza sativa* L. | Rice | Poaceae | Herb | Seed | Cooked | 0.01 | 0.18 |
| *Phyllanthus amarus* Schumach. & Thonn. | Carry me seed | Phyllanthaceae | Herb | Whole plant | Juice | 0.11 | 0.25 |
| *Portulaca oleracea* L. | Purslane | Portulacaceae | Herb | Leaves | Cooked | 0.02 | 0.18 |
| *Portulaca umbraticola* Kunth | Wing pod purslane | Portulacaceae | Herb | Leaves | Decoction | 0.02 | 0.22 |
| *Senna alata* (L.) Roxb. | Candle bush | Fabaceae | Shrub | Leaves | Decoction | 0.09 | 0.25 |
| *Senna auriculata* (L.) Roxb. | Avaram | Fabaceae | Shrub | Flower | Cooked | 0.09 | 0.18 |
| *Sida acuta* Burm.f. | Wire weed | Malvaceae | Herb | Leaves | Paste | 0.03 | 0.22 |
| *Sida rhombifolia* L. | Rhombus-leaved Sida | Malvaceae | Herb | Leaves | Juice | 0.04 | 0.18 |
| *Solanum nigrum* L. | Black nightshade | Solanaceae | Shrub | Leaves | Juice | 0.06 | 0.18 |
| *Solanum torvum* Sw. | Turkey berry | Solanaceae | Shrub | Fruit | Cooked | 0.04 | 0.22 |
| *Solanum trilobatum* L. | Indian nettle | Solanaceae | Shrub | Leaves | Cooked | 0.05 | 0.22 |
| *Sphagenticola trilobata* (L.) Pruski | Creeping Ox-eye | Asteraceae | Climber | Leaves | Paste | 0.02 | 0.18 |
| *Tabernaemontana divaricata* R.Br. ex Roem. & Schult. | Pinwheel flower | Apocynaceae | Shrub | Leaves | Paste | 0.03 | 0.18 |
| *Tamarindus indica* L. | Tamarind | Fabaceae | Tree | Fruit | Cooked | 0.03 | 0.25 |
| *Tithonia diversifolia* (Hemsl.) A. Gray | Tree marigold | Asteraceae | Shrub | Leaves | Paste | 0.02 | 0.14 |
| *Tribulus terrestris* L. | Puncture vine | Zygophyllaceae | Herb | Leaves | Decoction | 0.02 | 0.28 |
| *Trichodesma zeylanicum* (Burm.f.) R.Br. | Camel bush | Boraginaceae | Herb | Leaves | Juice | 0.01 | 0.18 |
| *Tridax procumbens* L. | Coat buttons | Asteraceae | Herb | Leaves | Paste | 0.09 | 0.25 |

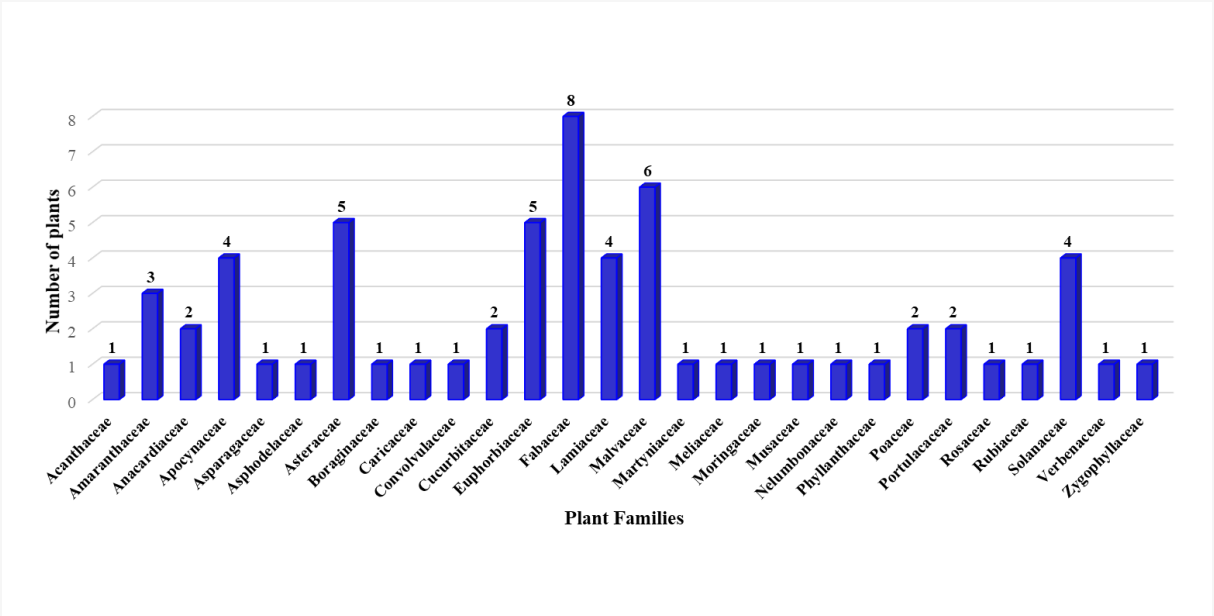


**Figure 2.** Number of species documented in each family.

**Slika 2.** Število dokumentiranih vrst v vsaki družini.

The social relevance of the local floristic resources is demonstrated by the fact that every species that was reported grew naturally in the region (Ribeiro et al., 2010). Within the life form category, the study area's herbaceous group were reported to have the greatest number of species, followed by shrubs (Wagh and Jain, 2018). However, in the present investigation, most of the plants were shrubs that were utilized to cure diverse medical conditions.

Numerous innate features of shrubs are crucial in their regular participation in ethnobotanical studies. Their size being smaller compared to trees makes them simpler to gather and manage. Numerous bushes demonstrate strong resilience and the ability to thrive in different environmental conditions, such as arid climates, low-quality soil, and diverse sunlight intensities. This flexibility is especially important in areas with unreliable weather patterns or damaged environments (Bekele et al., 2022). Several factors shape the important role of shrubs in research on traditional plant use. Their value comes from characteristics such as how easy they are to collect, their adaptability to various environments, and their ability to produce beneficial compounds, all of which make them valuable resources for human societies. Their significance in indigenous societies is enhanced by their numerous applications in healing, sustenance, and practical uses.

## Plant parts (%) used for the preparation of medicine

Treatments for a range of ailments were achieved by using different parts, such as leaves (65%), whole plant (11%), fruits (8%), roots (5%), flowers (3%), seeds (5%), and bark (3%) (Table 2). Plant leaves are the main ingredient in complementary and alternative medicines, and they are easy to collect whenever required without harming the plant (Ganesan and Xu, 2017). The plant leaves, which are more succulent and contain more water, are what produce this phenomenon (Kannan et al., 2016). Since they are easier to gather than underground parts, flowers, fruits, seeds, bark, etc., leaves were expended more frequently (Sivasankari et al., 2024). Leaves are readily accessible and typically need minimal preparation before being utilized (Simbo, 2010). Many ethnobotanical studies highlight the widespread use of leaves, showing how convenient and valuable this readily available plant part is (Tugume et al., 2016). In addition, leaves play a crucial role in plants by acting as the primary organs for photosynthesis and the production of various secondary metabolites. Multiple studies have demonstrated the diverse pharmacological effects associated with leaf extracts, such as antimicrobial, anti-inflammatory, analgesic, and antidiabetic properties (Bieski et al., 2012).

## Mode of preparation and administration

Many methods were used to prepare plants and their parts, including cooking, decoction, paste, juice, raw, powder, oil, and soaking in water. Most medical preparations were administered internally, with plants being utilized in large percentages of decoction (24%), cooked preparations (24%), paste (22%), juice (14%), raw plant material (8%), powder (6%), and oil (2%) (Table 2). In line with earlier ethnobotanical research, the oral route of administration was the primary method of application for traditional medicines, followed by topical treatment (Rashid et al., 2015). The plant sources were heated along with water, sugar, and /or salt for both decoction and cooked versions. The medicinal mixture is taken orally after it has been heated. With the use of a blender or extractor, juice was extracted and then appropriately salted and/or sweetened before being consumed orally. The powder was made from dried plant material, while the paste was made from pulverized plant components that were applied topically (Ignacimuthu et al., 2008). With 80% of all preparations using this method, decoction is the most widely utilized. Notably, oral treatment, external application, and drug washing were the most often employed methods (Zhou et al., 2023). Most of the time, people drink leaf decoctions or apply pastes on the infected area to treat illnesses. Body aches, wounds, scabies, boils, and skin illnesses are among the ailments that can be treated externally with the paste, though internal application is more common than external application (Raj and Ayyanar, 2024).

The most commonly employed plant parts in the preparation remedies mentioned in the study are taken orally, while certain plant species are also used topically and inhaled. Almost 68% of the drugs were found to be administered orally compared to topical (27%) and inhalation (5%) methods (Table 2). The natural design of the human digestive system allows for the effective intake of nutrients and medications (Fabricant and Farnsworth, 2001). Numerous plant-derived bioactive compounds can be easily absorbed through the gut, making oral administration an effective way to ensure widespread distribution within the body. Preparation and delivery of oral medications are generally

less complex and require fewer specialized tools or knowledge than other approaches. This simplicity makes them more accessible to a wider population, including those with limited access to health care or specialized education. In addition, oral medications allow for ease of transportation and storage, making them particularly well-suited for those living in remote settings (Clement et al., 2015). Another reason why traditional medicine is so widely used is the ease of preparation, administration, and storage of oral medications. As suggested, topical treatments such as poultices or creams may be more effective in treating skin infections or injuries (Oladunmoye and Kehinde, 2011).

### Use Value (UV)

*Acalypha indica* L. was found to be the most commonly used species with the highest UV of 0.13, and most people used this plant to treat various disorders, particularly skin problems. *Phyllanthus amarus* exhibited a UV of 0.11. Other plants with high UV in the present study were *Euphorbia hirta* and *Azadirachta indica* with UV of 0.10 each, *Tridax procumbens, Senna alata, Senna auriculata, Abutilon indicum* and *Abrus precatorius* of UV 0.09. Some species recorded very low UV ranging from 0.04 to 0.01, as they were reported only by very few informants (Table 2). *Acalypha indica* has a major role in traditional medicine, as it is used to heal skin disorders. It also has anti-inflammatory, antibacterial, and antioxidant qualities. According to the usage reports, the greater demand for the local medicinal plant collectors' species in the local marketplaces can be linked to their frequent use in the treatment of various ailments (Shrinitha and Aruna, 2023). When a plant species has a higher use value, it means that it is more common in a certain location, which increases its familiarity and likelihood of being collected in comparison to rare plants (Pandey et al., 2024). Local residents have identified *Acalypha indica* as a beneficial remedy for a variety of healing needs. Various parts of the plant are used for different therapeutic purposes, such as treating worms, ulcers, bronchitis, asthma, wounds, bacteria, and additional ailments (Zahidin et al., 2017). Plant recorded with high UV indicates their further investigation using diverse extraction techniques that are required to determine the impact of particular phytochemicals on a range of illnesses. There may remain numerous undiscovered pharmacological characteristics (Dattaray, 2022). The flora that displays high utility in the studied area seems to indicate that the local inhabitants are aware of their prospective medicinal benefits (Rehman et al., 2022). Such awareness is vital for the long-term conservation and exploitation of the valuable resource. Our discoveries call for further research on the bioactive molecules of these plants and any potential applications in modern drugs.

### Relative Frequency Citation (RFC)

RFC showed the frequencies of plant parts used for therapeutic purposes in the provinces under study. The most common application of medicinal plants was *Acalypha indica* (0.33; skin problems), *Tribulus terrestris* (0.28; diuretic, aphrodisiac), *Euphorbia hirta* (0.25; asthma), *Senna alata* (0.25; skin disorders), *Phyllanthus amarus* (0.25; jaundice) and *Azadirachta indica* (0.22; anti-inflammatory) (Table 2). High RFC in the study area reflects that this plant is extensively used by local healers and communities. Herbal drugs are often used in the treatment of cutaneous diseases (Seebaluck et al., 2015). The leaves of *Acalypha indica* contain various bioactive compounds, such as steroids, triterpenoids, alkaloids, saponins, and flavonoids, that have demonstrated different pharmacological activities, including anti-inflammatory and antimicrobial activities (Kasrina and Zukmadini, 2021). The herb is among the most well-known herbal remedies, widely used to treat a wide range of conditions (Nyirenda and Chipuwa, 2024). *Acalypha indica* has been extensively studied as a traditional medicinal plant, with academic research focusing on its healing properties and chemical compounds. This plant has shown potential in the treatment of various health conditions due to its desirable properties. This research has enlightened various pharmacological effects ranging from anti-cancer, anti-inflammatory, anthelmintic, antibacterial, anti-diabetic, anti-hyperlipidemic, anti-obesity, anti-venom, and wound healing properties (Chekuri et al., 2020).

## Conclusions

The study concludes by offering fresh insights into the traditional uses of medicinal plants in the 25 villages of Thiruthuraipoondi, located in the Tiruvarur district of Tamil Nadu, South India. Our findings highlight the fact that the villagers' extensive knowledge of native medicinal plants serves as their main source of healthcare. Surprisingly, the study found that although a large percentage of youth are

aware of the existence of traditional medicine, they seek the opinion of a medical doctor for minor health complaints. Findings from this research will provide long-lasting, profound insights into the traditional utilization of medicinal plants and further assist in the conservation of indigenous knowledge and the impetus of future generations towards taking on traditional healing practices. Our study demonstrated that the indigenous population utilizes an immense array of medicinal plants for healing purposes in treating health disorders and diseases. The exhaustive recording of sixty-three species of plants, covering twenty-eight families of medicinal plants used across the generations, greatly enriches the knowledge gathered on the folk medicine practices of the community. Care should be given according to the dosage level while consuming traditional medicines, depending on the age and health of the patient. Overuse or improper preparation could lead to adverse effects.

## Author contributions

Conceptualization, S.G.; methodology, S.G., G.D., S.P.; software, S.G.; validation, S.G., G.D., S.P.; formal analysis, S.G., G.D., S.P.; investigation, S.G.; data curation, G.D., S.P..; writing—original draft preparation, S.G., G.D., S.P.; writing—review and editing, S.G., G.D., S.P. All authors have read and agreed to the published version of the manuscript.

## Acknowledgement

The authors would like to thank the villagers of Thiruthuraipoondi for extending their support in plant data collection.

## Funding

This research received no external funding.

## Conflicts of Interest

The authors declare no conflict of interest.

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Original Research

# Cyanobacterial control in simulated natural water bodies conditions by commercially available ultrasound: biomass reduction and cyanotoxin degradation

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## Abstract

Low-frequency and low-intensity commercially available ultrasound to control algal growth in natural water bodies was studied. To evaluate the efficiency of ultrasound on cyanobacteria growth rate reduction and, microcystins release, and degradation, a large-scale lab experiment with 150 L of high-density *Microcystis aeruginosa* suspension simulating natural conditions was conducted at different times of ultrasonication: 0, 15 min, one h, five h, 24 h, and 48 h. The first effect of ultrasonication on biomass reduction was noticed at 24 h of continuous ultrasonication, with the highest reduction rates of 97% and 93% for cell count and chlorophyll-*a*, respectively, at 48 h of continuous ultrasound treatment. The growth inhibition test showed biomass reduction in the samples exposed to ultrasonication for at least one hour with increasing effect from here on. The most efficient in *M. aeruginosa* reduction was the longest tested ultrasound treatment of 48 h with growth inhibition of 96%, followed by 24-h ultrasound treatment with 50%, and 5-h ultrasound treatment with 17% growth inhibition after 9 days of incubation. At five hours of ultrasonication, a sharp increase in dissolved microcystins in the medium was observed as a result of ultrasound-induced stress, followed by a drop of dissolved microcystins under the detection limit at 24 h of continuous ultrasonication. This study showed that commercially available ultrasound devices are highly efficient for cyanobacterial bloom control already at relatively low times of exposure, one to two days, with no health risks due to increased dissolved toxins after continuous ultrasound treatment for 24 h or more.

## Keywords

algae control; cyanobacteria; ultrasound; cyanotoxins; *Microcystis aeruginosa*

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**Citation:** Govindaraj, S., Dhandapani G., Pappaiyan, S., (2025). Ethnobotanical investigation of therapeutic plants in Thiruthuraipoondi, Tamil Nadu. Acta Biologica Slovenica 68 (3)

**Received:** 20.02.2025 / **Accepted:** 24.04.2025 / **Published:** 28.04.2025

**https://doi.org/10.14720/abs.68.3.21912**

## Zaviranje rasti cianobakterij v naravnih vodnih telesih s komercialno dostopnim ultrazvokom: zmanjšanje biomase in razgradnja cianotoksinov

**Izvleček**

V študiji smo raziskovali nizko frekvenčni komercialno dostopni ultrazvok z nizko intenziteto za namen nadzora prekomerne razrasti alg v naravnih vodnih telesih. Za ovrednotenje učinkovitosti ultrazvoka za zmanjšanje biomase cianobakterij ter sproščanja in razgradnje mikrocistinov smo izvedli laboratorijski poskus s 150 L suspenzije potencialno toksičnega seva cianobakterije *Microcystis aeruginosa* z visoko gostoto in simulacijo naravnih pogojev ter različnimi časovnimi intervali uporabe ultrazvoka: 0, 15 min, 1 h, 5 h, 24 h in 48 h. Prvi učinek ultrazvoka na zmanjšanje biomase cianobakterij je bil opazen po 24 urah neprekinjene uporabe ultrazvoka. Najvišje zmanjšanje števila celic (97 %) in koncentracije klorofila-a (93 %) smo ugotovili po 48 urah neprekinjene uporabe ultrazvoka. Študija zaviranja rasti, z devet dnevno inkubacijo, je pokazala zmanjšanje biomase *M. aeruginosa* v vzorcih, ki so bili vsaj eno uro izpostavljeni ultrazvočni obdelavi z naraščajočim učinkom pri daljši izpostavljenosti. Največje zmanjšanje biomase *M. aeruginosa* (96 %) smo ugotovili pri najdaljši testirani ultrazvočni izpostavljenosti kulture in sicer 48 ur. Sledila ji je 24-urna izpostavljenost s 50 % zmanjšanjem biomase in 5-urna izpostavljenost s 17 % zmanjšanjem biomase. Po petih urah ultrazvočne obdelave smo opazili močno povišano koncentracijo raztopljenih mikrocistinov v mediju kot rezultat ultrazvočnega stresa, ki mu je po 24 urah neprekinjene ultrazvočne obdelave sledil padec raztopljenih mikrocistinov pod mejo zaznavnosti. Študija je pokazala, da so komercialno dostopne ultrazvočne naprave zelo učinkovite za nadzor cvetenja cianobakterij že pri relativno kratkih časih izpostavljenosti, enega do dveh dni, brez zdravstvenih tveganj zaradi povečanega sproščanja raztopljenih cianotoksinov po 24 urah ali več neprekinjene uporabe.

**Ključne besede**

nadzor alg; cianobakterije; ultrazvok; cianotoksini; *Microcystis aeruginosa*

# Introduction

Algal blooms can impact the environmental health of water resources and influence water use (Zhu et al., 2021). Toxic cyanobacteria blooms, in particular, due to the production of toxins (cyanotoxins), can have negative impacts on human and animal health, and thus, they can impair the recreational value of the water bodies and drinking water quality (Huisman et al., 2018) and consequently threatens water security and safety in Europe and globally (Ho et al., 2019). Cyanobacterial blooms increase largely due to anthropogenic eutrophication, which is emphasized by climate change, with extreme weather events like heavy rains causing an increase in nutrient runoff from land, further exacerbating eutrophication (Sinha et al., 2017). Global warming and draughts are extending temperature stratification in lakes, strengthening water column stability, which favours buoyant, potentially toxin-forming cyanobacteria over non-buoyant, harmless algae (Paerl & Huisman, 2008). The presence of cyanobacteria in water bodies can cause significant economic losses to businesses, such as water companies, losses to fisheries, and impacts on water-related recreational activities and tourism (Hamilton et al., 2013; Sanseverino et al., 2016).

In case of excessive growth (blooms) some cyanobacteria taxa produce toxins in quantities causing toxicity in mammals, including humans (e.g., Van Apeldoorn et al., 2007), together with volatile organic compounds such as geosmin and 2-methylborneol causing unacceptable taste and odour of drinking water and fish (Jüttner & Watson, 2007; Robin et al., 2006; Yoshinaga et al., 2006). There are many different types of cyanotoxins; however, microcystins (MCs), cylindrospermopsins, anatoxins and saxitoxins are causing the most public health risks (Chorus & Welker, 2021; Qi et al., 2014). Microcystin-LR (MC-LR), the most common MC variant, has been classified as Group 2B, possibly carcinogenic to humans (Lyon, 2010). Once in cells, MCs cause protein phosphatase (PP1, PP2A and PP5) inhibition, resulting in destabilization of the cytoskeleton followed by cellular apoptosis and necrosis (Mackintosh et

al., 1990). High acute doses cause haemorrhage in the liver, but at low doses (<20 µg/kg bw) and with repeated long-term exposure, phosphatase inhibition induces cellular proliferation, hepatic hypertrophy and tumour-promoting activity (Chorus & Welker, 2021). There is a growing body of evidence indicating harmful MC-related neurological and reproductive effects, but the data are not yet robust enough to use as a basis for guideline development (Chorus & Welker, 2021).

Based on available toxicological data, the World Health Organization (WHO) in 2003 established a health safety guideline value for drinking water with lifetime exposure of 1 µg/L for MC-LR equivalents (Chorus & Welker, 2021; WHO, 2003, 2017). In 2021, WHO has upgraded values for the main four groups of cyanotoxins (e.g., 0.7 µg/L for cylindrospermopsins, 1 µg/L for MCs, 3 µg/L for anatoxins and 0.3 µg/L for saxitoxins) and predicted different exposure scenarios; previously mentioned guideline values for lifetime drinking water exposure, short-term drinking water exposure (e.g., 12 µg/L of MCs,) as well as values for recreational exposure (e.g., 24 µg/L of MCs). MC-LR is also included in the revised Drinking Water Directive (Directive (EU) 2020/2184) adopted in December 2020. Also, the Bathing Water Directive (Directive 2006/7/EC) refers to cyanobacteria proliferation as a problem, suggesting appropriate monitoring of bathing water quality and adequate fast managing measures. Exposure to cyanotoxins occurs via drinking contaminated water, swimming (showering) in contaminated water, consumption of fish or shellfish farmed in contaminated water, or consumption of food crops irrigated with contaminated water (Svirčev et al., 2017). According to Testai et al. (2016), contamination of food items with cyanotoxins, for example, through crop spray irrigation, is considered by the European Food Safety Authority (EFSA) as an emerging issue.

Management for preventing or suppressing cyanobacteria blooms can include nutrient-loads reduction, hydrodynamics regulation, chemical-algaecides addition, flocculation and harvesting (Zhou et al., 2018). Chemical-algaecides are usually used in emergencies due to their fast and efficient operation; however, they can have severe side effects on aquatic organisms and the aquatic ecosystem (Huisman et al., 2018). Removing cyanobacteria from water through conventional water treatment processes such as coagulation, flocculation, and filtration is not easy because of their small size and low gravity (Kong et al., 2019). Moreover, water treatment processes that include the use of potassium permanganate or chlorine may even release toxins from the cyanobacteria cells into the water, which can enter the human food chain through drinking water supplies (Rajasekhar et al., 2012) since also cyanotoxins may not effectively be removed and degraded by conventional water treatment techniques, especially during a bloom event (He et al., 2014). Thus, alternative approaches for cyanobacteria bloom prevention and suppression are a necessity.

The application of ultrasonic waves as a non-chemical technique for the reduction of cyanobacterial blooms in fresh water and wastewater has become popular in the last decades (Chorus & Welker, 2021). Several studies reported that ultrasound (US) efficiently reduces the growth rate of cyanobacteria by collapsing the gas vesicles during cavitation, inhibiting cell division, or inflicting immediate damage on photosynthetic activity (e.g., Ahn et al., 2003; Kazunori Nakano et al., 2001; Zhang et al., 2006a). However, the US is known to be able to induce the lysis of cyanobacteria cells, which causes the release of intracellular content into the water (Rajasekhar et al., 2012; Zhang et al., 2006b). Song et al. (2005), among others, reported that the US is also effective in degrading cyanotoxins. An additional positive is that the US proved to have minimal impact on green algae or on algal cells which lack gas vacuoles (Rajasekhar et al., 2012; Tang et al., 2004).

Lab-scale studies on the growth rate reduction efficiency of cyanobacteria by ultrasonication (e.g., Huang et al., 2020; Kong et al., 2019; Li et al., 2019; Lürling et al., 2014; Rumyantsev et al., 2021; Wu et al., 2020) are numerous, while studies on cyanotoxin degradation by ultrasonication (e.g., Chen et al., 2020; Lürling et al., 2014) are a little bit less common. However, there is often a disconnect between the research activities and the commercial products since the research activities mostly focus on high-intensity US devices with cavitation effect, whereas commercially available US devices for controlling algal blooms in natural water bodies are low-intensity for minimizing the risk of non-target organisms and the environment. Moreover, there is a lack of peer-reviewed scientific publications on trials in natural or semi-natural conditions using commercially available US units. Therefore, the aim of our study was to investigate the efficiency of commercially available low-intensity US in controlling cyanobacterial blooms and their toxins in the natural water environment. A large-scale laboratory experiment simulating natural conditions was conducted with a strain of toxic *M. aeruginosa* at high-density conditions (simulating cyanobacterial bloom) in a 150 L volume setting (simulating

shallow reservoir or lake) and with the use of low-intensity and low-frequency commercially available US device. The research questions were a) what is the minimum time of ultrasonication affecting *M. aeruginosa* growth, b) what is the time dynamics after US treatment in the surrounding medium or when to expect the increase and decrease of cyanotoxins in the water column, and c) what happened with cyanotoxin concentrations outside and inside the cyanobacterial cells.

# Materials and Methods

## Cultivation of *Microcystis aeruginosa*

*Microcystis* is the most dominant colonial bloom-forming genus responsible for toxic blooms in eutrophic lakes worldwide (Fang et al., 2018). *M. aeruginosa* was used as a test organism in this study. Toxic strain of *M. aeruginosa* (PCC 7806) was purchased as a live culture from the algae bank of the Pasteur Institute (Paris, France) and cultivated according to Stanier et al. (1971) at an ambient temperature of 25 °C (Fang et al., 2018) using nutrient medium BG-11 (Merck, Germany). The starter culture of *M. aeruginosa* was cultivated in aseptic conditions, first in Erlenmeyer flasks (from 100 to 5000 mL) and later in covered glass aquariums. For *M. aeruginosa* cultivation, tubular fluorescent lamps (FLUORA L36W/77, OSRAM, Germany) and a 16 h/8 h light/dark cycle were used. Light irradiance was measured with a light meter (MS-1300, Voltracft, Italy). *M. aeruginosa* culture with low cell concentration (<104 cells/mL) was cultivated for three weeks under low illumination intensity (<40 µmol photon/m$^2$/s); when *M. aeruginosa* culture reached exponential phase of growth (106 cells/mL), it was further cultivated at higher illumination intensity (60 µmol photon/m$^2$/s) (Fang et al., 2018) for another two weeks until it reached final density of 108 cells/mL (inoculum).

## Experimental design

For the experiment conduction a custom-made polyvinyl chloride (PVC) foam pond (width 70 cm, length 180 cm, height 45 cm) with volume of approximately 350 L coated with PVC foil to ensure water tightness was used (Fig. 2). The experiment was conducted at an ambient temperature of 25 °C with illumination of 60 µmol photon/m$^2$/s (FLUORA lights 33W/77, Osram, Germany) in 16 h/8 h light/dark cycle.

To obtain the needed culture volume of approximately 150 L, dense *M. aeruginosa* culture (inoculum) was diluted to $10^6$ cells/mL by adding fresh nutrient medium BG-11 (Merck, Germany). Diluted *M. aeruginosa* culture was transferred into the PVC pond for US treatment. Commercially available LG Sonic (www.lgsonic.com) US device (power 25 W, frequency output 20-100 kHz) was immersed into the *M. aeruginosa* culture and switched on for the next 48 h.

## Monitoring of the system

400 mL of sample was siphoned 10 cm below the water surface at different ultrasonication times (0, 15 min, one h, five h, 24 h, and 48 h) and extracted. This way, *M. aeruginosa* cells, which sank to the bottom of the container due to the destruction of gas vesicles caused by US treatment, were not captured in the sample. The ultrasonication times were selected based on our preliminary experiments (not published) and the findings of Kong et al. (2019). 100 mL of the extracted sample was used for cyanobacterial growth assessment (cell count and chlorophyll-*a* analyses) and MCs analyses. The rest 300 mL of the extracted sample was used for a growth inhibition test. All experiments were performed in triplicates.

## Cell count and chlorophyll-*a* concentration

Cell count was performed according to a protocol of Moheimani et al. (2013) by using a light microscope (CX31RBSF, Olympus, Japan) and a Neubauer chamber (Celeromics, France) with a 0.1 mm depth and a 0.0025 mm$^2$ total counting surface.

Chlorophyll-*a* was analyzed according to Vollenweider (1969). In short, a 5 mL sample was centrifuged at 8000 rpm for 10 min with a UNIVERSAL 320 centrifuge (Hettich Zentrifugen, Germany). The supernatant was discarded, and the sample was re-suspended in 8 mL of methanol (Sigma-Aldrich, USA). The suspension was kept in a water bath at 50 °C for one hour and centrifuged at 4000 rpm for 10 min. Chlorophyll-*a* concentration was determined spectrophotometrically and calculated using the following equation:

$$\text{Chlorophyll } a \left[\frac{\mu g}{mL}\right] = \frac{13.9 \times (E_{665} - E_{750}) \times 8}{V_{sample} \times l}$$

$E_{665}$ and $E_{750}$ are the absorbance of the chlorophyll-*a* suspension in methanol at 665 nm and 750 nm, respectively, and l is the width of the cuvette used.

## Growth inhibition test

For the growth inhibition test 100 mL of sample taken at each time of ultrasonication (0, 15 min, one h, five h, 24 h, and 48 h) was added to the 100 mL of nutrient medium BG-11 (Merck, Germany) and transferred into a 250 mL Erlenmeyer flask provided with illumination of 60 µmol photon/$m^2$/s (FLUORA lights 33W/77, Osram, Germany) in 16 h/8 h light/dark cycle for the following 9 days. Continuous mixing was provided by magnetic stirrers (IKA, Germany, 250 rpm). The growth inhibition test was performed in triplicates at an ambient temperature of 25 °C according to the OECD guidelines (OECD, 2011). Chlorophyll-*a* concentration as an indicator of *M. aeruginosa* growth was analysed in each Erlenmeyer flask on days 1, 2, 3, 5 and 9 using a Nanocolor VIS spectrophotometer (Macherey-Nagel, Germany). The relationship between cell count with a Neubauer chamber (Celeromics, France) under a light microscope (CX31RBSF, Olympus, Japan) and measured chlorophyll-*a* concentration was determined. In 200 mL of nutrient medium, 40 mL of *M. aeruginosa* inoculum was added and cultivated for 7 days at the conditions described in Section 2.1. Afterwards, the dilution with the nutrient medium in ranges of 1%, 2.5%, 5%, 7%, 10%, 20%, 30%, 40%, 50%, 60%, 80%, and 100% was performed. A linear relationship (R2=0.9892) between cell count and chlorophyll-*a* was obtained (data not shown). The chlorophyll-*a* concentration of the cyanobacterial suspension was therefore used as a means of monitoring cell concentration and, hence, cell growth (Moheimani et al., 2013). Growth inhibition was calculated according to the following equation:

$$\text{Growth inhibition [\%]} = \frac{(\text{No. of cells at time 0} - \text{No. of cells at specific time of ultrasonication})}{\text{No. of cells at time 0}} \times 100$$

## Microcystins concentration

For the analyses of cyclic peptides, a well-established high-performance liquid chromatography (HPLC) method (Sedmak et al., 2008) was used, which is an optimized Harada method (Harada et al., 1988). All the samples were first lyophilised, extracted, and purified. Approximately 100 mL of dense cyanobacterial culture was lyophilised (Alpha Christ, France). Freeze-dried cyanobacteria (exactly 50 mg) were extracted three times with 5% aqueous acetic acid (3 x 20 mL) for 30 min while stirring. The mixture was frozen to increase sedimentation. The extracts were centrifuged at 4000 rpm for 10 min. The combined supernatants were applied to preconditioned 500 mg reversed-phase disposable columns (LiChrolut RP-18, Merck). The columns containing the extract were washed with 20 mL of 10% methanol, and the cyclic peptides eluted with 2 mL methanol (LiChrosolv, Merck), evaporated to dryness under nitrogen stream, and the residues, eluted from the columns dissolved in the buffer for HPLC analysis.

Samples were then analysed using the analytical HPLC method (Waters Corporation), using isocratic elution with methanol: 0.05 M phosphate buffer 58:42 (v/v) pH 3.0. The extracts were separated on an analytical Hibar Pre-Packed RT 125-4 LiCrospher 100 RP-18 (5 µm) column (Merck), flow rate 1 mL/min, using HPLC/PDA (Waters) to visualise cyclic peptides. Millenium 32 software (Ver.3.0, Waters) was used to run the hardware and to process the data.

The cyclic peptides were identified and visualised with a photodiode array detector (PDA). The column eluate was monitored at wavelengths (λmax) 238 in order to locate and distinguish MCs from other biologically active substances from cyanobacteria. From the individual peaks, the amounts of the cyclic peptides were calculated by comparison of the integrated peak areas with the values from the calibration curves that were standardised by previously isolated cyclic peptides in pure form.

## Statistical analyses

One-way analysis of variance (ANOVA) with Tukey's post hoc test and a linear correlation were used for statistical analyses. The normality of data was checked using the Lilliefors test; all datasets were distributed normally. The analyses were conducted using the SPSS statistical package.

The algal reduction rate was used to characterize the reduction effect of the US irradiation on *M. aeruginosa* biomass and MCs concentration and was calculated as follows:

$$\text{Reduction rate [\%]} = \frac{Cc - Ct}{Cc} \times 100$$

Cc and Ct are the measured parameters of the control group (time 0; before sonication) and the experimental (test) group at the same time, respectively. The MCs reduction rate was used to characterize the effect of the US irradiation on MCs concentration released from *M. aeruginosa* cells, where 100% reduction represents MCs concentration at the end of the experiment, where no dissolved MCs were detected by HPLC.

# Results and Discussion

## Impact of ultrasonication on *Microcystis aeruginosa* biomass

*M. aeruginosa* biomass at different times of ultrasonication (0, 15 min, one h, five h, 24 h, and 48 h) determined as cell count and chlorophyll-*a* concentration are shown in Fig. 1. Initial cell concentration in the water column before the start of the US treatment was 1 x $10^6$ cells/mL with initial chlorophyll-*a* concentration of 0.09 µg/L (Fig. 1).

In the first five h of continuous ultrasonication, there was no marked change in the number of *M. aeruginosa* cells or chlorophyll-*a* concentration in the water column (Fig. 1). After 24 h of ultrasonication, cell concentration in the water column decreased by 4-fold to 0.25 x $10^6$ cells/mL and chlorophyll-*a* concentration decreased by 2.3-fold to 0.04 µg/L, indicating efficient reduction of *M. aeruginosa* in the water by continuous 24-h US treatment. After 48 h of US treatment, cell concentration in the water column decreased by 28-fold to 4 x $10^4$ cells/mL, while chlorophyll-*a* concentration dropped close to the detection limit, showing that the vast majority of the *M. aeruginosa* cells were sedimented. Reduction rates of *M aeruginosa* increased with ultrasonication time (Fig. 2), which is congruent with other studies (Kong et al., 2019; Peng et al., 2020; Rumyantsev et al., 2021). The highest reduction rates reached in our study were 97% for cell count and 93% for chlorophyll-*a* at 48 h of continuous US treatment. However, at 24 h of continuous ultrasonication, reduction rates achieved were 78% and 59% for cell count and chlorophyll-*a*, respectively. Kong et al. (2019) reached an 86% reduction rate of *M.*

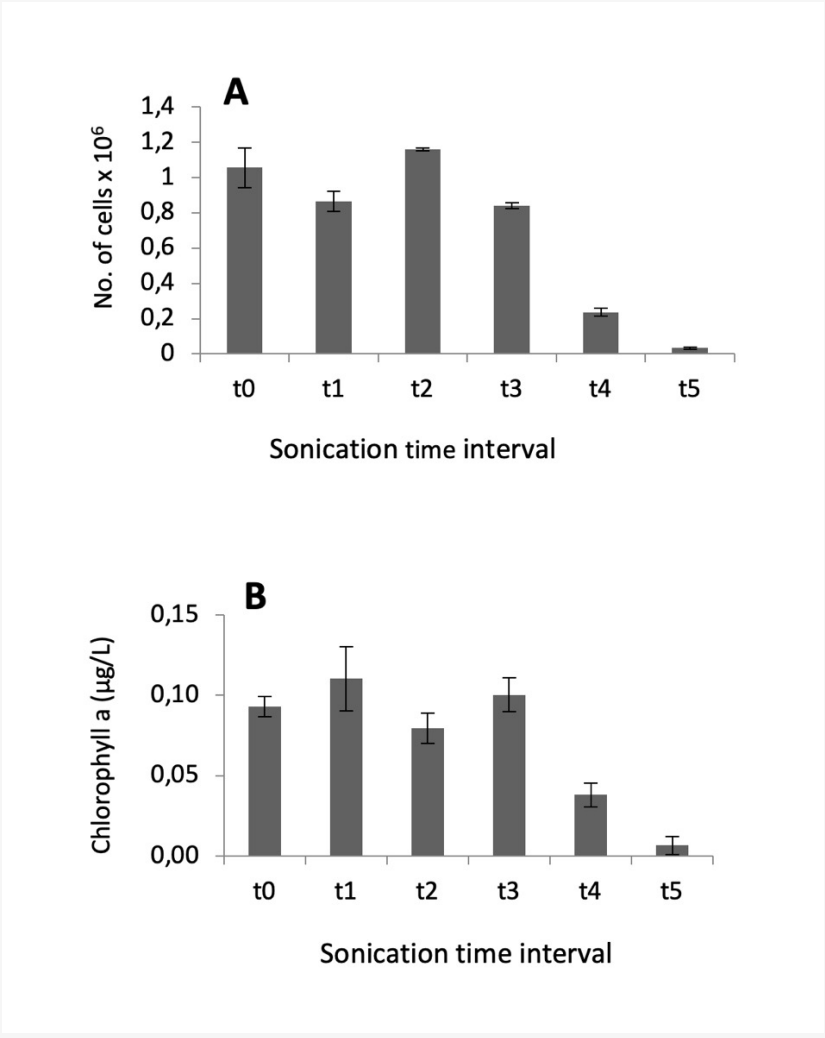


**Figure 1.** Cell count (A) and chlorophyll-*a* concentration (B) of *Microcystis aeruginosa* at different times of continuous ultrasound treatment: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

**Slika 1.** Število celic (A) in koncentracija klorofila-*a* (B) pri različnih časih neprekinjene obdelave cianobakterije *Microcystis aeruginosa* z ultrazvokom: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

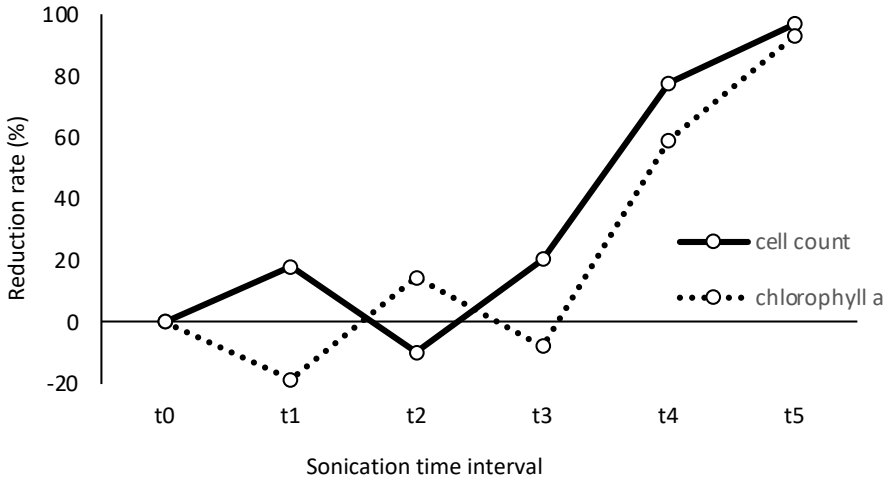


**Figure 2.** Reduction rate of *Microcystis aeruginosa* at different times of continuous ultrasound treatment: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

**Slika 2.** Stopnja zmanjšanja cianobakterije *Microcystis aeruginosa* pri različnih časih neprekinjene obdelave z ultrazvokom: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

*aeruginosa* measured as cell count after 20 min of US exposure, while Li et al. (2019) reported on >90% *M. aeruginosa* reduction rate measured as turbidly after only 5 min of US exposure; however, they both studied high-intensity cavitation US units. Acoustic cavitation is a phenomenon where usually low frequency (20–100 kHz) US is causing intense heat of 4,500–7,500 °C accompanied by pressure of around 10,000 Bar (Klemenčič and Krivograd Klemenčič, 2021a). On the other hand, Rumyantsev et al. (2021), who studied low-intensity US (20-200 kHz), found a reduction in cyanobacteria *Synechocystis* biomass after 12 to 15 days of continuous US operation.

The results of ANOVA analysis showed a statistically significant difference ($p<0.05$) in measured parameters of *M. aeruginosa* (cell count, chlorophyll-*a*, reduction rate) in the water column between longer (24 h and 48 h) and shorter ultrasonication times (0, 15 min, one h, and five h). Moreover, there was a statistically significant difference ($p<0.05$) in measured parameters also between 24 h and 48 h of ultrasonication, whereas the difference between shorter ultrasonication times was not statistically significant. Visual inspection showed that after 24 h of continuous ultrasonication, the colour of the water in the experimental pond changed from blue-green to brown-green with foam present on the surface, indicating a shift of *M. aeruginosa* culture to the death phase, while after 48 h of continuous ultrasonication, the water colour turned into brown with even more foam present on the surface, indicating decay of cyanobacteria in the experimental pond.

High-intensity US tend to inactivate *M. aeruginosa* cells due to mechanical destruction and formation of free-radical oxidation as a result of the cavitation effect, which could cause severe damage to the structure and physiological function of algae cells and could damage the cell membrane, wall, and organelle (Kong et al., 2019). On the other hand, the main mechanism of low-intensity US affecting *M. aeruginosa* is, according to Rumyantsev et al. (2021), the US causes stress-state of cyanobacteria, which leads to a sharp increase in the consumption of energy to synthesize exopolysaccharides so that mucous membranes and proteins can grow to restore extracellular protein structures destroyed by US. The two simultaneously working mechanisms of biosynthesis deplete the accumulated and irreplaceable energy, resulting in the death of cyanobacteria.

Cavitation US (high-intensity, high-frequency) is recommended for drinking water treatment purposes (Li et al., 2019). In natural water ecosystems, the use of low-intensity (or low-power) US is widely accepted as an environmentally

friendly method for cyanobacterial control (Rumyantsev et al., 2021) also because it isn't expected to have an impact on non-target organisms such as fish and daphnids (Klemenčič & Krivograd Klemenčič, 2021b). High-frequency US irradiation has a small impact distance within the water; therefore, the ultrasonic frequency should be low for application in large bodies of water such as lakes or reservoirs (Huang et al., 2020). Therefore, low-intensity and low-frequency US units are recommended for controlling algal (cyanobacterial) blooms in natural environments (Klemenčič & Krivograd Klemenčič, 2021b) as is the US unit used in our research.

## Impact of ultrasonication on *Microcystis aeruginosa* growth inhibition

The results of the growth inhibition test are shown in Fig. 3. The ANOVA analysis showed a statistically significant difference ($p<0.05$) in *M. aeruginosa* cell count and reduction rate between longer (24 h, 48 h) and shorter times of ultrasonication (15 min, one h). Moreover, there was also a statistically significant difference ($p<0.05$) in measured parameters between 24 h and 48 h of ultrasonication, whereas the difference between shorter ultrasonication times was not statistically significant. Our results showed that up to one hour of US treatment did not affect cell growth at all and that after one hour of ultrasonication, the effect of US treatment on *M. aeruginosa* growth increased with the time of ultrasonication. These results are congruent with the research of other authors (e.g., Li et al., 2019), who also reported that the duration of ultrasonication is among the most important parameters influencing algal reduction. The most efficient in *M. aeruginosa* reduction was the longest tested US treatment of 48 h with a growth inhibition of 96%, followed by 24-h US treatment with 50%, and 5-h US treatment with 17% growth inhibition at the end of the 9-day growth inhibition test (Fig. 3). Huang et al. (2020) who also tested *M. aeruginosa* reduction by low-intensity and low-frequency US observed similar reduction efficiencies; however, at significant lower ultrasonication times up to 10 min which can be the consequence of low volume of 0.5 L algal suspension used compared to 150 L of algal suspension used in our experiment. It is well known that US operational parameters determined from short-term lab tests may not work for the field ultrasonication for algal reduction in a different size of water and in a longer-term operation (Purcell et al., 2013). That is why, in our experiment, a larger volume of algal suspension was applied, simulating shallow water bodies in a semi-natural environmental condition.

Nevertheless, that 48-h of ultrasonication significantly inhibited *M. aeruginosa* growth, the cells were still able to re-grow after US treatment and thus, long-term treatment (>48 h) when using low-intensity US units is required in order to maintain a low number of *M. aeruginosa* cells in the water column. Low-intensity US irradiation (below the cavitation threshold) achieves algal control mainly through mechanical impacts on water; thus the algal reduction rate is relatively low, and the damage to the algal cells can be repaired, as concluded by Huang et al. (2020). *M. aeruginosa* cells complete cell repair within 36–48 hours after low-frequency and low-intensity ultrasonic irradiation. However, even in the case of high-intensity US (above the cavitation threshold), not all *M. aeruginosa* cells break down directly, and the cell wall can keep integrity or suffer some damage, and such cells can recover their activity spontaneously (Kong et al., 2019). It is important to mention that in our re-growth experiment, 250 mL Erlenmeyer flasks were used, and due to continuous mixing, all the cells in the sample were illuminated, which is different from the natural environment. In natural water bodies, US treatment usually causes *M. aeruginosa* cells to sink to the bottom of the water body, where the light intensity is usually too low to support the growth of phototrophic organisms such as cyanobacteria and algae.

## Impact of ultrasonication on microcystins

MCs content was evaluated as a peak surface from an HPLC chromatogram and calculated per dry weight in order to assess the MCs content in different compartments, dissolved MCs in media and MCs in cells (Fig. 4) after different times of US exposure. MCs content (µg/g DW) in the cells (pellet) was more or less constant during the whole experiment, with differences that were not statistically significant (black rectangles in Fig. 4), which is similar to the findings of Lürling et al. (2014). Our results showed a sharp increase in dissolved MC concentrations in aquatic medium starting after 15 min and stopped after five h of ultrasonication. The increase in cyanotoxin concentrations in the media after the application of US has also been reported by other authors (Lürling et al., 2014; Rajasekhar et al., 2012; Rumyantsev et al., 2021; Zhang et al., 2006b). According to Zhang et al. (2006b), the use of

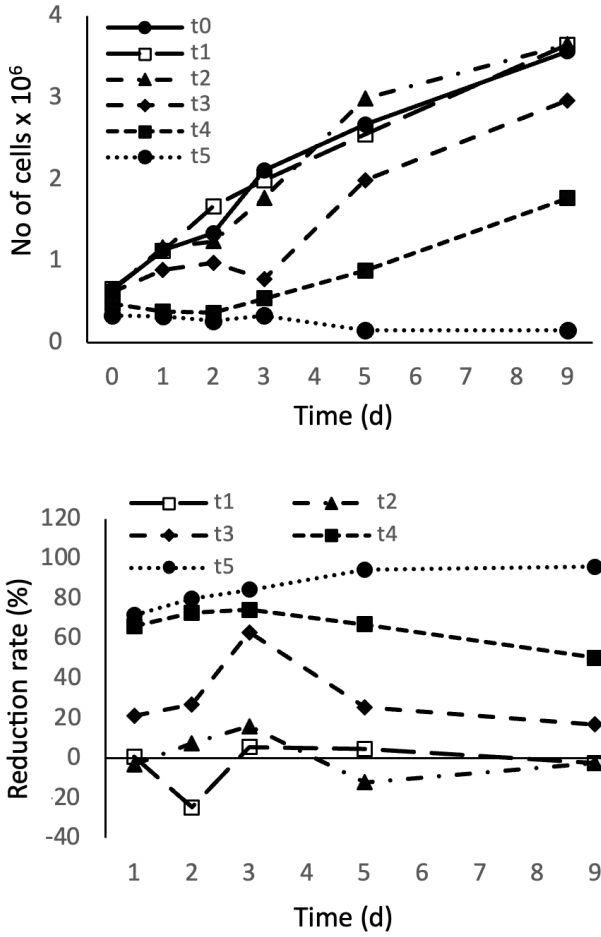


**Figure 3.** Inhibition of *Microcystis aeruginosa* growth after different times of ultrasound treatment: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h of ultrasonication (n=3).

**Slika 3.** Inhibicija rasti cianobakterije *Microcystis aeruginosa* po različnih časih obdelave z ultrazvokom: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

high-intensity cavitation-producing ultrasound (80 W, 80 kHz) for 5 min increased the extracellular MCs concentration from 0.87 µg/L to 3.11 µg/L in the experiment of *M. aeruginosa* removal by ultrasonication. Lürling et al. (2014) reported on minor release of MCs into the water in the lab-scale experiment of US treatment of *M. aeruginosa* toxic strain using commercial US units (power and frequency of US and time of sonication not reported). Rumyantsev et al. (2021), which used low-intensity US (40–300 kHz) to treat a toxic strain of *Synechocystis* sp., reported a decrease in the concentration of cells in the experimental containers from the 12th to the 15th day of US exposure and on increased synthesis of toxins. Rajasekhar et al. (2012) reported that 5 min sonication (20 KHz) of *M. aeruginosa* suspension at 0.32 W/mL, or for a longer exposure time (>10 min) at a lower power intensity (0.043 W/mL), led to an increase in MCs level in the experimental containers. The increase in cyanotoxin concentration can be the consequence of the damage caused by the US to the algal cells or the collapse of the cells (Zhang et al., 2006b), which is usually the case in high-intensity US applications. However, in some cases, high-intensity US can be so powerful that algal cells are destroyed within minutes with no release of cyanotoxins in the media (Chen et al., 2020). In the case of low-intensity US application, the biosynthesis of toxins can increase after US exposure due to increased stress from cyanobacteria (Rumyantsev et al., 2021) rather than due to cell lysis. This is also indicated by our study since the results of *M. aeruginosa* biomass clearly show no sedimentation of cells (Figs. 1, 2) during the occurrence of a rise in dissolved MCs (from 15 min to 5 h), indicating the absence of major cell damages or cell lysis. According

to Rumyantsev et al. (2021), the cell wall and the mucous membrane of cyanobacteria become thicker under the influence of the US, which is connected with the biosynthesis of toxins. The results of Rumyantsev et al. (2021) indicate that toxigenic cyanobacteria protect themselves against US irradiation by growing mucous membranes and starting the biosynthesis of toxins and their release into the aquatic medium. The reason for the high increase of dissolved MCs in our experiment could be that in the samples also, dissolved MCs from sedimented cells which were not sampled were captured; however, this effect was negligible since the results of cell count and chlorophyll-*a* concentration show almost no sedimentation of *M. aeruginosa* cells after five h of ultrasonication (Figs. 1 and 2). Nevertheless, ultrasonication can temporarily increase the levels of dissolved MCs in the water. The MCs exposed to the US tend to be less toxic, as reported by Hudder et al. (2007), who found that US irradiation of MC-LR effectively reduces hepatotoxicity in mice.

At 24 h of continuous ultrasonication, the concentration of dissolved MCs in the aquatic medium dropped under the detection limit and stayed at that level also after 48 h of continuous ultrasonication. Other authors also reported a decrease in dissolved cyanotoxin concentrations following the initial increase as a result of US treatment (Rajasekhar et al., 2012; Rumyantsev et al., 2021). However, our results are inconsistent with the study of Lürling et al. (2014), where a decrease in cyanotoxin concentrations after US treatment was not observed (continuous operation of commercially available US for 5 days); however, in their study, the levels of dissolved MCs were the same during the experiment indicating no effects of US treatment on MCs synthesis or degradation. In natural water bodies, ultraviolet light (UV) and bacterial degradation are the main mechanisms of MC reduction once MCs are released out of the cells in the water (Edwards et al., 2008; Kaya & Sano, 1998). According to Tsuji et al. (1994), the photochemical breakdown of MCs in full sunlight can take 2 to 6 weeks or even more for a breakdown greater than 90%. A more rapid breakdown under sunlight has been reported in the presence of naturally occurring humic substances, which can act as photosensitisers, with approximately 40% of the MCs degraded per day under summer conditions of insolation (Welker & Steinberg, 1999). In our experiment, tubular fluorescent lamps were employed, which, according to the producer information (OSRAM, Germany), emit

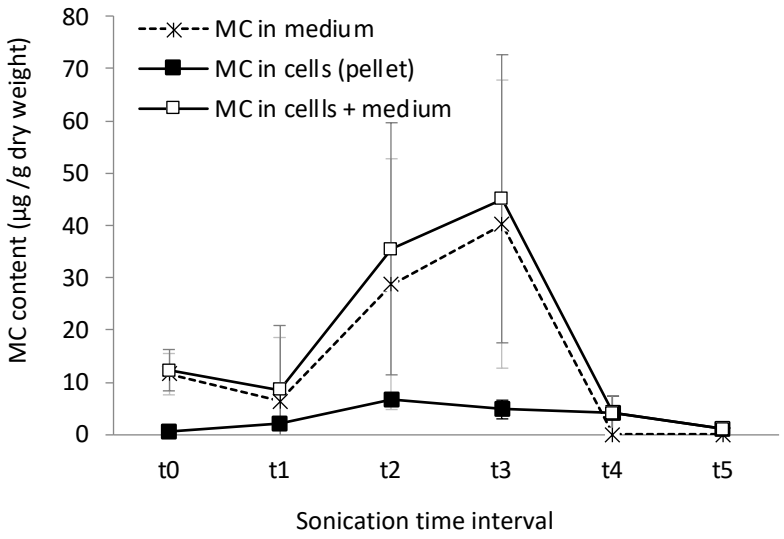


**Figure 4.** Microcystins (MC) concentration (per dry weight) in the *Microcystis aeruginosa* culture (cells+medium), medium and in the cells (pellet) at different times of continuous ultrasound treatment: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h of ultrasonication (n=3).

**Slika 4.** Koncentracija mikrocistinov (MC) (podana kot suha teža) v kulturi cianobakterije *Microcystis aeruginosa* (celice+medij), medij in v celicah (peleti) pri različnih časih neprekinjene obdelave z ultrazvokom: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h of ultrasonication (n=3).

significantly less UV than sun-light does (wavelength range 380 nm and higher) and thus, it can be hypothesised that the effect of photo-oxidation on MCs was minimal. Bacterial degradation of MCs is often characterised by an initial lag phase lasting from 2 days to more than several weeks with little loss of MCs (Edwards et al., 2008). Once the bacterial biodegradation process commences, the reduction of MCs can be fast, with half-lives of 0.2–5 days for different MCs (Tsuji et al., 2006). Therefore, we can conclude that deterioration and complete cessation of dissolved MCs in the aquatic medium after 24 h of ultrasonication in our experiment is the consequence of US irradiation with photooxidation and bacterial degradation having only minimal impact. According to Song et al. (2005), the principle of US degradation for algal toxins is to degrade MC-LR by attacking the benzene ring and the cracks in the peptide bonds.

Our results are also presented as the reduction rate of dissolved MCs (Fig. 5, where 100% reduction represents MCs concentration where no dissolved MCs were detected). Negative reduction is interpreted as an increase of dissolved MCs in the medium, which is a consequence of ultrasonication-caused reduction or leaking of MCs from the *M. aeruginosa* cells (Fig. 5).

## Visual observation of *Microcystis aeruginosa* cells exposed to ultrasonication

*M. aeruginosa* cells were visually observed under a light microscope (Nikon Eclipse TE 300, Japan) at 600x magnification, and their diameter was measured in order to detect cell size changes after US treatment. According to Turner et al. (2000), cell size change is a direct indicator of cell disruption, and smaller-sized cells can indicate disruption of cells as the effect of US treatment. However, no visual changes or changes in the diameter of the cells were observed. Nevertheless, we cannot conclude, based on these results, that *M. aeruginosa* cells were not damaged by the US because, according to Kong et al. (2019), the US can break up the colloidal sheath outside the cell wall without cell disintegration. In the research of Kong et al. (2019), damaged algal cells released the cytoplasm through a broken breach, but most cells retained an intact peripheral structure, although the internal structure was destroyed. Moreover, *M. aeruginosa* cells are very small, with a diameter of up to 8 µm and for more precise visual observation of such small organisms, electronic microscopy or flow cytometer analyses would be more suitable.

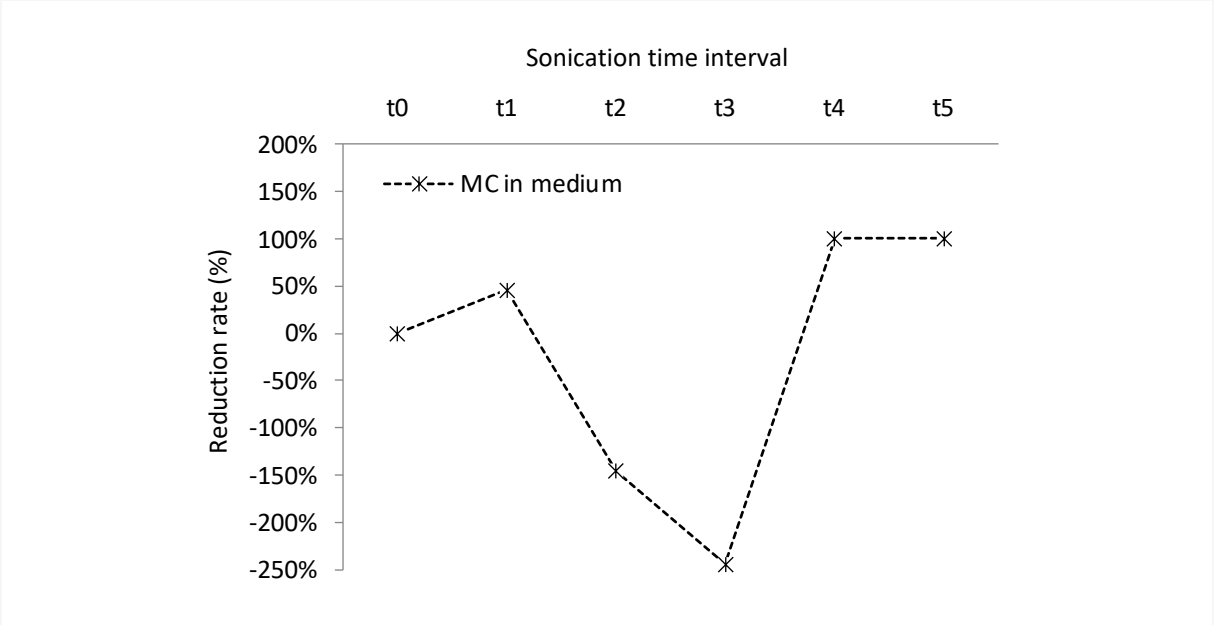


**Figure 5.** The reduction rate of dissolved microcystins (MC) at different times of continuous ultrasound treatment: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

**Slika 5.** Stopnja zmanjšanja raztopljenih mikrocistinov (MC) pri različnih časih neprekinjene obdelave z ultrazvokom: t0=0 min, t1=15 min, t2=1 h, t3=5 h, t4=24 h, and t5=48 h (n=3).

# Conclusions

In this research, we studied the effect of commercially available low-frequency and low-intensity ultrasound (US) devices to control algal blooms in natural water bodies regarding cyanobacterial biomass reduction and microcystins (MCs) release and degradation at different times of ultrasonication. In order to come close to natural conditions, a volume of 150 L of dense *Microcystis aeruginosa* suspension was used as a test field. The results showed no biomass reduction in the first five hours of US treatment; however, at 24 hours of continuous US treatment, the reduction in *M. aeruginosa* biomass achieved was 78% and 59% for cell count and chlorophyll-*a*, respectively, with the highest reduction rates of 97% for cell count and 93% for chlorophyll-*a* at 48 h of continuous US treatment. The results of the growth inhibition test showed that the effect of US treatment on *M. aeruginosa* growth increased with the time of ultrasonication and that *M. aeruginosa* cells can repair themselves from the damage caused by ultrasonication. Ultrasonication up to one hour did not affect cell growth at all, while the most efficient in *M. aeruginosa* reduction was the longest tested US treatment of 48 h with growth inhibition of 96%, followed by 24-h US treatment with 50%, and 5-h US treatment with 17% growth inhibition at the end of the 9-day re-growth period.

There are still reservations about using US technology to control cyanobacterial blooms due to the possible release of toxins in the water and related health risks. The results of our study indeed showed an increase in dissolved MCs in the water during the first five hours of ultrasonication, which was related to US-induced stress since no cell lysis was observed; however, after the initial increase, the dissolved MCs concentration dropped under the detection limit at 24 h of continuous US operation and stayed low till the end of the experiment. This indicates the high effectiveness of US in MCs degradation since photooxidation and bacterial degradation, which are the main mechanisms of dissolved MCs reduction in a natural environment, are usually much more time-consuming processes.

We can conclude that low-frequency and low-intensity commercially available US units are very effective in controlling cyanobacterial blooms already at relatively low exposure times of one to two days. The minimal recommended treatment time with such US devices, based on our results, is one day or 24 h to allow ultrasonic technology to degrade dissolved toxins and to avoid possible toxin-related health issues.

## Author Contributions

Conceptualization, A.K.K. and T.E.; methodology, A.K.K. and T.E.; investigation, A.K.K. and T.E.; resources, A.K.K.; data curation, A.K.K. and T.E.; writing—original draft preparation, A.K.K.; writing—review and editing, A.K.K. and T.E.; project administration, A.K.K.; funding acquisition, A.K.K. All authors have read and agreed to the published version of the manuscript.

## Funding

The research was performed in the frame of the 7FP Dronic project “Application of an unmanned surface vessel with ultrasonic, environmentally friendly system to (map and) control blue-green algae (Cyanobacteria)”. The authors also acknowledge the financial support from the Slovenian Research Agency (research core funding No. P2-0180). The authors are grateful for all the support.

## Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research

# Identification of *Helicobacter pylori* virulence factors and assessment of their pathogenic potential and pathogenic activity in patients with gastrointestinal disorders

Basheer Ayyed Ahmed [1,*], Nuha J. Kandala [1]

## Abstract

*Helicobacter pylori* (*H. pylori*) is found everywhere (approximately 50%) in people in the world, causing various types of gastrointestinal diseases ranging from mild inflammation, ulcers, and sometimes, stomach cancer. This pathogen carries a diverse set of genes encoding several virulence factors. In this research, we look forward to isolating bacteria and measuring them biochemically and molecularly to determine bacterial virulence factors that have an effective role in cancer generation. Different techniques and tests have been used for *H. pylori* detection. Biopsy samples were taken from all participants by endoscopy, which was used for rapid urease test (RUT), isolating in a pure form, examining it under an optical microscope, and conducting biochemical tests for confirmation. Out of 100 participants in the current study, 60 patients had various gastric diseases, and forty of the participants (control) were almost healthy. The median age of the control group was 42 years, while the median age of the patient group was 38. Among the patients, 53 were seropositive for *H. pylori*, and the presence of the virulence genes *cag*A and *vac A* was assessed in *H. pylori* seropositive patients. The *cag*A gene was detected in 32 (100%) seropositive patients, with the highest prevalence observed in gastritis patients (46.9%, n= 15), followed by gastric cancers and duodenal ulcers (both 21.9%, n= 7), and gastric ulcers (9.4%, n= 3). The *vac A* gene was present in 25 (100%) seropositive patients, with the highest prevalence found in gastritis patients (56%, n= 14), followed by gastric cancers (24%, n= 6), gastric ulcers (16%, n = 4), and duodenal ulcers (4%, n = 1). Interestingly, a small proportion of patients (12.8%, n = 6) with gastric cancers tested seronegative for *H. pylori*, while one patient with gastritis (2.1%, n= 1) was also seronegative. None of the seronegative patients harboured the *cag*A or *vac A* virulence genes. At the molecular level comes the effect of infection with *H. pylori*, which possesses very strong virulence factors (*Cag* A protein and *Vac* A toxin), creating an environment conducive to cancer.

## Keywords

*Helicobacter pylori*, virulence factors, gastrointestinal disease, gastric cancer

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**Citation:** Ahmed, B. A., Kandala N. J., (2025). Identification of *Helicobacter pylori* virulence factors and assessment of their pathogenic potential and pathogenic activity in patients with gastrointestinal disorders. Acta Biologica Slovenica 68 (3)

**Received:** 09.03.2025 / **Accepted:** 28.04.2025 / **Published:** 29.04.2025

**https://doi.org/10.14720/abs.68.3.22043**

## Identifikacija dejavnikov virulenčnosti *Helicobacter pylori* in ocena njihovega patogenega potenciala in patogene aktivnosti pri bolnikih z gastrointestinalnimi motnjami

**Izvleček**

*Helicobacter pylori* (*H. pylori*) se nahaja povsod (približno 50 %) pri ljudeh po vsem svetu in povzroča različne vrste gastrointestinalnih bolezni, od blagih vnetij, razjed do raka želodca. Ta patogen nosi raznoliko skupino genov, ki kodirajo več dejavnikov virulence. V tej raziskavi želimo izolirati bakterije in jih biokemično in molekularno karakterizirati, da bi določili dejavnike virulence bakterij, ki imajo učinkovito vlogo pri nastanku raka. Za odkrivanje *H. pylori* so bile uporabljene različne tehnike in testi, biopsijski vzorci so bili odvzeti vsem udeležencem z endoskopijo, ki je bila uporabljena za hitri ureazni test (RUT), izolirani v čisti obliki, pregledani pod optičnim mikroskopom in opravljeni biokemični testi za potrditev. Od 100 udeležencev v tej študiji je imelo 60 pacientov različne želodčne bolezni, 40 udeležencev (kontrolna skupina) pa je bilo skoraj zdravih. Povprečna starost kontrolne skupine je bila 42 let, povprečna starost skupine pacientov pa 38 let. Med pacienti je bilo 53 oseb seropozitivnih za *H. pylori*, pri seropozitivnih pacientih za *H. pylori* pa je bila ocenjena prisotnost genov virulence *cag*A in *vac A*. Gen *cag*A je bil odkrit pri 32 (100 %) seropozitivnih bolnikih, najvišja prevalenca pa je bila ugotovljena pri bolnikih z gastritisom (46,9 %, n = 15), sledili so bolniki z rakom želodca in duodenalnim razjedami (oba 21,9 %, n = 7) ter bolniki z želodčnimi razjedami (9,4 %, n = 3). Gen *vac A* je bil prisoten pri 25 (100 %) seropozitivnih bolnikih, najvišja prevalenca pa je bila ugotovljena pri bolnikih z gastritisom (56 %, n = 14), sledili so bolniki z rakom želodca (24 %, n = 6), želodčnimi razjedami (16 %, n = 4) in duodenalnimi razjedami (4 %, n = 1). Zanimivo je, da je bil majhen delež bolnikov (12,8 %, n = 6) z rakom želodca seronegativen za *H. pylori*, medtem ko je bil tudi en bolnik z gastritisom (2,1 %, n = 1) seronegativen. Noben od seronegativnih bolnikov ni bil nosilec genov virulence *cag*A ali *vac A*. Na molekularni ravni se pojavi učinek okužbe z *H. pylori*, ki ima zelo močne virulenčne faktorje (beljakovina *Cag* A in toksin *Vac* A) in ustvarja okolje, ki spodbuja nastanek raka.

**Ključne besede**

*Helicobacter pylori*, dejavniki virulence, gastrointestinalne bolezni, rak želodca

# Introduction

*Helicobacter pylori* (*H. pylori*), a spiral-shaped, Gram-negative bacteria formally known as *Campylobacter*, is mostly found in the stomach and is a microaerophile (Prasad et al., 2022). Changing shape Depending on the required physiological activity, *H. pylori* can adopt different shapes that could alleviate its long-term survival in fluctuating environmental conditions: temperature or pH levels change abruptly over time, prolonged periods without food, being exposed to an antibiotic (Salama et al., 2013). The bacterium migrates to the stomach epithelial cells and penetrates through mucosa by its flagella-driven motility (Kao et al., 2016). *H. pylori* is associated with the development of mucosa-associated lymphoid tissue (MALT) lymphoma at various sites other than the stomach and several organs such as the oesophagus, colon, rectum and ocular adnexa (Huang et al., 2016). The infection is usually asymptomatic, but it can occasionally cause gastritis or ulcers in the stomach and proximal duodenum (Blaser, 2006; Hosseini et al., 2024). No treatment exists that consistently clears the pathogen, which infects approximately half of humanity. The increased comprehension of the pathophysiology and pathogenesis mechanisms of *H. pylori* throughout the years has allowed better management of neoplasms (Blaser, 2006). The virulence factors of bacterium include colonization factors, including BabA, SabA, OipA and HopQ and effector proteins that are required for gastric pathogenicity, such as Cag A, And Vac A HtrA (Sharndama and Mba, 2022). The bacteria bottle up large quantities of urease, an enzyme that allows them to cope with the strong acidity in their stomach habitat (Ansari and Yamaoka, 2019; Zaman et al., 2024). There, the urease-catalyzed hydrolysis of urea generates ammonia ($NH_3$) and carbonic acid($H_2CO_3$), consequently creating a suitable, nearly neutral micro-environment around *H. pylori* (Athmann et al., 2000). *H. pylori* injects *Cag*A protein and

peptidoglycan into host epithelial cells using a Type IV Secretion System (T4SS), leading to the development of gastric cancer (Schuelein et al., 2011; Backert et al., 2015). *Cag*A activates oncogenic signalling pathways, including Ras-ERK MAP kinases and Wnt-Beta (Hatakeyama, 2014; Lang et al., 2016). Upon tyrosine phosphorylation, *Cag*A interacts with Src homology 2-phosphatase (SHP-2) and the adapter protein Grb2 that subsequently influences cell adhesion, proliferation (Higashi et al., 2002), IL-8 expression (Saito et al., 2010) and elongation. It was found that just Vac A has pathogenicity among them and is the one which forms vacuoles in host cells, responsible for lesions called vacuolization cytotoxic effect and apoptosis (Maleki Kakelar et al., 2019). The Vac A exhibits a variation in polymorphic forms when different strains with distinct alleles produce diverse effects that optimize bacterial survival by inhibiting host lysosomal and autophagic killing of bacterial cells (Abdullah et al., 2019; Abid and Rana, 2020). Among the actions of Vac A localized to cellular compartments are endosomes, mitochondria, Golgi apparatus and the endoplasmic reticulum, whereby its interactions disrupt normal functioning (Viala et al., 2004; Capurro et al., 2019). *H. pylori* was known to have a crucial effect in the induction of genetic and epigenetic disturbances, for instance, DNA methylation resulting in cancer evolution (Davalos and Esteller, 2023; Muhammad et al., 2023). Both findings may partially be explained by the association of *cag*A and vac A positivity in *H. pylori* strains with further gene methylation (Zhang et al., 2016) through mechanisms such as AKT-NF-κB p53-independent pathway that lead to silencing tumour suppressor genes. Indeed, DNA methylation has been proposed as a candidate marker for the early detection of cancer (Locke et al., 2019; Zhao et al., 2020).

This study aimed to diagnose and isolate the bacteria in a pure form and to investigate (molecularly) the fact that they possess highly effective virulence factors (*cag*A and *vac A*) that play an important role in the development of stomach cancer.

# Materials and Methods

## Clinical specimens

This study includes 100 specimens divided into a control group (40 samples) of approximately healthy people who were diagnosed as not suffering from any digestive system disorders. Their ages ranged between (18-76) years old, males and females. This study also included (60 samples), ranging in age (from 19-71) years old from males and females, from patients who showed symptoms and signs in the digestive system (diarrhoea, vomiting, weight loss, and indigestion). The interviews with the patients were performed in a face-to-face procedure. The questionnaire used several questions to collect data regarding each patient's demographics, clinical picture, sickness history, prior treatments, and family history. Through the specialist physicians, an endoscopy of esophagogastroduodenoscopy (OGD) was performed in the digestive system unit/Fallujah Hospital/ Anbar/ Fallujah in the period from 14-March-2023 to 15-Sep-2023. This study was approved by the College of Science Research Ethics Committee/University of Baghdad (CSEC/0723/0055). They were accurately diagnosed by a physician through histological examination as suffering from a stomach disorder. A biopsy was also taken for the purpose of detecting, isolating, and molecularly diagnosing *H. pylori*. One biopsy has been directly placed into a rapid urease (Himedia/ India) medium at the time of endoscopy. The specimens were submerged in the medium and incubated aerobically at room temperature. The test result can be observed after 10 min, one hr., two hrs. and 24 hrs. of incubation. The development of a pink-red or red-violet colour indicates a positive result, according to Radif and Saleh, (2024). From the patient samples that showed a positive test for bacteria, we were able to isolate 10 pure isolates, all from patients with acute gastritis, and they were examined by biochemical methods, as well as examined by optical microscopy, in addition to molecular diagnosis. The rest of the samples that showed a positive result for the bacteria were examined using molecular methods only.

## Isolation and characterization of *H. pylori*

All culture media and chemical solutions were prepared and sterilized in order to isolate *H. pylori* in a pure manner and conduct biochemical tests for the bacteria. The preparation of solutions for both classic and differentiated cultures has been done in accordance with manufacturer orders. The autoclave (HIRAYAMA / USR) was used to sterilize the culture media, and it ran for 15 minutes at 121 °C and 1 bar of pressure. Rapid Urease Test Broth, which does not autoclave, for heat-sensitive solutions like urea solution and crystal violate solution, 0.22 µm Millipore filters were utilized for filtration. It was necessary to alter the pH of the solutions

using either 1M NaOH or 1M HCL (Crescent/Saudi Arabia).

### Molecular detection of virulence genes

For the molecular diagnosis of bacteria, the DNA was extracted using a G-spin DNA extraction kit and intron/ biotechnology. After extracting and purifying the genomic DNA, its concentration was measured using the Nanodrop device (Korea/ Nabi), and Purity was measured under the 260/280 column (A good purity ranges from 1.80-2.00) (Jassim and Kandala, 2021).

The polymerase reaction process was performed using primers manufactured by (Integrated DNA Technologies/ USA). Forward 5'GATAGGGATAA*CAG*GCAAGC - 3', Reverse 5'-GGGGGTTGTATGATATTTTC - 3' for *cag*A gene (Yua et al., 2017), and Forward 5'-TGGGTAATGTGTGGATGGGC - 3', Reverse 5'- ATTGATGCGCGATTGACTGC- 3' for *vac A* gene (Parihar and McNamara, 2021), were used for molecular diagnosis to detect the presence of *H. pylori* virulence factors.

In addition to a 2X Master mix solution, iNtRON's Maxime PCR PreMix Kit comes in a variety of PreMix Kit types based on experience level. The product known as the Maxime PCR PreMix Kit (i-Taq) is what is used to mix all of the components for one rxn PCR in one tube, including reaction buffer, dNTPs mixture, and i-Taq DNA Polymerase. Subsequently, a primer set, D.W., and a template DNA were added. After completing the polymerase reaction technique, an electrophoresis system (agarose gel), Labnet International/USA, was used to investigate the results of the technique.

### Statistical analysis

GraphPad Prism 8 (GraphPad Software, CA, USA) was used to perform the statistical analysis. Continuous variables, such as age, were presented as median and interquartile range (IQR), while categorical variables, including sex, smoking status, comorbidities (chronic disease include Diabetes and blood pressure), residence, *H. pylori* infection, and medical treatment, were presented as frequencies and percentages. Fisher's exact test was employed to compare the distribution of categorical variables between the control and patient groups. Using odds ratios (ORs) and matching 95% confidence intervals (CIs), the relationship between the presence of the virulence genes *cag*A and *vac A* in *H. pylori* seropositive patients and the likelihood of developing gastrointestinal illnesses was evaluated. For all statistical tests, a *p*-value of less than 0.05 was considered statistically significant.

## Results

### Demographic and clinical characteristics

Out of 100 participants in the current study, 60 patients had various gastric diseases (Table 1). The median age of the control group was 42 years (Interquartile range, IQR=25.3), while the median age of the patient group was 38 years (IQR=21.8), with no significant difference between the groups ($p$=0.813). In addition, the distribution of sex was similar in both groups, with 37.5% males and 41.7% females in the control group and 46.7% males and 53.3% females in the patient group ($p$=0.414). The proportion of smokers was also relatively similar in both groups, with 30% of participants being smokers and 70% being non-smokers ($p$=0.999). Although the patient group had a higher percentage of individuals with comorbidities (40%) compared to the control group (22.5%), this difference did not reach statistical significance ($p$=0.084). The distribution of residence was also similar between the groups, with 57.5% of the control group and 55% of the patient group residing in urban areas, while 42.5% and 45% resided in rural areas, respectively ($p$=0.840). A notable difference was observed in the prevalence of *H. pylori* infection between the two groups. While none of the participants in the control group tested positive for *H. pylori* infection, a significant proportion (88.3%) of the patient group was found to be infected ($p$=0.001). Lastly, the proportion of participants receiving medical treatment was comparable between the control group (10%) and the patient group (63.3%), with no significant difference observed ($p$=0.834).

The frequency and percentage of patients with various gastric conditions in the patient group are presented in Figure 1A. Gastritis was the most prevalent condition, affecting 51.7% of patients, followed by gastric cancer (23.3% of patients), duodenal ulcers (13.3% of patients), and gastric ulcers (11.7% of patients). Furthermore, the distribution of these conditions by sex is depicted in Figure 1B. Notably, gastritis was equally prevalent among males (16 patients) and females (15 patients), while duodenal ulcers were more common in females (6 patients) compared to males (2 patients), but without any significant differences. Gastric cancer affected an equal number of males and females (7 patients each), and gastric ulcers showed a similar distribution, with three male and four female patients. Moreover, the age-wise distribution of these gastric conditions is illustrated in Figure 1C. Interestingly, gastritis was more

prevalent in patients aged 40 years or younger (21 patients) compared to those over 40 years (10 patients). In contrast, gastric cancer was more common in patients over 40 years (10 patients) than in those aged 40 years or younger (4 patients). Gastric ulcers and duodenal ulcers showed a similar distribution across both age groups, with 3 and 4 patients aged 40 years or younger and four patients each in the over-40 years age group, respectively.

**Table 1.** The study participants' demographic and clinical features.

**Tabela 1.** Demografske in klinične značilnosti udeležencev študije.

| | Controls (n= 40) | Patients (n= 60) | *P* |
|---|---|---|---|
| **Age, years** | 42 (25.3) | 38 (21.8) | 0.813 |
| **Sex** | | | |
| **Male** | 15 (37.5) | 28 (46.7) | 0.414 |
| **Female** | 25 (41.7) | 32 (53.3) | |
| **Smoking status** | | | |
| **Smoker** | 12 (30) | 18 (30) | 0.999 |
| **Non-smoker** | 28 (70) | 42 (70) | |
| **Comorbidities (chronic disease including Diabetes and blood pressure)** | | | |
| **Present** | 9 (22.5) | 24 (40) | 0.084 |
| **Absent** | 31 (77.5) | 36 (60) | |
| **Residence** | | | |
| **Urban** | 23 (57.5) | 33 (55) | 0.840 |
| **Rural** | 17 (42.5) | 27 (45) | |
| ***H. pylori* infection** | | | |
| **Positive** | 0 (0) | 53 (88.3) | 0.001 |
| **Negative** | 40 (100) | 7 (11.7) | |
| **Medical treatment** | | | |
| **Yes** | 24 (10) | 38 (63.3) | 0.834 |
| **No** | 16 (90) | 22 (36.7) | |

The age is presented as median (and interquartile range, IQR), and categorical data (sex, residence, comorbidities, and smoking status) are presented as frequencies (and percentages). P-values were calculated using Mann-Whitney's U test for age and Fisher's exact test for categorical data.

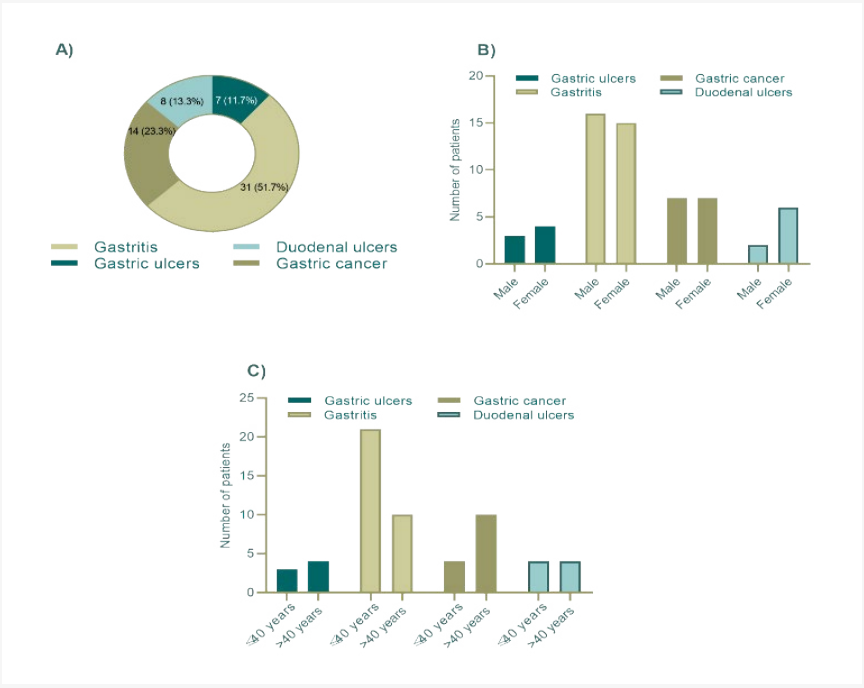



**Figure 1.** Distribution of gastric conditions among patients: A) Frequency and percentage of patients with gastric ulcers, gastritis, gastric cancer, and duodenal ulcers, B) Distribution of gastric conditions by sex, and C) Age-wise distribution of gastric conditions, categorized into patients aged 40 years or younger and those over 40 years.

**Slika 1.** Razporeditev želodčnih bolezni med bolniki: A) Pogostost in odstotek bolnikov z želodčnimi razjedami, gastritisom, rakom želodca in duodenalnimi razjedami, B) Razporeditev želodčnih bolezni po spolu in C) Razporeditev želodčnih bolezni po starosti, razvrščena v skupine bolnikov, starih 40 let ali manj, in bolnikov, starejših od 40 let.

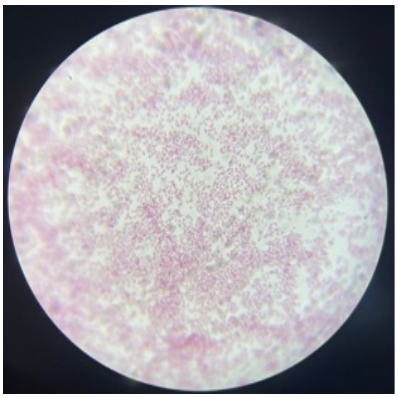
## *H. pylori* virulence factors and risk of gastric disease

Among the patients, 53 (100%) were seropositive for *H. pylori*, while none of the healthy controls tested positive; 10 of the bacterial infections were isolated in pure form, and biochemical tests were conducted on them. In this study, all bacterial isolates (10 strains that were isolated in pure form) of *H. pylori* were characterized by morphological, microscopical and biochemical tests.

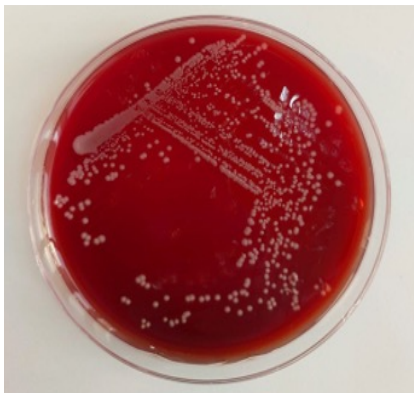
Bacteriological methods were used to accurately identify *H. pylori* bacteria, as the morphology of the colonies (shape and colour) was identified. To observe the shape and arrangement of the bacterial cells, they were examined microscopically using Gram stain. Under the optical microscope, the bacteria appeared small and spiral-shaped, and some of them were straight Gram-negative (Pokhre et al., 2019), Figure 2A. On Skirrow Colombia blood agar, which is considered selective and differential because it allows the growth of bacteria (*H. pylori*) and prevents the growth of other competing bacteria, as it contains selective supplements (Skirrow) that prevent the growth of bacteria. *H. pylori* appeared after they were incubated and provided with appropriate conditions of temperature and oxygen concentration, small, white-grey, round and convex colonies (Degnan et al., 2003), Figure 2B.

## Biochemical Identification

Several biochemical tests were done to characterize *H. pylori*. The results of biochemical tests are summarized in the (Table 2). According to the biochemical properties, all strains (10) that were isolated in pure form gave a positive result in the catalase test. The development of oxygen gas bubbles on the glass slide during the catalase test demonstrated the bacteria's capacity to manufacture the catalytic enzyme needed to convert the hydrogen peroxide reagent into water and oxygen (Al-Sulami et al., 2012). Figure 3A-D. Positive urease test (appearance of pink colour). A rapid urease test was performed on biopsies taken directly from patients, and it gave a positive result for the patients (the appearance of a pink-red colour) (Radif and Saleh, 2024).



A)



B)

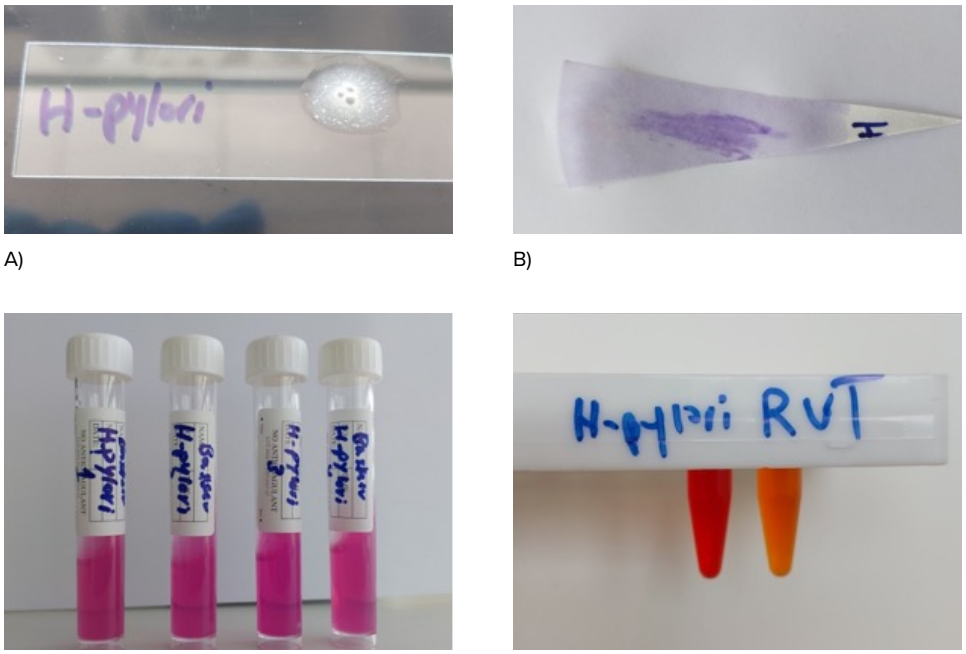
**Figure 2.** Appearance of *H. pylori*. A) Examined under light microscope (40X Magnification). B) Grown on Skirrow Colombia blood agar.

**Slika 2.** Izgled *H. pylori*. A) Pregledano pod svetlobnim mikroskopom (40-kratna povečava). B) Gojeno na Skirrow Colombia krvnem agarju.

**Table 2.** Biochemical tests for characterization of *H. pylori*.

**Tabela 2.** Biokemični testi za karakterizacijo *H. pylori*.

| **Bacteria** | **Biochemical Tests** | | | | |
|---|---|---|---|---|---|
| *H. pylori* | Catalase | Oxidase | RUT | Urease | H2S |
| | + | + | + | + | + |




A) B)

C) D)

**Figure 3.** Biochemical identification of *H. pylori*: A) Positive catalase test (appearance of bubbles), B) Positive oxidase test (appearance of blue colour), C) Positive urease test (appearance of pink colour), D) *H. pylori* from left to right: Positive RUT test for *H. pylori* (appearance of pink-red colour), while the negative RUT test appearance of a yellow-orange colour.

**Slika 3.** Biokemična identifikacija *H. pylori*: A) Pozitivni katalazni test (pojav mehurčkov), B) Pozitivni oksidazni test (pojav modre barve), C) Pozitivni ureazni test (pojav rožnate barve), D) *H. pylori* od leve proti desni: Pozitivni RUT test za *H. pylori* (pojav rožnato-rdeče barve), medtem ko je negativen RUT test rumeno-oranžne barve.

Urease is the enzyme that hydrolyzes urea in ammonia ($NH_3$) and carbamate, which is spontaneously decomposed to give another $NH_3$, together with the carbonic acid $H_2CO_30$. Carbonic acid is further decomposed into carbon dioxide ($CO_2$) and water molecules. RUT positivity (pink-red colour) and RUT negativity; yellow-orange colour (Radif and Saleh, 2024).

## Molecular Identification

Among the research subjects, the prevalence of *H. pylori* seropositivity and the presence of the virulence genes *vac A* and *cag*A were examined (Table 3). Among the patients, 53 (32 *cagA+* and 25 *vac A+*) were seropositive for *H. pylori*, while none of the healthy controls tested positive. The highest prevalence of *H. pylori* seropositivity was observed in patients with gastritis (56.6%, n= 30), followed by gastric cancers and duodenal ulcers (both 15.1%, n= 8), and gastric ulcers (13.2%, n= 7). The results of the PCR technique for the genes of the bacteria are shown in Figure 4, as they were shown after performing the electrophoresis (2 % agarose) and using Red Safe Stain in order to show the results of the electrophoresis after using the Transilluminator.

The presence of the virulence genes *cag*A and *vac A* was assessed in *H. pylori* seropositive patients. The *cag*A gene was detected in 32 (100%) seropositive patients, with the highest prevalence observed in gastritis patients (46.9%, n= 15), followed by gastric cancers and duodenal ulcers (both 21.9%, n= 7), and gastric ulcers (9.4%, n= 3). The *vac A* gene was present in 25 (100%) seropositive patients, with the highest prevalence found in gastritis patients (56%, n= 14), followed by gastric cancers (24%, n= 6), gastric ulcers (16%, n = 4), and duodenal ulcers (4%, n = 1). Interestingly, a small proportion of patients (12.8%, n = 6)

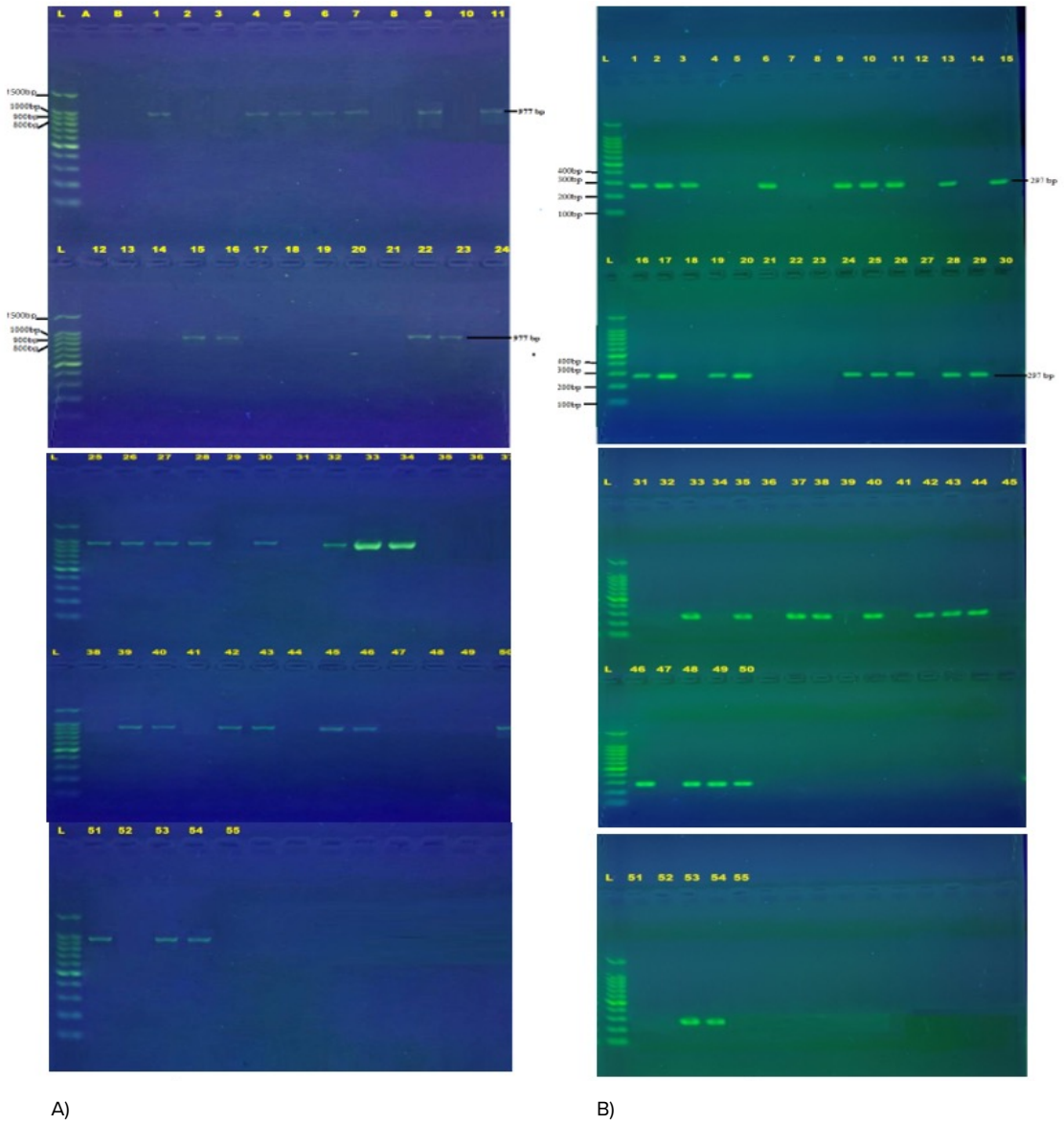



**Figure 4.** Agarose gel (2 % agarose at 5 volt/cm2.) 1x TBE buffer for 1 hour. stained with Red Safe® for the detection of genes (*vac*A and cagA). A) *vac*A gene detected as in lane L: DNA ladder (100 bp step), lane A, B Negative control, lane (1-55) It indicates the patient's sample number, the appearance of the band (PCR product 977 bp) indicates the presence of the gene, while the absence of the band indicates that the strain lacks a virulence factor. B) *cag*A gene detected as lane L: DNA ladder (100 bp step), lane (1-55). It indicates the patient's sample number, the appearance of the band (PCR product 297 bp) indicates the presence of the gene, while the absence of the band indicates that the strain lacks a virulence factor.

**Slika 4.** Agarozni gel (2 % agaroz pri 5 volt/cm2.) 1x TBE pufra za 1 uro. obarvan z Red Safe® za odkrivanje genov (*vac*A in cagA). A) Gen *vac*A odkrit v pasu L: DNA lestvica (100 bp korak), pas A, B Negativna kontrola, pas (1-55) Označuje številko vzorca pacienta, pojav pasu (PCR produkt 977 bp) kaže na prisotnost gena, medtem ko odsotnost pasu kaže, da sev nima virulenčnega faktorja. B) Gen *cag*A, zaznan v progi L: DNA lestvica (100 bp korak), proga (1-55) označuje številko vzorca pacienta, pojav pasu (PCR produkt 297 bp) označuje prisotnost gena, odsotnost pasu pa pomeni, da sev nima virulenčnega faktorja.

with gastric cancers tested seronegative for *H. pylori*, while one patient with gastritis (2.1%, n= 1) was also seronegative. None of the seronegative patients harboured the *cag*A or *vac A* virulence genes.

The association was investigated between the presence of virulence genes *cag*A and *vac A* in *H. pylori* seropositive patients and the risk of developing various gastric diseases, including gastric ulcers, gastritis, gastric cancers, and duodenal ulcers (Table 4). To evaluate the degree of the connection, odds ratios (ORs) and accompanying 95% confidence intervals (CIs) were estimated.

Among the 53 *H. pylori* seropositive patients, 32 (60.4%) harboured the *cagA* gene, while none of the 40 *H. pylori* seropositive controls tested positive for this virulence factor. The presence of *cagA* was significantly associated with an increased risk of developing gastric diseases (OR = ∞95% CI: 6.25 - ∞). Subgroup analysis revealed that the presence of *cagA* was strongly associated with an increased risk of gastric cancers and duodenal ulcers (both 87.5%, OR = ∞, 95% CI: 7.05 - ∞ and 28.3 - ∞, respectively), followed by gastritis (50%, OR = ∞, 95% CI: 9.94 - ∞) and gastric ulcers (42.9%, OR = ∞, 95% CI: 6.25 - ∞). The *vac A* gene was detected in 25 (47.2%) *H. pylori* seropositive patients, while none of the seropositive controls harboured this virulence factor. The presence of *vac A* was significantly associated with an increased risk of developing gastric diseases (OR = ∞, 95% CI: 4.97 - ∞). Subgroup analysis showed that the presence of *vac A* was strongly associated with an increased risk of gastric cancers (75%, OR = ∞, 95% CI: 7.25 - ∞), followed by gastric ulcers (57.1%, OR = ∞, 95% CI: 4.08 - ∞), gastritis (46.7%, OR = ∞, 95% CI: 5.27 - ∞), and duodenal ulcers (12.5%, OR = ∞, 95% CI: 0.49 - ∞).

**Table 3.** Prevalence of *H. pylori* seropositivity and virulence genes *cag*A and *vac A* in healthy controls and patients with gastric diseases.

**Tabela 3.** Razširjenost seropozitivnosti za *H. pylori* in virulenčnih genov *cag*A in *vac A* pri zdravih kontrolnih osebah in bolnikih z želodčnimi boleznimi.

| | N | Seropositive *H. pylori* | | Seronegative *H. pylori* | |
|---|---|---|---|---|---|
| | | *n* (%) | *n* (%) *cagA*$^+$/*vac A*$^+$ | *n* (%) | *n* (%) *cagA*$^+$/*vac A*$^+$ |
| Controls | 40 | 0 (0) | 0 (0) | 40 (85.1) | 0/0 |
| Patients | 60 | | | | |
| Gastric ulcers | | 7 (13.2) | 3 (9.4)/4 (16) | 0 (0) | 0/0 |
| Gastritis | | 30 (56.6) | 15 (46.9)/14 (56) | 1 (2.1) | 0/0 |
| Gastric cancers | | 8 (15.1) | 7 (21.9)/6 (24) | 6 (12.8) | 0/0 |
| Duodenal ulcers | | 8 (15.1) | 7 (21.9)/1 (4) | 0 (0) | 0/0 |
| Total | 100 | 53 (100) | 32 (100)/25 (100) | 47 (100) | 0 (0) |

The data is presented as frequencies and percentages (%) for each category. The presence of cagA and vac A genes was assessed only in *H. pylori* seropositive patients.

**Table 4.** Association between the presence of virulence genes *cagA* and *vac A* in *H. pylori* seropositive patients and the risk of developing gastric diseases.

**Tabela 4.** Povezava med prisotnostjo genov virulenčnosti *cagA* in *vac A* pri seropozitivnih bolnikih s *H. pylori* in tveganjem za razvoj želodčnih bolezni.

| | *CagA* (+ve) | | | Vac A (+ve) | | |
|---|---|---|---|---|---|---|
| | *H. pylori* cases | *H. pylori* control | *OR* (95% CI)* | *H. pylori* cases | *H. pylori* control | *OR* (95% CI)* |
| All patients | 32/53 (60.4) | 0/40 (0) | ∞ (6.25 - ∞) | 25/53 (47.2) | 0/40 (0) | ∞ (4.97 - ∞) |
| Gastric ulcers | 3/7 (42.9) | 0/40 (0) | ∞ (6.25 - ∞) | 4/7 (57.1) | 0/40 (0) | ∞ (4.08 - ∞) |
| Gastritis | 15/30 (50) | 0/40 (0) | ∞ (9.94 - ∞) | 14/30 (46.7) | 0/40 (0) | ∞ (5.27 - ∞) |
| Gastric cancers | 7/8 (87.5) | 0/40 (0) | ∞ (7.05 - ∞) | 6/8 (75) | 0/40 (0) | ∞ (7.25 - ∞) |
| Duodenal ulcers | 7/8 (87.5) | 0/40 (0) | ∞ (28.3 - ∞) | 1/8 (12.5) | 0/40 (0) | ∞ (0.49 - ∞) |

For each category, the data is shown as the percentage of total instances or the number of positive cases. Calculating odds ratios (ORs) and accompanying 95% confidence intervals (CIs) allowed for the evaluation of the association's strength. The symbol "∞" denotes an infinite odds ratio, implying a substantial correlation between the probability of acquiring the associated gastric disease and the virulence gene's existence.

## Discussion

The distribution of sex was similar in both groups, with 37.5% males and 41.7% females in the control group and 46.7% males and 53.3% females in the patient group ($p$=0.414). The recent study by Xing et al. (2024) found substantial gender variations in GC results, demonstrating that gender affects both epidemiological and clinical outcomes. Males had a greater rate of GC and differentiated cancers. However, females survive better, especially in early-stage disorders (Parihar and McNamara, 2021). The proportion of smokers was also relatively similar in both groups, with 30% of participants being smokers and 70% being non-smokers ($p$=0.999). This is an indication that smoking was not considered a risk for gastric ailments, and this is not consistent with several current studies, including the meta-analysis established that cigarette smoking serves as an independent risk factor for gastric cancer, especially in the gastric cardia region (Rota et al., 2024). Although the patient group had a higher percentage of individuals with comorbidities (40%) compared to the control group (22.5%), this difference did not reach statistical significance ($p$=0.084). The distribution of residence was also similar between the groups, with 57.5% of the control group and 55% of the patient group residing in urban areas, while 42.5% and 45% resided in rural areas, respectively ($p$=0.840). This result contradicts what Minhas et al. (2021) reached, as they ultimately concluded that rurality correlates with increased mortality from stomach cancer. A notable difference was observed in the prevalence of *H. pylori* infection between the two groups. While none of the participants in the control group tested positive for *H. pylori* infection, a significant proportion (88.3%) of the patient group was found to be infected ($p$=0.001). These results confirm many previous and current results and demonstrate the danger of the bacteria in stomach infection and its complications (Alshareef et al., 2023; Kebede and Ashenafi, 2024; Zama et al., 2024). Lastly, the proportion of participants receiving medical treatment was comparable between the control group (10%) and the patient group (63.3%), with no significant difference observed ($p$=0.834). This is consistent with several studies in the field that have determined an association between these two genes and bacteria contributing to chronic inflammation, which can ultimately result in carcinogenesis (Khatoon et al., 2016; Burz et al., 2024). Several epidemiologic and molecular studies have reported a decreased incidence of gastric cancer in patients who underwent eradication therapy for *H. pylori* (Ansari and Yamaoka, 2019). *H. pylori* carcinogenicity is well-studied and extensively reviewed; the expression of individual virulence factors (e.g., *Cag*A or other genotypic traits) distinctly associate with specific *H. pylori*-induced molecular disturbances, cellular behaviours, malignant transformation potentials (Wroblewski and Peek, 2016; Muhammad et al., 2019). *H. pylori*-induced inflammatory environment with increased cell infiltration, upregulated levels of oxygen species (ROS), cytokines and growth factors might also influence DNA methylation in gastric epithelial cells to result in oncogenic modifications (Qi et al., 2020).

## Conclusion

This study demonstrated that gastric cancer is a complex disease resulting from the influence of numerous environmental and genetic factors. Based on our findings, environmental factors such as lifestyle, poor nutrition, and smoking, as well as the presence of other chronic diseases along with infection with *H. pylori*, lead to chronic inflammation that predisposes to cancer. The current study revealed the association between the effect of infection with *H. pylori* contributing to chronic inflammation, which can ultimately result in carcinogenesis and two virulence factors genes (*cag*A and *vac A*), Cag A protein which deteriorates the organization inside the cell that plays an important role in cancer generation because it disrupts the main signalling pathways, and this leads to irregular cell growth, also toxin Vac A dangerous as it works to create an environment conducive to cancer because it enhances the proliferation of epithelial cells in the stomach and inflammation.

### Author Contributions

Conceptualization, B.A.A.; Methodology, B.A.A.; Validation, B.A.A.; Investigation, B.A.A.; Resources, B.A.A.; Data curation, B.A.A.; Writing—original draft preparation, B.A.A.; Writing—review and editing, B.A.A.; Visualization, N.J.K.; Supervision, N.J.K.; Project administration, N.J.K.; Funding acquisition, N.J.K. All authors have read and agreed to the published version of the manuscript.

### Ethics approval

This study was conducted with the approval of the College of Science Research Ethics Committee/ University of Baghdad (CSEC/0723/0055).

### Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research

# *Teucrium polium* L.: A natural source of antioxidants and a potential UV protector

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## Abstract

*Teucrium polium* L., commonly known as "El Kheyata," is a medicinal plant widely used in traditional Algerian medicine. In this study, we evaluated the total phenolic content (TPC) and biological activities of three extracts derived from its aerial parts: methanolic (MeOH), dichloromethane (DCM), and petroleum ether (EDP) extracts. The functional groups of these extracts were characterised using UV-VIS-NIR spectroscopy. Antioxidant potential was assessed through multiple assays, including 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,20-Azinobis (3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS), Reducing power assay (FRAP), and O-phenanthroline, while the sun protection factor (SPF) was measured to evaluate photoprotective effects. Our results demonstrated that *T. polium* contains significant concentrations of phenolic and flavonoid compounds, correlating with its notable biological activities. UV-VIS-NIR spectroscopy detected the presence of conjugated compounds, especially flavonoids and phenolic acids, in the MeOH and DCM extracts. The EDP extract, on the other hand, is rich in lipophilic compounds, such as terpenes. The results demonstrated that the MeOH extract stood out for its superior antioxidant activity compared with the DCM and EDP extracts. In line with this antioxidant richness, MeOH extract also demonstrated a powerful photoprotective effect, with SPF values ranging from 15.45 ± 0.45 to 32.96 ± 0.69, suggesting effective UV absorption. These results show that *T. polium* is a promising source for pharmaceutical applications (sunscreens, antioxidant supplements), pending further studies on its pharmacokinetic profile and potential drug interactions.

## Keywords

antioxidant activity, sun protection factor, UV-VIS spectroscopy, *Teucrium polium* L., secondary metabolites

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**Citation:** Lamari, S., Dekak, A., Boukhedir, S., Bensouici, C., Hamel, T., Kadi, I., (2025). *Teucrium polium* L.: A natural source of antioxidants and a potential UV protector. Acta Biologica Slovenica 68 (3)

**Received:** 24.10.2024 / **Accepted:** 20.05.2025 / **Published:** 23.05.2025

**https://doi.org/10.14720/abs.68.3.20040**

## *Teucrium polium* L.: Naravni vir antioksidantov in potencialni zaščitnik pred UV-žarki

**Izvleček**

*Teucrium polium* L., splošno znan kot „El Kheyata“, je zdravilna rastlina, ki se pogosto uporablja v tradicionalni alžirski medicini. V tej študiji smo ocenili skupno vsebnost fenolov (TPC) in biološke aktivnosti treh izvlečkov, pridobljenih iz nadzemnih delov rastline: metanolnih (MeOH), diklorometanovih (DCM) in petrol eterovih (EDP) izvlečkov. Funkcionalne skupine teh izvlečkov smo karakterizirali z UV-VIS-NIR spektroskopijo. Antioksidativni potencial smo ocenili z več preskusi, vključno z 2,2-difenil-1-picrilhidrazilom (DPPH), 2,20-azinobis (3-etilbenzotiazolin-6-sulfonska kislina) (ABTS), preskusom redukcijske moči (FRAP) in O-fenantrolinom, medtem ko smo za oceno fotoprotektivnih učinkov izmerili sončni zaščitni faktor (SPF). Naši rezultati so pokazali, da *T. polium* vsebuje znatne koncentracije fenolnih in flavonoidnih spojin, kar je v skladu z njegovimi izjemnimi biološkimi lastnostmi. UV-VIS-NIR spektroskopija je zaznavala prisotnost konjugiranih spojin, zlasti flavonoidov in fenolnih kislin v izvlečkih MeOH in DCM. Izvleček EDP pa je bogat z lipofilnimi spojinami, kot so terpeni. Rezultati so pokazali, da se ekstrakt MeOH odlikuje po svoji izjemni antioksidativni aktivnosti v primerjavi z ekstrakti DCM in EDP. V skladu s to antioksidativno bogastvom je ekstrakt MeOH pokazal tudi močan fotoprotektivni učinek, z vrednostmi SPF od 15,45 ± 0,45 do 32,96 ± 0,69, kar kaže na učinkovito absorpcijo UV-sevanja. Ti rezultati kažejo da je *T. polium* obetaven vir za farmacevtske aplikacije (sončna krema, antioksidativni dodatki), vendar so potrebne nadaljnje študije o njegovem farmakokinetičnem profilu in potencialnih interakcijah z zdravili.

**Ključne besede**

antioksidativna aktivnost, zaščitni faktor proti soncu, UV-VIS spektroskopija, *Teucrium polium* L., sekundarni metaboliti

# Introduction

The use of medicinal plants in various forms, raw or prepared, has expanded considerably. The World Health Organisation (WHO) estimates that 80% of the global population depends on traditional medicine and phytotherapy for health care, which seems an acceptable solution (Hachlafi et al., 2020). A large number of molecules with fascinating therapeutic properties are found in medicinal plants. Beyond their traditional use, modern science is increasingly interested in these natural treasures, deciphering the mechanisms of action of their components and confirming their therapeutic potential (Vaou et al., 2021). As part of a strategic approach to medicinal plant research, valorisation does not stop with ethnopharmacological and biological knowledge of plants, but also with phytochemical research, which is essential to capture all the information required for valorisation (Parisi et al., 2023). In fact, natural bioactive molecules are the subject of a great deal of research, and new impetus is being given to the exploitation of secondary metabolites in general, and polyphenols in particular, both in the health sector and with regard to pernicious diseases (cancer), and in the agri-food industry (Ugolini et al., 2021). These compounds are widely sought for their biological properties: antioxidant, antibacterial, anti-inflammatory and anti-allergenic, highlighting their dual importance in medicine and nutrition, medicinal plant species not only contain pharmacologically important phytochemicals but also have unique nutritional compositions (Maroyi, 2018). Moreover, medicinal plants are considered to be a potential source of natural bioactive molecules and have been the subject of many studies for their antioxidant properties (Gürbüz et al., 2018). Antioxidants eliminate free radicals that damage proteins, amino acids, lipids and DNA. This type of damage could be considered the root cause of inflammation, ageing, and many diseases such as cancer. Although we have intrinsic defences that can scavenge free radicals, an external source of antioxidants is needed when an imbalance occurs between producing and eliminating them (oxidative stress). Due to consumer preferences and health concerns associated with the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are suspected of mutagenic, carcinogenic, and teratogenic effects with long-term use, the demand for natural antioxidants is very high today. Simultaneously, there is a growing interest in natural products that offer protection from the sun's

UV rays, which are responsible for skin ageing and skin cancer (Brewer, 2011). The Sun Protection Factor (SPF) is an indicator of a product's effectiveness in absorbing UV rays. The European Commission's 2006 guideline classifies SPF values into minimum, moderate, strong, and very strong sun protection. Consumers can use SPF ratings to make informed selections when choosing sunscreen that offers adequate protection against UV radiation (Bouzana et al., 2025). Sunscreens with SPF levels between 2 and 12 provide limited sun protection; those with SPF ratings between 12 and 30 offer moderate protection, and sunscreens with SPF ratings between 30 and 50 provide strong protection, while those over 50 offer potent protection (Mishra, 2024). Recent studies have shown that certain plant extracts have significant SPF activity (Sharifi-Rad et al., 2022). *Teucrium polium* L., a plant belonging to the Lamiaceae family, is used in traditional medicine for its anti-inflammatory, antibacterial and antioxidant properties (Asadi & Farahmandfar, 2020; Elmasri et al., 2015). This herbaceous plant is renowned for its high content of phenolic compounds, which are known for their antioxidant properties (Stankovic et al., 2011). The aim of this study is to assess the antioxidant potential of *T. polium*. Using various in vitro methods, to determine its SPF activity. This study will contribute to our understanding of the therapeutic and cosmetic potential of this promising medicinal plant.

# Materials and Methods

## Plant collection

In April 2022, a total of 15 samples of *T. polium* aerial parts were manually collected from a single site in Souk Ahras, northeastern Algeria (36.112862° N, 8.084243° E), specifically in the Taoura region. Professor Hamel Tarek, a botanist at Badji Mokhtar Annaba University, authenticated the plant species. The specimens, consisting of the aerial parts (leaves, stems, and flowers), were meticulously cleaned and dried. The dried aerial parts were ground using an electric blender and then stored in paper bags until use.

## Extraction

According to a study by Diallo et al. (2004), the extraction process involved using three solvents with increasing polarity: petroleum ether (EDP), dichloromethane (DCM), and methanol (MeOH). The amount of solvent used had to be enough to cover the plant material (0.5L of solvent for 50g of drugs in our case). Extraction was carried out at ambient conditions for 24 hours with continuous stirring. Afterwards, the residue was concentrated using a Rotavap (Büchi) evaporator.

## Total Phenolic Content (TPC)

The total phenolic content of MeOH, DCM, and EDP extracts was determined by the Folin-Ciocalteu method according to Müller et al. (2010). This involved mixing 20 µL of each sample with 100 µL of Folin Ciocalteu reagent and 75 µL of 7.5% sodium carbonate solution, allowing it to stand at ambient temperature for two hours before measuring absorbance at 765 nm using a microplate reader, Perkin Elmer Enspire. The phenolic compounds were measured as gallic acid equivalents per milligram of solid dry extract (µg GAE/mg E).

## Total Flavonoid Content (TFC)

The total flavonoid content in extracts was determined utilising methods described by Topçu et al. (2007). To start, we mixed 20µL of each sample dilution with 10µL of 10% aluminium nitrate, 10µL of 1 M potassium acetate, and 130µL of methanol. This mixture was left at room temperature for 40 minutes. The amount of flavonoids was calculated by measuring the absorbance at 415 nm. The results were reported as Quercetin equivalents per milligram of extract (µg QE/mg E).

## Spectrophotometric Characterisation of the Extracts

The spectrophotometric measurements of the three extracts prepared were carried out on a SHIMADZU UV-VIS-NIR spectrophotometer, using a quartz cell with a 10 mm optical path. The measurement was performed between 200 and 3600 nm.

## In vitro antioxidant activity

The tests were conducted in triplicate using a microplate reader, Perkin Elmer Enspire and 96-well microplates. The standard antioxidants used were butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ascorbic acid, while methanol was the negative control, and the results

were expressed as IC50 values, which represent the concentration of a compound needed to inhibit a biological process by 50%, and A0.50 values, which indicate the concentration of an antioxidant necessary for 50% scavenging of free radicals.

## 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The DPPH radical scavenging assay offers valuable insights into the antioxidant potential of compounds by evaluating their ability to donate hydrogen atoms and neutralise free radicals (Dewi et al., 2012). When the DPPH radical, which is initially purple in solution, contacts an antioxidant capable of donating a hydrogen atom, it is reduced, resulting in the creation of a yellow diphenylpicrylhydrazine (Moon et al., 2014).

We have used the technique described by Blois (1958). A 40µL volume of each extract prepared in several concentrations (800; 400; 2OO; 100; 50; 25; 12.5 µg/mL) were combined with 160 µL of DPPH solution. The reaction mixture was allowed to react for 30 minutes at ambient temperature in dark conditions. The samples' absorbance was measured at 517 nm.

## 2,20-Azinobis (3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS) radical scavenging activity

The ABTS test has emerged as the most widely used technique for assessing antioxidant activity. The method relies on using spectrophotometric measurements to track alterations in cation radicals due to their interaction with antioxidants (Benso et al. 2016). The ABTS test was conducted according to the method of Re et al. (1999). A total of 40 µL of extract at various concentrations (800; 400; 2OO; 100; 50; 25; 12.5 µg/mL was mixed with 160 µL of ABTS solution. Following a 20-minute incubation period, the absorbance was quantified at 517 nm. The percentage of DPPH free radical scavenging and ABTS activity was determined using the following formula:

I% = [(AC - AS) / AC] × 100

AC: Absorbance of control; AS: Absorbance of the sample.

## Reducing power assay (FRAP)

The FRAP test plays a role in antioxidant studies by offering insights into how antioxidants interact with electron transfer processes and combat damage (Firuzi et al., 2005). In this evaluation method, antioxidants react with potassium ferricyanide ($Fe^{3+}$), converting it to potassium ferrocyanide ($Fe^{2+}$). The resulting $Fe^{2+}$ ions then combine with chloride to form a ferrous and ferric ion complex (Xiao et al., 2020). This assessment quantitatively measures the activity by evaluating the reducing power of samples through the formation of a blue complex (Ganesan et al., 2017).

The FRAP assay of *T. polium*'s extracts was evaluated using the reducing power method previously described by Oyaizu (1986). To 10 µL of sample solution (extract or standard) in methanol at varying concentrations (200; 100; 50; 25; 12.5; 6.25; 3.125 µg/mL), 40 µL of phosphate buffer (pH = 6.6) was added. This was followed by the addition of 50 µL of potassium ferricyanide (1%). For twenty minutes, the mixture was incubated at 50 °C. After that, absorbance was measured at 700 nm after adding 50 µL of trichloroacetic acid solution (10%), 40 µL of distilled water, and 10 µL of ferric chloride solution (0.1%) in that order.

## O-phenanthroline assay

In this assay, phenanthroline forms a complex with iron ions ($Fe^{2+}$), which can be oxidised by radicals present in the test sample (Nkhili et al., 2014). Antioxidants in the sample work by neutralising these radicals, thereby preventing oxidation of the phenanthroline iron complex. The spectrophotometric assessment of antioxidants' ability to prevent oxidation relies on the change in the colour intensity of the phenanthroline iron complex. The higher the colour intensity, the more potent the antioxidant activity (Hossain et al., 2013).

The phenanthroline method was performed according to the protocol described by Szydłowska-Czerniak et al. (2008). A total of 10 µL of various dilutions (200; 100; 50; 25; 12.5; 6.25; 3.125 µg/mL) of sample solutions (extract or standard) in methanol were combined with 50 µL FeCl3 (0.2%). 30 µL of O-phenanthroline (0.5%) was then added, and the mixture was adjusted with 110 µL of methanol. The absorbance was determined at 510 nm after a 20-minute incubation period at 30°C.

## Sun Protection Factor activity

The SPF was calculated using the methodology described by Mansur et al. (1986). 1mg/mL concentrations of each extract were prepared and dispensed into 200 µL wells of

a 96-well microplate. The absorbance was measured in the 290 to 320 nanometer range every 5 nm (UV-B).

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

EE: erythemal effect spectrum
I: Solar intensity spectrum
Abs: absorbance of sunscreen product
CF: correction factor (= 10)
The parameters EE X I are constants that are determined by Sayre et al. (1979).

### Statistical analysis

The results were presented as the mean value ± the standard deviation of three concurrent measurements. An analysis of variance (ANOVA) was performed to compare the means, followed by Tukey's post hoc test. The statistical analysis was conducted utilising GraphPad Prism software version 8.4 (Software, San Diego, CA, USA). Significant differences were noted when the p-value was less than 5%.

## Results

### Total phenolic and flavonoid content

The methanolic extract stands out with the highest concentration of total phenolic (291.94 µg GAE/mg) and flavonoids (198.47 µg QE/mg). This suggests that methanol is the most efficient solvent of the three solvents for extracting these bioactive compounds from the source material. While lower than the MeOH extract, both DCM and EDP extracts contain considerable amounts of phenolic and flavonoid content. The results are summarised in Table 1 and presented in Figure 1.

### Characterisation of the extracts by UV-VIS spectroscopy analysis

**Methanolic extract**

The MeOH extract of *T. polium* analysed showed the presence of functional groups such as aromatic rings (C=C), hydroxyl groups (-OH), and carbonyls (-C=O) presented in Figure 2. Notably, in the 200-400 nm range (strong absorption around 230-270 nm), flavonoids and phenolic acids are known for their antioxidant properties. A high absorption was also observed at around 664 nm. Compounds that absorb in this range often belong to plant pigment families, such as carotenoids, which have long conjugated chains (C=C double bonds) that are responsible for this absorption. Terpene structures, conjugated aromatic rings, and hydroxyl groups associated with these molecules are the major functional groups in this region. Furthermore, weak absorption near 1078 nm, this region is less common for small organic molecules. However, some complex compounds, such as polysaccharides or lipids, can exhibit electronic transitions in this region. The associated functional groups would be long carbon chains (C-H) or O-H bonds in complex structures. In contrast, results beyond 1000 nm, especially in the near infrared, indicate the presence of more complex compounds or macromolecules. This spectrum reflects the chemical diversity of the MeOH extract.

**Dichloromethane extract**

As shown in Figure 3, the DCM extract contains predominantly lipophilic compounds such as terpenoids, certain sterols and other apolar compounds. The absorption peak around 230-250 nm is a strong indication of such compounds. Compared with the MeOH extract, a difference in the type of secondary metabolites extracted can be expected, with DCM favouring less polar compounds.

**Table 1.** Total phenolic and flavonoid content.

**Tabela 1.** Skupna vsebnost fenolov in flavonoidov.

| Extracts | Total phenolic (µg GAE/mg) | Flavonoids (µg QE/mg) |
|---|---|---|
| MeOH extract | 291.94±0.50 | 198.47±1.92 |
| DCM extract | 76.058±1.47 | 151.25±1.45 |
| EDP extract | 76.35±0.50 | 38.47±1.68 |

MeOH: methanolic extract, DCM: Dichloromethane extract, EDP: Petrelium ether extract, total phenolics (µg gallic acid/mg extract), total flavonoids (µg quercetin/mg extract). Results are expressed as mean ± SD

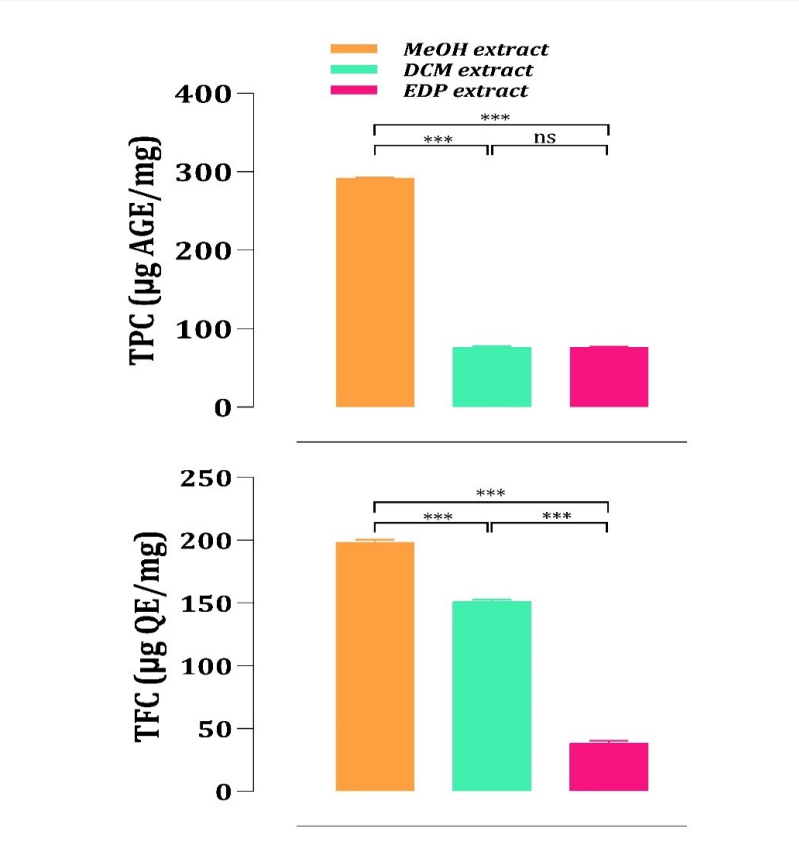


**Figure 1.** Total phenolic and flavonoid content. Results are expressed as mean ± SD. One-way ANOVA was used for statistical analysis, followed by Turkey's post hoc test (*).

**Slika 1.** Skupna vsebnost fenolov in flavonoidov. Rezultati so izraženi kot povprečje ± SD. Za statistično analizo je bila uporabljena enosmerna ANOVA, ki ji je sledil Turkeyjev post hoc test (*).

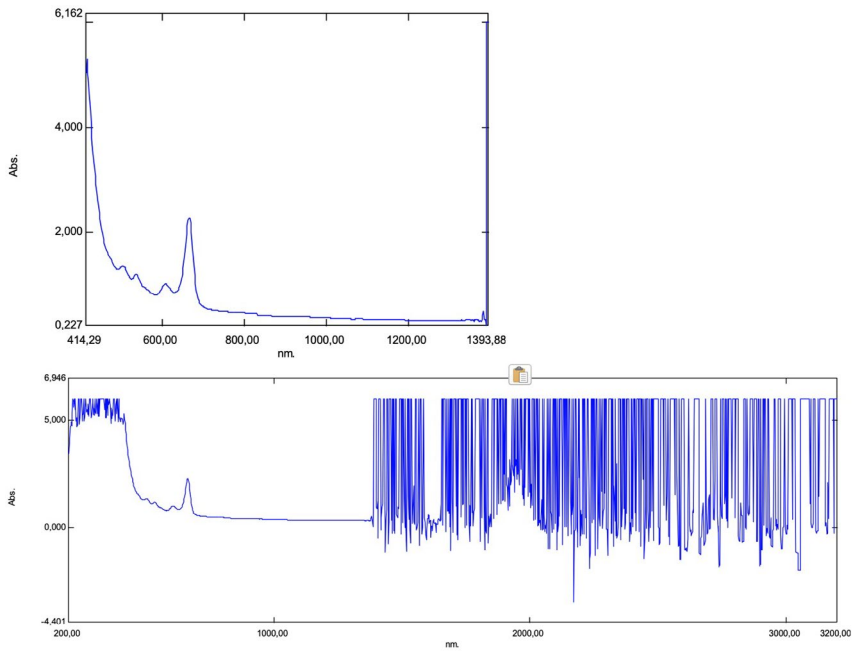


**Figure 2.** Characterisation of the methanolic extract by UV-VIS spectroscopic analysis.

**Slika 2.** Karakterizacija metanolnega ekstrakta z UV-VIS spektroskopsko analizo.

**Petrolium ether extract**

The EDP extract of *T. polium* contains non-polar compounds, including terpenes, hydrocarbons, and other lipids, as illustrated in Figure 4. The principal peak observed in the 200-230 nm region is consistent with the conjugated systems present in these compounds. The differences between the EDP, MeOH, and DCM extracts are readily apparent, as the latter two solvents are capable of dissolving compounds with greater polarity. This phenomenon is indicative of the unique properties of petroleum ether, which is utilised for the extraction of apolar molecules.

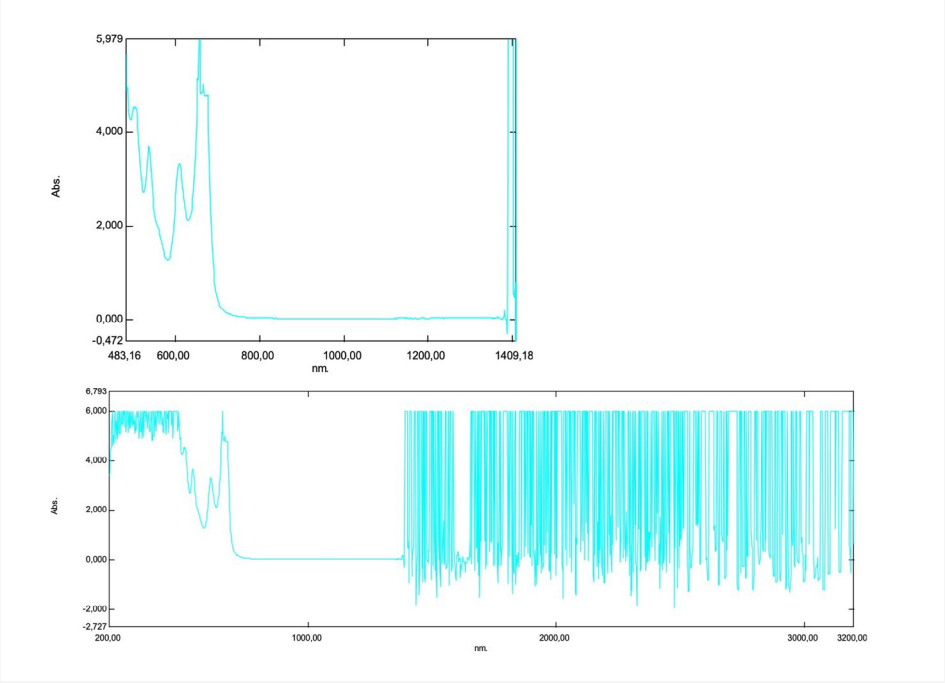


**Figure 3.** Characterisation of the Dichloromethane extract by UV-VIS spectroscopic analysis.

**Slika 3.** Karakterizacija ekstrakta diklorometana z UV-VIS spektroskopsko analizo.

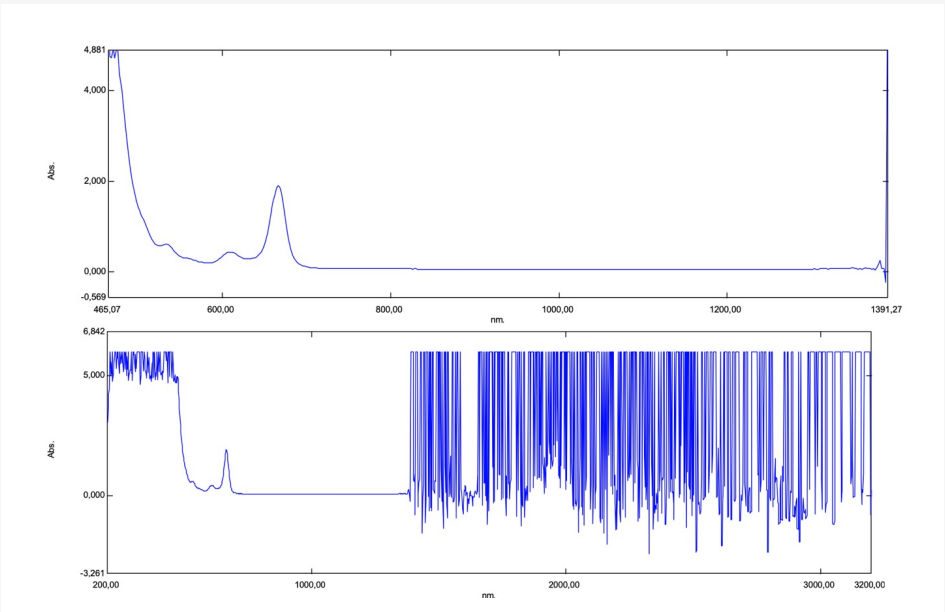


**Figure 4.** Characterisation of the Petroleum ether extract by UV-VIS spectroscopic analysis.

**Slika 4.** Karakterizacija ekstrakta petrol etra z UV-VIS spektroskopsko analizo.

## In vitro antioxidant activity

The antioxidant activity of *T. polium* has been evaluated using four distinct techniques: DPPH, ABTS, FRAP, and phenanthroline tests. The data obtained are shown in Figure 2, expressed in IC50 (µg/mL) for the DPPH and ABTS tests and reported in terms of absorbance A0.50 (µg/mL) for FRAP and O-phenanthroline techniques. The antioxidant activity of the extracts was evaluated and compared to the standards, namely BHA, BHT and ascorbic acid. The IC50 and the A0.50 were calculated based on various evaluated concentrations. The compound with a lower IC50 or A0.50 showed significant antioxidant activity.

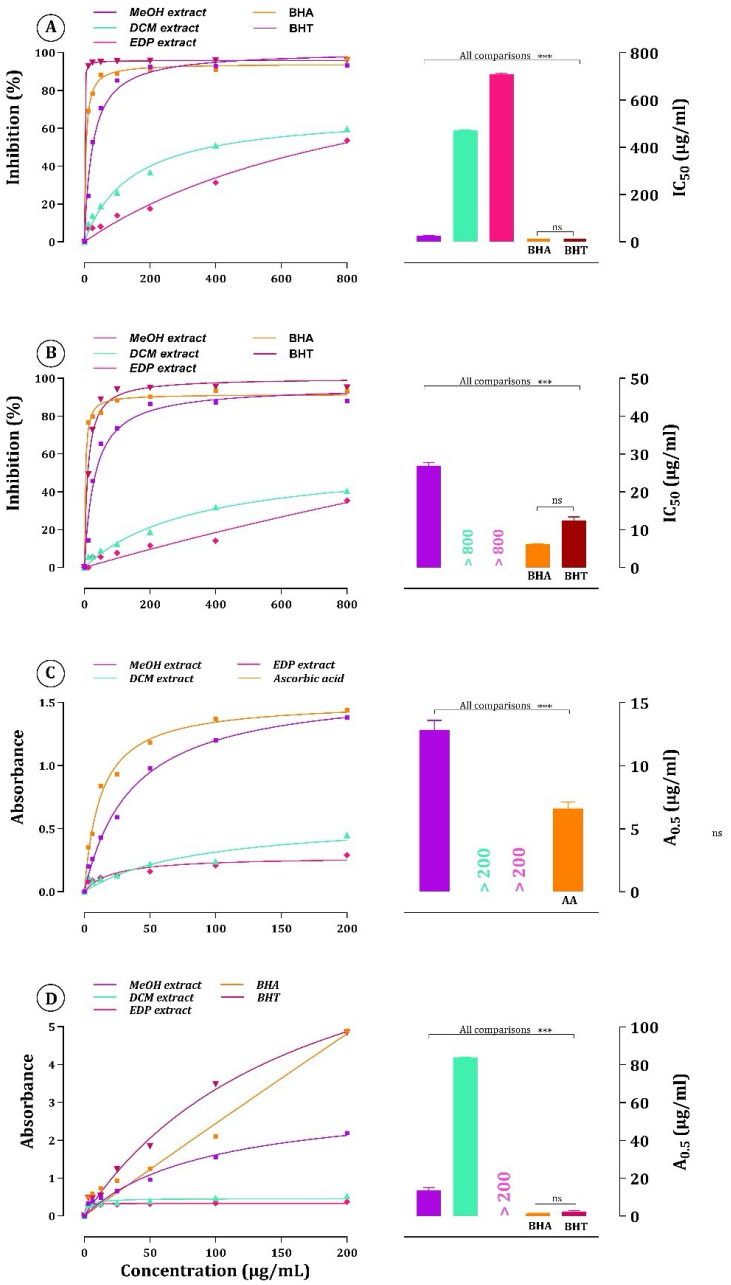


**Figure 5.** Antioxidant activities expressed as IC50 values of the studied extracts and standards determined by ABTS (A), DPPH (B), FRAP (C) and phenanthroline (D) assays. Results are expressed as mean ± SD. One-way ANOVA was used for statistical analysis, followed by Turkey's post hoc test. (*) for comparison with BHA and (#) for comparison with BHT or ascorbic acid.

**Slika 5.** Antioksidativne aktivnosti, izražene kot vrednosti IC50 preučevanih izvlečkov in standardov, določene z analizami ABTS (A), DPPH (B), FRAP (C) in fenantrolin (D). Rezultati so izraženi kot povprečje ± S.D. Za statistično analizo je bila uporabljena enosmerna ANOVA, ki ji je sledil Turkey-jev post hoc test. (*) za primerjavo z BHA in (#) za primerjavo z BHT ali askorbinsko kislino.

## DPPH scavenging activity

The DPPH assay results reveal a clear concentration-dependent antioxidant activity in all tested extracts. The MeOH extract showed the highest antioxidant activity among the different extracts, with an IC 50 of 26.91± 0.80 mg/mL and achieving over 80% inhibition at 50 µg/mL and nearly matching BHA at higher concentrations, as shown in Table 2. However, it is important to note that the DCM and EDP extracts exhibit significantly moderate antioxidant activity at higher concentrations.

## ABTS radical scavenging activity

Among the extracts tested, the MeOH extract exhibited the antioxidant efficacy, showing an IC50 value of 26.47 mg/mL (refer to Table 3), while DCM and EDP followed with IC50 values of 471.12 and 709.59 mg/mL, respectively.

## Reducing power assay

The MeOH exhibited stronger reducing power activity compared to the DCM and EDP extracts, even matching the effectiveness of ascorbic acid at low concentrations ($A_{0.5}$: 13.81 ± 2.50). In contrast, the DCM and EDP extracts showed significantly lower antioxidant activity in this assay (refer to Table 4).

## O-phenanthroline assay

The results are presented in Table 5. The potent activity (Abs 1.55±0.15) was observed in the MeOH extract at 100 mg/mL, surpassing the effectiveness of BHA and BHT. The DCM extract displayed moderate activity, while the EDP extract showed the lowest activity with an absorbance of 0.37±0.02 at 200µg/mL.

## Sun Protection Factor activity

Table 6 and Figure 6 present the sun protection factor (SPF) values of different extracts, indicating that the studied extracts offer varying amounts of sun protection. The MeOH extract demonstrated superior SPF values (32.96±0.69), while the DCM and EDP extracts showed limited efficiency (SPF = 15.45±0.45, 11.40±0.20), respectively.

## Correlation between the phenolic compounds and biological activities of *T. polium*.

Correlation coefficients range from -1 to 1, with positive values (green) indicating a positive correlation, negative values (red) demonstrating a negative correlation, and values close to 0 (yellow) revealing an absence of correlation (refer to Figure 7). Correlation analysis highlights the varying roles of phenolic compounds in the biological activities of *T. polium*, depending on the different extracts. The MeOH extract stood out for its strong relationship between phenols, antioxidant activity and SPF, while the DCM extract showed a moderate positive correlation with TPC and antioxidant activities. In contrast, the EDP extract showed the weakest association between phenols and biological activities tested.

**Table 2.** DPPH radical scavenging activity of extracts and standards (BHT, BHA).

**Tabela 2.** Aktivnost izvlečkov in standardov (BHT, BHA) pri lovljenju DPPH radikalov.

| Samples | % Inhibition in DPPH assay | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 12.5 µg | 25 µg | 50 µg | 100 µg | 200 µg | 400 µg | 800 µg | $IC_{50}$ µg/mL |
| MeOH extract | 14.38± 1.37 | 45.70± 1.34 | 81.26±1.02 | 83.53±0.18 | 86.34±0.45 | 87.11±0.47 | 87.95± 0.27 | 26.91± 0.80 |
| DCM extract | 5.67± 1.97 | 5.91± 2.07 | 8.89±0.31 | 12.95±2.74 | 18.85±1.65 | 31.80±2.24 | 40.51±2.33 | >800 |
| EDP extract | 0.06± 2.07 | 5.73± 2.77 | 5.55±2.38 | 7.70±0.27 | 11.58±0.31 | 14.08±1.73 | 35.38±1.86 | >800 |
| BHA | 76.55± 0.48 | 79.89± 0.26 | 81.73±0.10 | 84.18±0.10 | 87.13±0.17 | 89.36±0.19 | 90.14±0.00 | 6.14±0.41 |
| BHT | 49.09± 0.76 | 72.63± 2.06 | 88.73±0.89 | 94.00±0.31 | 94.97±0.08 | 95.38±0.41 | 95.02±0.23 | 12.99±0.41 |

The concentrations corresponding to 50% inhibition are designated as IC50 values. Linear regression analysis was employed to determine the IC50 values, which are presented as mean ± SD (n = 3). BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene

**Table 3.** Comparison of ABTS radical scavenging activity between extracts and standards.

**Tabela 3.** Primerjava aktivnosti izločanja radikalov ABTS med ekstrakti in standardi.

| Samples | % Inhibition in ABTS assay | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 12.5 µg | 25 µg | 50 µg | 100 µg | 200 µg | 400 µg | 800 µg | $IC_{50}$ µg/mL |
| MeOH extract | 24.37±1.75 | 52.81±1.06 | 85.65±0.36 | 92.28±0.24 | 92.44±0.24 | 92.75±0.63 | 93.11±0.09 | 26.47±0.54 |
| DCM extract | 9.51±0.78 | 13.75±1.73 | 18.98±0.90 | 26.08±0.90 | 36.75±1.89 | 51.00±0.18 | 59.65±1.79 | 471.12±2.96 |
| EDP extract | 7.02±1.63 | 7.33±1.00 | 8.11±1.59 | 13.91±1.35 | 17.53±1.03 | 31.26±2.34 | 53.59±2.33 | 709.59±4.52 |
| BHT | 69.31±0.40 | 79.23±1.44 | 88.14±1.18 | 89.76±3.05 | 90.85±1.74 | 90.95±0.61 | 96.68±0.49 | 1.29±0.30 |
| BHA | 92.93±1.44 | 94.78±0.32 | 94.95±0.92 | 95.42±0.26 | 95.58±0.46 | 95.83±0.15 | 95.96±0.10 | 1.81±0.10 |

The concentrations corresponding to 50% inhibition are designated as IC50 values. Linear regression analysis was employed to determine the IC50 values, which are presented as mean ± SD (n = 3). BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene

**Table 4.** Results of the reducing power assay for extracts and ascorbic acid.

**Tabela 4.** Rezultati preskusa redukcijske moči za izvlečke in askorbinsko kislino.

| Samples | Absorbance in the reducing power assay | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3.125 µg | 6.25 µg | 12.5 µg | 25 µg | 50 µg | 100 µg | 200 µg | $A_{0.5}$ µg/mL |
| MeOH extract | 0.20±0.01 | 0.26±0.03 | 0.43±0.08 | 0.59±0.13 | 0.98±0.06 | 1.20±0.05 | 1.38±0.10 | 13.81±2.50 |
| DCM extract | 0.12±0.02 | 0.08±0.03 | 0.10±0.02 | 0.13±0.02 | 0.22±0.03 | 0.24±0.02 | 0.45±0.04 | >200 |
| EDP extract | 0.08±0.01 | 0.09±0.01 | 0.11±0.01 | 0.13±0.01 | 0.16±0.00 | 0.21±0.00 | 0.29±0.01 | >200 |
| Ascorbic acid | 0.35±0.05 | 0.46±0.03 | 0.84±0.12 | 0.93±0.30 | 1.18±0.34 | 1.37±0.20 | 1.44±0.21 | 6.77±1.15 |

The concentrations corresponding to 0.50 absorbance are designated as A0.50 values. Linear regression analysis was employed to determine the A0.50 values, which are presented as mean ± SD (n = 3).

**Table 5.** O-phenanthroline assay results for extracts compared to BHA and BHT.

**Tabela 5.** Rezultati preskusa z O-fenantrolinom za ekstrakt v primerjavi z BHA in BHT.

| Samples | Absorbance in the Phenanthroline assay | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3.125 µg | 6.25 µg | 12.5 µg | 25 µg | 50 µg | 100 µg | 200 µg | $A_{0.5}$ µg/mL |
| MeOH extract | 0.33±0.01 | 0.38±0.02 | 0.48±0.04 | 0.66±0.07 | 0.96±0.06 | 1.55±0.15 | 2.19±0.42 | 13.50±1.50 |
| DCM extract | 0.30±0.01 | 0.31±0.01 | 0.33±0.01 | 0.36±0.01 | 0.40±0.02 | 0.47±0.05 | 0.53±0.09 | 83.67±0.29 |
| EDP extract | 0.29±0.01 | 0.29±0.01 | 0.30±0.01 | 0.30±0.01 | 0.32±0.00 | 0.34±0.02 | 0.37±0.02 | >200 |
| BHA | 0.49±0.01 | 0.59±0.01 | 0.73±0.02 | 0.93±0.01 | 1.25±0.04 | 2.10±0.05 | 4.89±0.06 | 0.93±0.07 |
| BHT | 0.47±0.01 | 0.47±0.01 | 0.53±0.03 | 1.23±0.02 | 1.84±0.01 | 3.48±0.03 | 4.84±0.01 | 2.24±0.17 |

The concentrations corresponding to 0.50 absorbance are designated as A0.50 values. Linear regression analysis was employed to determine the A0.50 values, which are presented as mean ± SD (n = 3). BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene

**Table 6.** Photoprotective activity of different extracts of *Teucrium polium*.

**Tabela 6.** Fotoprotektivna aktivnost različnih izvlečkov *Teucrium polium*.

| λ (nm) | CFx EE(λ)xI(λ)x Ab (λ) | | |
|---|---|---|---|
| | MeOH | DCM | EDP |
| 290 | 0.48± 0.01 | 0.32±0.003 | 0.289±0.007 |
| 295 | 2.66±0.94 | 1.46±0.11 | 1.20±0.02 |
| 300 | 10.05±0.94 | 4.49±0.11 | 3.48±0.05 |
| 305 | 10.49±0.18 | 4.86±0.15 | 3.53±0.06 |
| 310 | 6.002±0.11 | 2.75±0.09 | 1.89±0.03 |
| 315 | 2.68±0.04 | 1.25±0.04 | 0.289±0.01 |
| 320 | 0.57±0.010 | 0.27±0.01 | 0.17±0.003 |
| SPF | 32.96±0.69 | 15.45±0.45 | 11.40± 0.20 |

Values are presented as mean ± SD of three parallel measurements. SPF: Sun Protection Factor; MeOH: methanolic extract; DCM: Dichloromethane extract; EDP: Petroleum ether extract.

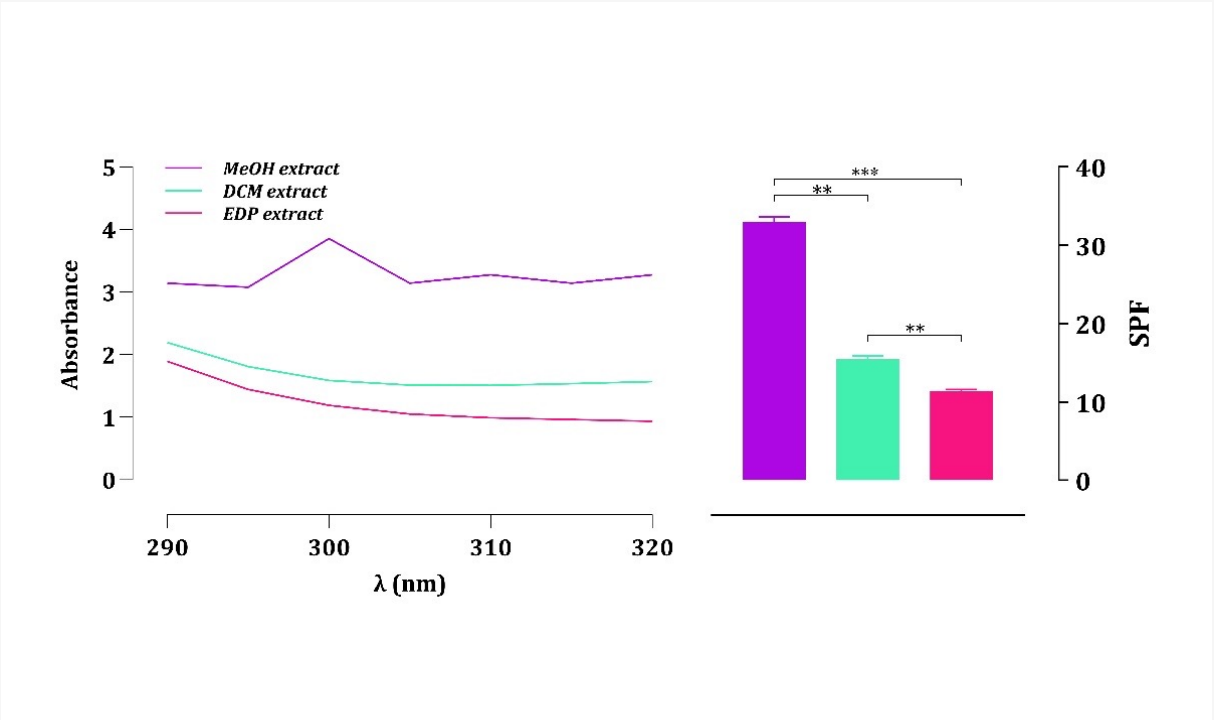


**Figure 6.** Values of the sun protection factor of the *T. polium* extracts. Results are expressed as mean ± SD. One-way ANOVA was used for statistical analysis.

**Slika 6.** Vrednosti zaščitnega faktorja izvlečkov *T. polium*. Rezultati so izraženi kot povprečje ± S.D. Za statistično analizo je bila uporabljena enosmerna ANOVA.

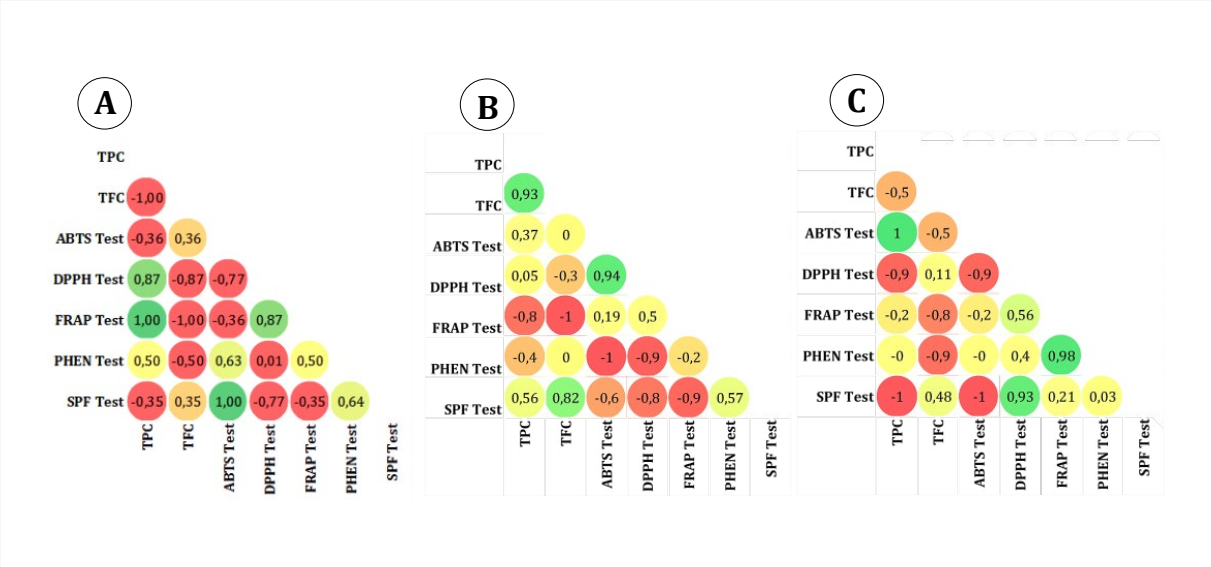


**Figure 7.** Correlation between the phenolic compounds and biological activities of *T. polium*, (A): MeOH extract, (B): DCM extract, (C): EDP extract.

**Slika 7.** Korelacija med fenolnimi spojinami in biološkimi aktivnostmi *T. polium*, (A): ekstrakt MeOH, (B): ekstrakt DCM, (C): ekstrakt EDP.

## Discussion

Phenolic compounds are an important category of secondary metabolites in plants. It has a significant pharmacological effect on human health. These compounds play crucial roles in plant defence mechanisms and have diverse pharmacological potentials (Basit et al., 2023). Based on the results obtained, we can deduce that *T. polium* is abundant in phenolic compounds, particularly flavonoids, making it a valuable natural source of bioactive molecules. These findings align with the results of previous studies (Khadhri et al., 2022). The obtained results of Total phenolic and flavonoid content in our study were higher compared to those of the MeOH extract of leaves of *T. polium* growing in Suva planina Mt, in Southeast Serbia (Stankovic et al., 2012). The quantitative differences in the phenolic content of plants are indeed affected by various factors such as climatic conditions, geographical origin, extraction methods, and environmental parameters (da Silva et al., 2022). Based on research, methanol was proven to be an efficient solvent for extracting phenolic compounds from plant materials (Vijayalaxmi et al., 2015). This is due to its capability to extract a higher amount of compounds compared to other solvents. The differences in the concentrations of phenolic and flavonoid compounds in the extracts can be attributed to the varying polarities of the solvents utilised. Methanol is highly polar, dichloromethane is moderately polar, and petroleum ether is nonpolar; this suggests that the phenolic compounds present in plants may exhibit degrees of polarity, leading to varying extraction efficiencies with each solvent (Rusu et al., 2018).

Differences in intensity between spectra indicate variations in composition or concentration, underlining the usefulness of this technique for characterising and comparing extracts. UV-Vis-NIR spectroscopy is thus proving a valuable tool for rapid, non-destructive analysis of extracts, offering key information on their chemical composition and optical properties (Sm & Kalaichelvi, 2017).

Various antioxidant tests assess the antioxidant capacity of plants, including DPPH, ABTS, FRAP, and phenanthroline (Bayrak & Yanardağ, 2021). These methods demonstrate how antioxidants can scavenge radicals and reduce metal ions, thus improving our understanding of their effectiveness in combating stress (Sariburun et al., 2010).

Several approaches have been used to assess the antioxidant potential of *T. polium* extracts. The MeOH extract exhibited the highest antioxidant activity across all experiments, surpassing other extracts and demonstrating comparable efficacy to synthetic antioxidants, like BHT and BHA. Our results totally agree with the studies of Noumi et al. (2020) and Benchikha et al. (2022). The findings indicate a potential for exploring this product as a source

of antioxidants. Future studies should delve deeper into identifying the molecules for MeOH potent properties and exploring its applications across various domains, including food preservation, cosmetics, and pharmaceuticals.

However, the DCM extract showed a modest degree of antioxidant activity, while the EDP extract had the lowest scavenging capacity. The results are consistent with research conducted by Qabaha et al. (2021) on the same species, which proved the antioxidant properties of the leaf extract.

The three extracts exhibited notable variations in the SPF values. The findings emphasise the capacity of the MeOH extract to serve as a very efficient UV shield in sunscreen compositions. Conducting additional research on the precise UV-absorbing elements of this extract and their modes of action would be advantageous in enhancing and creating more effective sun protection solutions.

## Conclusions

The study concludes by highlighting the promising antioxidant activity of *T. polium* extracts, in particular the MeOH extract, which outperformed other extracts in various in vitro tests. These results highlight the potential uses of *T. polium* as a natural source of antioxidants for medicinal purposes, which could have an impact on cosmetics and well-being in general.

### Author Contributions

Conceptualization, S.L. and A.D.; methodology, S.L.; software, S.L.; validation, A.D., T.H. and C.B.; formal analysis, I.K.; investigation, S.B.; resources, C.B.; data curation, S.L.; writing—original draft preparation, S.L.; writing—review and editing, S.L.; visualization, I.K.; supervision, A.D.; project administration, A.D., S.B. and C.B.; funding acquisition, C.B. All authors have read and agreed to the published version of the manuscript.

### Acknowledgement

We would like to thank MESRS (Ministry of Higher Education and Scientific Research, Algeria) for their financial support. We sincerely thank the Constantine Biotechnology Research Centre for their essential contribution to the success of this study, and our sincere thanks to the ENSET (Higher Normal School of Technological Education) in Skikda for making the spectrophotometer available to us.

### Funding

This research was not funded by any external sources.

### Conflicts of Interest

The authors declare that they have no conflict of interest.

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ABS

Original Research

# New Records and Ecological Insights on *Russula* (*Russulaceae*, Russulales) Species in Kosovo

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## Abstract

Among its members, the genus *Russula* is one of the largest ectomycorrhizal genera, with approximately 1,300 recognized species. Previous studies on macrofungi in Kosovo have documented 25 species, primarily from oak forests. This study adds 14 new species to the mycobiota of Kosovo, bringing the total number of *Russula* species documented in the country to 39. With the exception of *R. torulosa*, which was found at two locations, all other newly recorded species were observed at a single site. Additionally, new location records for previously known species are presented.

## Keywords

Basidiomycota, forest ecosystems, macrofungi, new records, fungal diversity

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**Citation:** Ramshaj, Q., Karadelev, M., (2025). New Records and Ecological Insights on *Russula* (*Russulaceae*, Russulales) Species in Kosovo. Acta Biologica Slovenica 68 (3)

**Received:** 17.04.2025 / **Accepted:** 20.06.2025 / **Published:** 27.06.2025

**https://doi.org/10.14720/abs.68.3.22539**

## Nove najdbe in ekološki vpogledi v vrste rodu *Russula* (*Russulaceae*, Russulales) v Kosovu

**Izvleček**

Rod *Russula* je eden največjih ektomikoriznih rodov, s približno 1300 priznanimi vrstami. Prejšnje študije o makroglivah na Kosovu so dokumentirale 25 vrst, predvsem iz hrastovih gozdov. Ta študija dodaja 14 novih vrst k mikobioti Kosova, s čimer se skupno število dokumentiranih vrst *Russula* v tej državi poveča na 39. Z izjemo *R. torulosa*, ki je bila najdena na dveh lokacijah, so bile vse druge novo zabeležene vrste opažene le na eni sami lokaciji. Poleg tega so predstavljeni novi lokacijski zapisi za že znane vrste.

**Ključne besede**

Basidiomycota, gozdni ekosistemi, makroglive, novi zapisi, raznolikost gliv.

# Introduction

The genus *Russula* Pers., recognized for its remarkable diversity, belongs to the family *Russulaceae* Lotsy (1907), one of the largest genera of ectomycorrhizal basidiomycetes (Buyck et al. 2018; He et al. 2024; Wisitrassameewong et al. 2020; Zhou et al. 2022; Li et al. 2023). It comprises over 2,000 species worldwide, with approximately 1,300 species currently accepted (Adamčík et al. 2018, 2019; Manz et al. 2021). Distinguished by its colour variation in sporocarps and obscure morphological and anatomical traits (Miller and Buyck 2002), the genus *Russula* primarily exhibits an agaricoid morphology (Buyck et al. 2008). From a morphological perspective, *Russula* is distinguished by its heteromerous context structure and amyloid spore ornamentation (Romagnesi 1967, 1985; Sarnari 1998, 2005; Li 2014). In recent years, phylogenetic studies utilizing ribosomal DNA sequences have emerged as the principal method for its classification (Miller and Buyck 2002). *Russula* species play a vital ecological role as symbionts of forest trees (Gardes and Bruns 1996; Buyck et al. 1996) and contribute significantly to fungal biomass (Richardson 1970) as well as serving as a food source for various animals and insects (Fogel and Trappe 1978; Rimšaitė 2007). Many species also hold economic value as globally recognized edible fungi (Miller and Buyck 2002). According to published data, the family *Russulaceae* exhibits considerable diversity across several Balkan and neighbouring countries. In Montenegro, 83 species have been recorded (Kasom and Karadelev, 2012), while North Macedonia and Croatia each report 87 species (Karadelev et al., 2018; Tkalčec and Mešić, 2003). In Greece, 29 species have been documented (Zervakis et al., 1998). In Bulgaria, 76 species (Denchev and Assyov, 2010) and Turkey have reported 68 species belonging to this family (Yılmaz Ersel and Solak, 2005).

These fungi establish symbiotic associations with a wide range of host plants, including broadleaved and coniferous trees (Das et al. 2017; Dutta et al. 2015), primarily species of *Pinus* sp., *Castanopsis* sp., *Picea* sp., *Larix* sp., *Fagus* sp., *Quercus* sp. and *Castanea* sp. (Hackel et al. 2022; Looney et al. 2022; Noffsinger et al. 2024). As essential components of forest ecosystems, they play a crucial role in nutrient cycling and soil stability, further enhancing ecosystem resilience. Beyond their ecological importance, *Russula* species have attracted sustained scientific interest due to their edibility, pharmaceutical potential, and bioactive properties (Clericuzio et al. 2012; Looney et al. 2018; Panda et al. 2021; Wang et al. 2009). The genus *Russula* holds a significant place in European mycological research, described by Persoon (1796) during the study of macrofungi in Northern Europe.

Research on the fungal diversity in Kosovo, including the genus *Russula*, began between 2017 and 2018 as part of the project "Fungi of Kosovo: Preparation of a Fungal Checklist, a Preliminary Red List, and a Proposal for Important Fungal Areas" (Karadelev 2018). This initiative laid the foundation for subsequent studies, including research on macrofungi in oak forests, which documented 25 *Russula* species (Ramshaj et al. 2021). Further exploration in the Sharr Mountains identified 18 species, including *Russula emetica* (Schaeff.) Pers., recorded for the first time in Koso-

vo's mycobiota (Ramshaj et al. 2022). The present study builds upon these efforts, with extensive fieldwork leading to the discovery of additional noteworthy and previously unreported *Russula* species. Given Kosovo's favourable climatic conditions for Basidiomycota but limited documentation of its basidiomycetous fungi, this paper contributes to bridging that knowledge gap by introducing 14 new records of *Russula* species in the country's mycobiota.

## Materials and Methods

During the period 2018–2022, mycological material was collected from various forested locations across Kosovo (Fig. 1, 2). Fresh *Russula* specimens were gathered in their natural habitats, and detailed field notes were recorded, documenting key macroscopic features relevant to field identification. The collected fungal species are presented in the following section, with the species list arranged alphabetically. Newly recorded species are marked with an asterisk (*) (see Results). For previously known species, only updated locality records are provided, along with relevant ecological data. A few specimens were fully dried for storage, while others were in a decayed condition at the time of collection or were left attached to their natural substrate.

All collected specimens were dried using an FDK24DW food dehydrator at temperatures ranging from 40 to 55°C and subsequently deposited in the Herbarium of the Faculty of Mathematics and Natural Sciences, University of Prishtina. Microscopic features were examined from dried material mounted in 3–5% potassium hydroxide (KOH), Melzer's reagent, Methylene Blue, or Congo Red to enhance structural visibility and assess the amyloid reaction of spores (Buyck 1989; Buyck et al. 2018; White et al. 1990). Other reagents like iron salts (FeSO4) and ammonia were also used. For the taxonomic identification of the collected fungi, key literature sources with identification keys were used, including Adamčík and Jančovičová (2013), Kibby (2017), Romagnesi (1967), Sarnari (2005), Horak (2005), Breitenbach and Kränzlin (2005) and Knudsen and Vesterholt (2012). The current taxonomic names were verified using the online nomenclatural database Index Fungorum (www.speciesfungorum.org) and MycoBank (www.mycobank.org).

**Figure 1.** Kuqishtë – *Picea abies* forest. Photo. Q. Ramshaj.

**Slika 1.** Kuqishtë – gozd smreke (*Picea abies*). Foto: Q. Ramshaj.

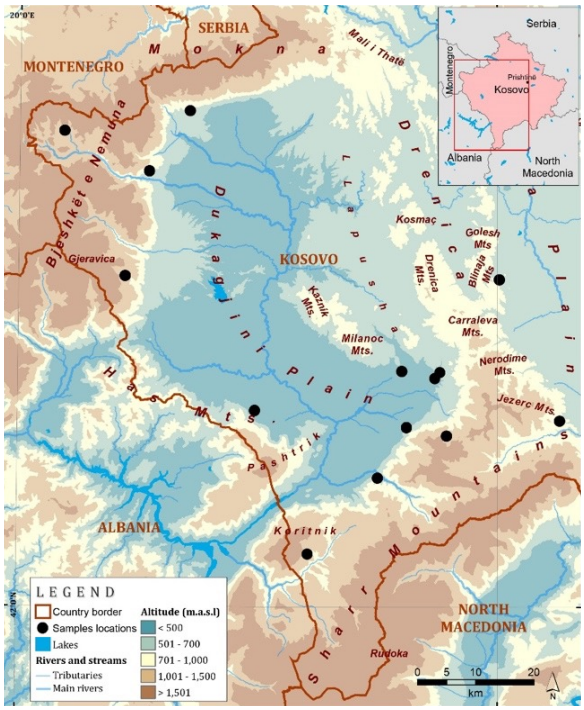


**Figure 2.** Map of sampling sites – areas of fungal collection.

**Slika 2.** Zemljevid mest vzorčenja – območja zbiranja gliv.

# Results

A total of 14 *Russula* species are reported for the first time, significantly expanding the documented fungal diversity of the country. With these additions, the total number of *Russula* species recorded in Kosovo now stands at 39. Of the identified species, 16 were classified as edible, 21 inedible, and 2 of them poisonous. In this study, most *Russula* species were recorded in oak forests (18 species). However, several species, such as *R. cyanoxantha*, *R. olivacea*, and *R. vesca*, exhibit a broader ecological range, occurring in both coniferous and deciduous forests. Regarding growth habits, the majority of species (25) were observed growing gregariously, often forming clusters, while 13 species displayed both solitary and gregarious growth habits. Only one species, *R. zvarae*, was found exclusively in a solitary growth pattern (see Table 1).

## List of Newly Recorded Species

***Russula albonigra* (Krombh.) Fr.,**
Material examined: Junik, in leaf litter, inside a *Picea abies* forest with Fagus sylvatica and *Abies alba*, 42°30'37.0"N, 20°13'20.7"E, at 1015 m a.s.l., northeast-oriented slope with shallow brown soils on micaschists, 28.06.2019, leg. QR, ST, KR and MK, det. QR and MK (No. 2019/150).

***Russula carpini* R. Girard & Heinem.**
Material examined: Gërqinë (Gjakovë), in leaf litter, in mixed broadleaf forests dominated by *Carpinus orientalis*, 42°18'13.5"N, 20°29'40.8"E, 380 m a.s.l., northeast oriented slope on brownized rendzina on compact limestone, 10.10.2020, leg. QR and GK, det. QR and MK.

***Russula cyanoxantha* (Schaeff.) Fr.**
Ref.: Ramshaj et al. (2021; 2022)
Material examined: Jabllanicë (Prizren), in leaf litter, in *Pinus nigra* forest mixed with *Quercus* sp., 42°11'58.0"N, 20°45'05.0"E, 520 m a.s.l., 12.11.2021, leg. QR, det. QR and MK. Koritnik (Dragash), in leaf litter, in beech forest, 42°04'54.26"N, 20°36'21.31"E, 1227 m a.s.l., 25.07.2021, leg. QR and PB, det. QR and MK. Mushtisht (Suharekë), in leaf litter, within Fagus sylvatica forest, 42°15'53.5"N, 20°53'41.5"E, 1084 m a.s.l., 24.09.2022, leg. QR, det. QR and MK.

**Table 1.** Growth habitat and associations of *Russula* species in Kosovo.

**Tabela 1.** Rastno okolje in povezave vrst *Russula* na Kosovu.

| Species | Growth habit | Associations |
|---|---|---|
| *Russula albonigra* | solitary/gregarious | conifer and beech forest |
| *Russula carpini* | solitary/gregarious | hornbeam forest |
| *Russula cyanoxantha* | solitary/gregarious | conifer, oak, beech forest |
| *Russula densifolia* | solitary/gregarious | oak forest |
| *Russula emetica* | gregarious | oak forest |
| *Russula favrei* | solitary/gregarious | conifer and beech forest |
| *Russula fragilis* | gregarious | oak forest |
| *Russula grata* | solitary/gregarious | oak forest |
| *Russula insignis* | gregarious | oak forest |
| *Russula integra* | gregarious | conifer and beech forest |
| *Russula mairei* | gregarious | beech forest |
| *Russula ochroleuca* | gregarious | oak and chestnut forest |
| *Russula olivacea* | gregarious | conifer, oak, beech |
| *Russula pectinata* | gregarious | hornbeam forest |
| *Russula rubroalba* | gregarious | oak forest |
| *Russula sanguinea* | gregarious | conifer forest |
| *Russula silvestris* | gregarious | oak and hornbeam forest |
| *Russula solaris* | gregarious | beech forest |
| *Russula torulosa* | gregarious | conifer and oak forest |
| *Russula vinosa* | gregarious | mixed forest |
| *Russula virescens* | gregarious | oak and beech forest |
| *Russula xerampelina* | gregarious | conifer forest |
| *Russula zvarae* | solitary | oak and hornbeam forest |

***Russula densifolia* Secr. ex Gillet**

Material examined: Reqan (Suharekë), in leaf litter, within *Quercus frainetto* forest, 42°21'14.1"N, 20°52'12.8"E, 467 m a.s.l., northeast oriented slope on reddish brown leached soil on reddish sediments, 25.09.2022, leg. QR, det. MK det. QR and MK.

***Russula emetica* (Schaeff.) Pers.**

Ref.: Ramshaj et al. (2022)

Material examined: Maja e Zezë (Pejë), in leaf litter, in *Castanea* forest, 42°40'22.0"N, 20°16'18.4"E, 699 m a.s.l., 29.06.2019, leg. QR, ST, KR and MK, det. QR and MK (No. 2019/386).

***Russula favrei* M.M. Moser**

Material examined: Junik, in leaf litter, inside a *Picea abies* forest with *Fagus sylvatica* and *Abies alba*, 42°30'37.0"N, 20°13'20.7"E, at 1015 m a.s.l., northeast oriented slope on shallow brown soils on micaschists, 28.06.2019, leg. QR, ST, KR and MK, det. QR and MK (No. 2019/154).

***Russula fragilis* Fr.**

Ref.: Ramshaj et al. (2021; 2022)

Material examined: Gërqinë (Gjakovë), in leaf litter, in mixed broadleaf forests dominated by *Carpinus orientalis*, 42°18'13.5"N, 20°29'40.8"E, 380 m a.s.l., 10.10.2020, leg. QR and GK, det. QR and MK. Jabllanicë (Prizren), in leaf litter, in *Pinus nigra* forest mixed with *Quercus* sp., 42°11'58.0"N, 20°45'05.0"E, 520 m a.s.l., 12.11.2021, leg. QR, det. QR and MK.

***Russula grata* Britzelm.**

Ref.: Ramshaj et al. (2021; 2022)

Material examined: Maja e Zezë (Pejë), in leaf litter, in

Castanea forest, 42°40'22.0"N, 20°16'18.4"E, 699 m a.s.l., 29.06.2019, leg. QR, ST, KR and MK, det. QR and MK (No. 2019/386).

***Russula insignis* Quél.**

Material examined: Semajë (Ferizaj), in soil, within *Quercus cerris* forest, 42°17'08"N, 21°07'60"E, 850 m a.s.l., north oriented slope on shallow brown soil on compact limestone, 01.06.2018, leg. TJ, KR and MK, det. MK.

***Russula integra* (L.) Fr.**

Material examined: Koshutan (Pejë), in leaf litter, within *Picea abies* forest, 42°44'02.0"N, 20°05'37.0"E, 1480 m a.s.l., east oriented slopes on shallow brown soil on compact limestone, 03.07.2022, leg. QR, det. QR and MK (No. 2022/599).

***Russula mairei* Peck**

Material examined: Mushtisht (Suharekë), in leaf litter, within *Fagus sylvatica* forest, 42°15'53.5"N, 20°53'41.5"E, 1084 m a.s.l., west oriented slopes on the river valley on shallow brown soil on schists, 24.09.2022, leg. QR, det. QR and MK (No. 2022/628).

***Russula ochroleuca* Fr.**

Ref.: Ramshaj et al. (2021)

Material examined: Maja e Zezë (Pejë), in leaf litter, in *Castanea* forest, 42°40'22.0"N, 20°16'18.4"E, 699 m a.s.l., 29.06.2019, leg. QR, ST, KR and MK, det. QR and MK (No. 2019/386).

***Russula olivacea* (Schaeff.) Fr.**

Ref.: Ramshaj et al. (2021; 2022)

Material examined: Korishë (Prizren), in leaf litter, in *Pinus nigra* and *Juniperus oxycedris* forest, 42°16'40.0"N, 20°48'41.9"E, 487 m a.s.l., 25.09.2022, leg. QR, det. QR and MK.

***Russula pectinata* Fr.**

Material examined: Gërqinë (Gjakovë), in leaf litter, in mixed broadleaf forests dominated by *Carpinus orientalis*, 42°18'13.5"N, 20°29'40.8"E, 380 m a.s.l., northeast oriented slope on brownized rendzina on compact limestone, 10.10.2020, leg. QR and GK, det. QR and MK.

***Russula rubroalba* (Singer) Romagn.**

Material examined: Zym (Prizren), in leaf litter, within *Quercus frainetto* forest, 42°29'83.3"N, 20°62'17.2"E, 360 m a.s.l., northeast oriented slope on loamy alluvium of Drini i Bardhë River bank, 10.10.2020, leg. QR and GK, det. QR and MK.

***Russula sanguinea* Fr.**

Material examined: Korishë (Prizren), in leaf litter, in *Pinus nigra* and *Juniperus oxycedris* forest, 42°16'40.0"N, 20°48'41.9"E, 487 m a.s.l., northwest oriented slope on brown rendzina on serpentine, 25.09.2022, leg. QR, det. QR and MK (No. 2022/632). Jabllanicë (Prizren), in leaf litter, in *Pinus nigra* forest mixed with *Quercus* sp., 42°11'58.0"N, 20°45'05.0"E, 520 m a.s.l., east oriented slopes on river valley, some 10 m above the river bed, on brownized ranker on schists, 12.11.2021, leg. QR, det. QR and MK.

***Russula silvestris* (Singer) Reumaux**

Ref.: Ramshaj et al. (2021)

Material examined: Maja e Zezë (Pejë), in leaf litter, in *Castanea* forest, 42°40'22.0"N, 20°16'18.4"E, 699 m a.s.l., 29.06.2019, leg. QR, ST, KR and MK, det. QR and MK (No. 2019/386). Mushtisht (Suharekë), in leaf litter, within *Fagus sylvatica* forest, 42°15'53.5"N, 20°53'41.5"E, 1084 m a.s.l., 24.09.2022, leg. QR, det. QR and MK.

***Russula solaris* Ferd. & Winge**

Material examined: Koshare (Gjakovë), in leaf litter, in beech forest, 42°45'57.63"N, 20°21'22.26"E, 1074 m a.s.l., southeast oriented slope on shallow brown soils on schists, 28.06.2019, leg. QR, ST, KR and MK, det. QR and MK.

***Russula torulosa* Bres.**

Material examined: Jabllanicë (Prizren), in leaf litter, in *Pinus nigra* forest mixed with *Quercus*, tree, 42°11'58.0"N, 20°45'05.0"E, 520 m a.s.l., east oriented slopes on river valley, some 10 m above the river bed, on brownized ranker on schists, 12.11.2021, leg. QR, det. QR and MK. Korishë (Prizren), in leaf litter, in *Pinus nigra* and *Juniperus oxycedrus* forest, 42°16'40.0"N, 20°48'41.9"E, 487 m a.s.l., northwest oriented slope on brown rendzina on serpentine, 25.09.2022, leg. QR, det. MK and QR (No. 2022/633).

***Russula vinosa* Lindblad**

Material examined: Reqan (Suharekë), in leaf litter, in mixed broadleaf forest (*Fraxinus* sp., *Robinia* sp., *Rubus* sp., *Crataegus* sp.), 42°21'47.0"N, 20°52'51.0"E, 470 m a.s.l., west oriented slope on brown leached soil on schists, 09.10.2019,

leg. QR and DS, det. QR and MK (No. 2019/260).

***Russula virescens* (Schaeff.) Fr.**
Ref.: Ramshaj et al. (2021; 2022)
Material examined: Mushtisht (Suharekë), in leaf litter, within *Fagus sylvatica* forest, 42°15'53.5"N, 20°53'41.5"E, 1084 m a.s.l., 24.09.2022, leg. QR, det. QR and MK.

***Russula xerampelina* (Schaeff.) Fr.**
Material examined: Koshutan (Pejë), in leaf litter, within *Picea abies* forest, 42°44'02.0"N, 20°05'37.0"E, 1480 m a.s.l., north oriented slopes on shallow brown soil on compact limestone, 03.07.2022, leg. QR, det. QR and MK.

***Russula zvarae* Velen.**
Ref.: Ramshaj et al. (2021; 2022)
Material examined: Kosovo, Peqan (Suharekë), in leaf litter, in *Quercus frainetto* forest, 42°21'53.7"N, 20°48'04.5"E, 378 m a.s.l., 23.04.2021, leg. QR, det. QR and MK (No. 2021/528).


**Figure 3.** Basidiocarps: a – *Russula carpini*; b – *R. cyanoxantha*; c – *R. emetica*; d – *R. fragilis*; e – *R. integra*; f – *R. mairei*; g – *R. rubroalba*; h – *R. sanguinea*; i – *R. torulosa*; j – *R. vinosa*; k – *R. xerampelina*; l – *R. zvarae*.

**Slika 3.** Trosnjaki: a – *Russula carpini*; b – *R. cyanoxantha*; c – *R. emetica*; d – *R. fragilis*; e – *R. integra*; f – *R. mairei*; g – *R. rubroalba*; h – *R. sanguinea*; i – *R. torulosa*; j – *R. vinosa*; k – *R. xerampelina*; l – *R. zvarae*.

## Discussion

Recognizing the diversity and distribution of macrofungal species is essential for understanding their ecological functions and interactions within forest ecosystems. In the following discussion, we examine individual species, beginning with *Russula albonigra*, classified within the section *Compactae* Fr. of the genus *Russula*, distinguished by its dense, firm texture and numerous lamellae. The fruiting bodies appear white in the early stages but darken to brown or black as they mature or when bruised (Shafer 1962). Originally described as *Agaricus alboniger* with a brief description and no designated holotype, it was later reassigned to *Russula* under its current name. This species is well-recognized and frequently documented across Europe (Krombholz 1845; Fries 1874). This species occurs in both deciduous and coniferous forests from summer to autumn. *R. albonigra* is considered rare in temperate-boreal regions. It has been recorded in Norway (only from Østfold), Denmark (VU), Finland (LC), Norway (NT), Sweden (NE), the Czech Republic (EN) and Slovakia (LR) (Knudsen et al. 2008; Holec and Beran 2006; Lizoň 2001). In North Macedonia, it has been recorded twice in Veles and once in Dobra Voda Mountain, within a *Quercetum frainetto-cerris* forest (Karadelev et al. 2018). In Greece, it has been recorded in association with *Castanea* (Maire and Politis 1940; Zervakis et al. 1998). From Turkey, it has also been recorded in habitats with *Fagus orientalis* and *Carpinus orientalis* forests (Doğan et al. 2021). Records also exist from Croatia, where it is listed in the Preliminary Checklist of Agaricales (Tkalčec and Mešić 2003). It has also been reported from Montenegro (Kasom and Karadelev 2012).

Another species within the section *Compactae* is *Russula densifolia*, distinguished by its surface, which initially appears whitish to brownish-orange before gradually turning greyish to reddish-brown and eventually blackening. It features narrow, crowded lamellae and grows solitarily or in small groups in rich soils, from summer to autumn, in deciduous, mixed, and coniferous forests (Ratheesh et al. 2019; Shaffer 1962). The species typically grows in deciduous forests, with *Fagus* and *Quercus*, also with *Picea*, *Pinus* and *Abies*, as partner trees (Delange et al. 2023; Romagnesi 1967). The species is common in the temperate zone, occasional in the hemiboreal zone and rare in the boreal zone (Knudsen et al. 2008). In the Balkan countries, *R. densifolia* has been reported so far from Bulgaria, Croatia and North Macedonia (Denchev and Assyov 2010; Tkalčec and Mešić 2003; Karadelev et al. 2018).

*Russula carpini* is a mycorrhizal species primarily associated with *Carpinus* (Knudsen and Vesterholt 2012). This species is easily recognizable, as it typically appears early in the year, starting in late spring, but is more commonly found during summer and autumn. The cap is initially convex, later becoming flattened, and varies in colour depending on its developmental stage, ranging from orange-brown to olive-brown, often with yellowish or reddish hues. The gills are white when young, gradually turning orange-ocher with age (Kränzlin 2005). *R. carpini* is a relatively rare species that has been recorded across several European countries. In France, it has been documented in regions such as Alsace (LC), Franche-Comté, Midi-Pyrénées and Poitou-Charentes (NT) (Courtecuisse 2009; INPN 2025). In the Czech Republic, it is categorized as Near Threatened (NT) (Holec and Beran 2006), and it has also been recorded in Denmark (VU) and Sweden (NT) (Knudsen et al. 2008). Further records of *R. carpini* are available from Slovenia (Boletus Informaticus 2025a). In Kosovo, the species has been recorded in lowland and hilly forests dominated by *Carpinus orientalis*, often in association with other broadleaf species such as *Cornus*, *Acer*, *Corylus* and *Prunus*.

*Russula favrei* is another record classified in the section *Polychromae* Maire but within the subsection *Xerampelinae* Singer. It features a purplish-brown cap that turns brown when bruised and is smooth and sticky when moist. This species fruits from late summer to early autumn and is commonly found in coniferous forests, particularly in association with *Pinus* and *Picea*. It typically grows on acidic to slightly calcareous soils enriched with humus or moss (Knudsen et al. 2008). This species has been reported as common in the mountainous areas of Central Europe (Courtecuisse and Duhem 1995; Ronikier and Adamčík 2009 a, b) and has been recorded in the montane vegetation belt of the Tatra National Park in Poland and Slovakia (Adamčík 2001; Ronikier and Adamčík 2009). Additionally, it was reported as a new record in Belarus (Kolmakow, 2015). Other reports of its presence come from France (Franche-Comté), where it is included in the Red List of Higher Mushrooms and classified as Near Threatened (NE) (Sugny et al. 2013). It has also been recorded in Slovenia as part of the central database of fungi (Boletus Informaticus 2025b).

*Russula rubroalba* has a cap measuring 50–90 (100) mm in diameter, initially hemispherical, later becoming

convex to the plane, often with a slightly depressed centre. The cap surface is smooth, dry, to slightly viscid when moist and varies in colour from purple-red to pinkish-red. The gills are white when young, turning ocher-yellow with maturity. The stipe is cylindrical, measuring 40–80 × 10–20 mm, and predominantly white. An ectomycorrhizal species, *R. rubroalba*, is primarily associated with *Fagus* and *Castanea* in deciduous forests, though it is occasionally found near *Quercus*. It thrives in acidic to basic soils and is typically found in leaf litter at low to mid-elevations, favouring warm areas. Occurs from spring to early summer, with specimens appearing singly or in small groups. *Russula rubroalba* is listed in the Checklist of Macrofungal Species of the Republic of Macedonia, where it has been reported from deciduous forests (Quercetum frainetto-cerris and beech forests). It is also listed in the Preliminary Checklist of Agaricales from Croatia (Tkalčec and Mešić 2003).

*Russula xerampelina*, from the same section *Polychromae* subsection *Xerampelinae* Singer, has a cap with a diameter of 50–90 mm, initially hemispherical, later plane, and sometimes undulating, developing a depressed centre. The cap surface is dry, smooth, and slimy when moist, and it is white to carmine-red. The stipe is white to red-flushed and may swell at the base, turning yellow when rubbed. The gills are white to cream and attached to the stipe. This species is commonly found in coniferous and mixed forests, primarily near *Pinus* and more rarely near *Picea*. It typically grows in damp soils at colline to montane elevations and occurs in autumn (Knudsen and Vesterholt 2012; Kränzlin 2005). *R. xerampelina* is a common species in temperate and boreal regions (Knudsen and Vesterholt 2012). It is included in the Checklist of Fungi from the Belarusian-Valdai Lake district, where it is assessed as common (Kolmakov 2015). In Greece, it is recorded in association with *Fagus* (Zervakis et al. 1998). In the Pieniny Mountains (Poland), it has been reported as a new species for this area, associated with coniferous trees (Chachuła et al. 2014), although it is considered common in Poland (Wojewoda 2003; Kujawa 2023). Additionally, it is listed in the Checklist of Macrofungal Species of North Macedonia (Karadelev et al. 2018) and in the Preliminary Checklist of Agaricales from Croatia (Tkalčec and Mešić 2003).

*Russula insignis* and *R. pectinata* belong together to the Ingratae section (Quel.) Maire. *R. insignis* has a hemispherical pileus when young, later becoming plane and often almost umbilicate. The pileus is dark grey-brown at the centre, gradually fading to a cream-yellowish shade toward the margin. The lamellae are white, turning yellow or brown with age (Knudsen et al. 2008; Kränzlin 2005). This species grows gregariously in hardwood and mixed forests, as well as in parks, often appearing in scattered small groups or forming dense clusters on disturbed, compacted soils, such as roadsides and along paths. It is primarily found in deciduous and mixed forests, frequently associated with beech (*Fagus*) and oak (*Quercus*), particularly on calcareous soils, as it is an obligate mycorrhizal species, especially with the *Quercus* tree. *R. insignis* typically fruits from summer to autumn (Doğan and Öztürk 2015; Walleyn and LeJeune 2005). This species is considered rare and is included in the Red List of Fungi in Poitou-Charentes as Least Concern (LC) (GEREPI 2019) and in Franche-Comté as Vulnerable (VU) (Sugny et al. 2013). *R. insignis* was first recorded in Bursa under *Quercus coccifera* and *Fagus orientalis* as a new addition to the mycobiota of Turkey (Doğan and Öztürk 2015) and was later reported from the Samanli Mountains under *Quercus* (Doğan et al. 2021). As an uncommon species growing with *Quercus*, *R. insignis* has also been reported from northern Belgium (Walleyn and LeJeune 2005) and Croatia (Tkalčec and Mešić 2003).

*Russula pectinata* is characterized by a convex to-plane pileus, measuring 30–40 mm, sometimes slightly depressed at the centre. The pileus surface is smooth, dry and lubricious when moist, with colouration ranging from ochraceous to yellow or olive brown, often exhibiting a darker centre. The lamellae are initially white, gradually turning ochre to yellowish with age, and may develop brownish stains. This species is usually described as growing in association with both broadleaf trees (especially *oaks*) and conifers, but it is primarily found with *Quercus*, as well as *Carpinus* and *Betula*. It prefers nutrient-poor, sandy, or calcareous soils and produces fruiting bodies from summer to autumn (Knudsen and Vesterholt 2012; Kränzlin 2005). *Russula pectinata* has been recorded in various European countries, including Greece, where it was found in association with *Pinus halepensis* and *Quercus pubescens* (Maire and Politis 1940). In North Macedonia, it has been reported from deciduous forests (*Fagus* and *Quercus*) as well as coniferous forests dominated by *Pinus peuce* (Karadelev et al. 2018). It has also been recorded in Croatia (Tkalčec and Mešić 2003). In Alsace, France, it is included in the Red List of Fungi (La Liste Rouge des Champignons Supérieurs Menacés en Alsace), where it is assessed as Near Threatened (NT) (Muller et al. 2014).

*Russula integra* and *R. vinosa* belong to *Russula* section *Polychromae* Maire, subsection *Integroidinae* (Romagn.) Bon. When young, *R. integra* has hemispherical pileus, later becoming convex and sometimes slightly depressed in the centre. Its colouration varies, displaying shades of brown, purple, olive and yellow. The stipe is white in youth, often with an orange-red base and may broaden at the base. The gills are white to cream, relatively dense and slightly attached. This species typically grows in coniferous and mixed forests, with occasional occurrences in deciduous forests. It is primarily found at montane elevations, forming associations with *Picea* and *Pinus* on rich, often moist soils, in summer to autumn (Knudsen et al., 2008; Kränzlin, 2005; Sarnari, 1998; Sarnari, 2005). *Russula integra* is a common species reported in both coniferous and deciduous forests in Belarus (Kolmakov 2015). In Alsace, France, it is classified as Least Concern (LC) in The Red List of Threatened Higher Fungi (Muller et al. 2014). It has been documented in association with *Abies cephalonica* (Diamandis and Minter 1983) and *Abies borisii-regis* (Diamandis 1985) and is listed in the Check-List of the Greek Macrofungi (Zervakis et al. 1998). Additionally, it is included in the Checklist of Macrofungal Species of the Republic of Macedonia (Karadelev et al. 2018). It is listed in the Preliminary Checklist of Agaricales from Croatia (Tkalčec and Mešić 2003) and has also been reported from Montenegro (Kasom and Karadelev 2012).

*Russula vinosa* is characterized by a hemispherical to campanulate-convex pileus, which later becomes plane and slightly depressed at the centre. The pileus surface is smooth, dry, and shiny, becoming lubricous when moist. Its colouration varies from reddish-brown to wine-red, often with a darker brown to blackish centre. The lamellae are initially white, gradually turning ochre to yellowish with age. The stipe is cylindrical and robust. This species is mycorrhizal, primarily associated with coniferous trees such as *Picea*, *Pinus*, and *Abies*. In mountainous areas, it also occurs with *Betula*. It prefers acidic or moist soils and appears from summer to autumn (Kränzlin 2005). *R. vinosa* is a protected species included in The IUCN Red List of Threatened Species, assessed under the IUCN Red List Category - LC (Least Concern) (Dahlberg 2019). It holds the same status in the Danish Red List (Læssøe 2018) and in the Red List of Finland (Red List 2019). In the Franche-Comté region, it is listed as Vulnerable (VU) (GEREPI, 2019), while in Alsace, it is assessed as Least Concern (LC) (Muller et al. 2014). *R. vinosa* is listed in the Checklist of Polish Larger Basidiomycetes and is also included in the Polish Red List (Wojewoda 2003). In North Macedonia, it has been recorded in association with *Pinus peuce*, *P. sylvestris* and *P. nigra* plantations (Karadelev et al. 2018).

*Russula mairei, R. sanguinea, R. solaris* and *R. torulosa* belong to the section *Russula* Pers. *Russula mairei* is characterized by a hemispherical to convex and plane pileus, 30– 60 (70) mm. The pileus surface is slightly tuberculate, dull when dry, shiny and viscid when it is moist and varies in colour from cherry-red to paler and whitish spotted. The lamellae are initially white in the early stage, turning pale, cream-coloured and narrow. The stipe is cylindrical to clavate or ventricose with white colour to white-pruinose. This species is mycorrhizal in beech forest or in mixed forest with *Fagus*, especially near the stem, usually grows gregarious, prefers acidic or neutral soils, colline to montane and occurs from late summer to autumn (Knudsen and Vesterholt 2012; Kränzlin 2005). *Russula mairei* is a mycorrhizal species commonly found in beech forests and mixed woodlands, particularly near the base of *Fagus* trees. It thrives in acidic to neutral soils at colline to montane elevations and typically fruits from late summer to autumn (Knudsen and Vesterholt 2012; Kränzlin 2005). This species is widespread, occurring not only under *Fagus* but also in mixed forests of the subalpine zone, where it associates with *Tilia*, *Corylus* and *Carpinus* (Sarnari 2005). *Russula mairei* represents a new record for Finland, where it was found under *Fagus sylvatica* (Bonsdorff et al. 2014). It is considered a common species in Poland (Wojewoda 2003; Kujawa 2023). In Greece, it has been recorded under *Fagus moesiaca* and *Quercus conferta* (Diamandis and Perlerou 1990), as well as under *Fagus orientalis* and *Quercus conferta* (Diamandis and Perlerou 1994). In North Macedonia, *R. mairei* has been documented in beech forest communities of *Calamintho grandiflorae-Fagetum* (Karadelev et al. 2018). In Poitou-Charentes (France), *R. mairei* is included in the Red List of Threatened Mushrooms (2018). It has been reported from Montenegro (Kasom and Karadelev 2012) and is also listed in the Preliminary Checklist of Agaricales from Croatia (Tkalčec and Mešić 2003).

*Russula sanguinea* is characterized by a cap measuring 30–80 mm in diameter, initially hemispherical when young, later becoming plane to slightly depressed at the centre. The surface is smooth, dull when dry, and silky or viscid when moist, sometimes developing cracks with age. Its colouration ranges from deep red to blood-red, occasionally fading to purple-red. The gills are initially white, turning

cream to pale ochre with maturity, moderately dense, and adnexed to slightly decurrent, often developing reddish or brownish stains when damaged. The stipe is cylindrical to slightly clavate, measuring 30–60 × 10–25 mm, firm and solid when young but becoming spongy with age. It is predominantly white, sometimes exhibiting pinkish or reddish tints. This species is mycorrhizal, commonly found in coniferous and mixed forests, particularly in association with *Pinus*. It also occurs in grassy areas, parks, and forest margins. *Russula sanguinea* thrives in both acidic and alkaline soils, often growing in sandy or humus-rich substrates. Occurs from summer to late autumn, with specimens appearing either scattered or in gregarious groups (Kränzlin 2005). Sarnari (2005) reports that *Russula sanguinea* is frequently found in association with *Pinus* across diverse landscapes, from lowland plains to mountainous regions, spanning the Mediterranean zone to subalpine elevations. Its range extends into northern Europe, where it also occurs beneath *Picea* and *Larix*. In the Russian Federation, this species is commonly encountered in pine forests mixed with birch, as well as in pine-wild strawberry forests with hazel, pine-bilberry formations, and pine-herb woodlands with maple, marking a newly documented presence in the Pskov region of Belarus (Kolmakov 2015). In Poland, it has been observed primarily growing with *Pinus sylvestris* on the forest floor, with occasional occurrences beneath *Larix decidua, Picea abies* and *Taxus baccata*; however, its conservation status remains unclear (Wojewoda 2003). In Montenegro, *R. sanguinea* is listed among the ectomycorrhizal fungi associated with *Pinus heldreichii* (Lazarević et al. 2011). Records from Greece indicate its presence in *Pinus nigra* and *Pinus brutia* woodlands, as documented in A Checklist of the Greek Macrofungi (Zervakis et al. 1998), as well as in Montenegro (Kasom and Karadelev 2012) and Croatia (Tkalčec and Mešić 2003). Furthermore, in North Macedonia, it is included in the national Checklist of Macrofungal species (Karadelev et al., 2018).

*Russula solaris* features a cap ranging from 20–50 (70) mm in diameter, initially hemispherical when young, later expanding to a plane shape or becoming slightly depressed at the centre. The cap surface is smooth, dry, and slightly viscid in moist conditions, occasionally developing fine cracks with age. Its colouration varies from bright yellow to golden yellow, sometimes with subtle orange hues. The gills are initially white, becoming pale cream with maturity, closely spaced, and adnexed to slightly decurrent, often developing ochre-yellow stains upon damage. The stipe is cylindrical to slightly clavate, measuring 25–40 × 6–10 mm, initially firm but becoming soft and hollow with age. It is predominantly white, tending to discolour yellow over time. *Russula solaris* is a mycorrhizal species frequently found in deciduous and mixed forests, forming associations with *Quercus* and *Fagus*. It prefers alkaline to neutral soils, often occurring in leaf litter, humus-rich substrates, or mosses. Fruiting from summer to autumn, it typically appears scattered or in gregarious groups (Kränzlin 2005). *Russula solaris* is an ectomycorrhizal species primarily associated with *Fagus* (beech), and it is recognized for its conservation significance across various European regions. It is classified as Vulnerable (VU) in the Red List of Fungi (Macromycetes) of the Czech Republic (Deckerová 2006). In Poland, it has been recorded growing on the forest floor in deciduous and mixed woodlands, particularly under Fagus (Wojewoda 2003). The species is considered Endangered (EN) in Bulgaria, as listed in the national Red List of fungi (Gyosheva et al. 2006). In France, its conservation status varies by region: it is categorized as Least Concern (LC) in Alsace, Endangered (EN) in Franche-Comté, and Data Deficient (DD) in Poitou-Charentes (Muller et al. 2014; Sugny et al. 2013; GEREPI 2019). Additionally, *R. solaris* has been recorded in North Macedonia, where it is listed in the national Checklist of Macrofungal species (Karadelev et al. 2018) and has also been reported from Montenegro (Kasom and Karadelev 2012) and Croatia (Tkalčec and Mešić 2003).

*Russula torulosa* is characterized by a cap measuring 50–100 mm in diameter, initially hemispherical, later becoming convex to plane, often with a depressed centre. The cap surface is smooth, shiny, and slightly viscid when moist. Its colouration ranges from deep purplish-red to dark wine-red, sometimes fading to pinkish hues with age. The gills are white when young, turning cream to pale ochre at maturity, and are moderately crowded. The stipe is cylindrical, measuring 30–50 × 15–20 mm, firm when young but becoming spongy with age. It is predominantly white, often exhibiting reddish to vinaceous tints, particularly near the base. An ectomycorrhizal species, *R. torulosa* is commonly found in coniferous and mixed forests, primarily in association with *Pinus*. It thrives in base-rich yet nutrient-poor soils, frequently occurring in pine needle litter in warm locations. Fruiting occurs from summer to late autumn, with specimens appearing scattered or in small groups. Notable for its acrid taste, this species is considered inedible. *Russula torulosa* has been documented in various forest ecosystems across Europe and adjacent regions.

In Turkey, it has been recorded in the Samanlı Mountains, growing on the ground in association with *Fagus orientalis*, *Carpinus orientalis* and *Castanea sativa* (Doğan et al., 2021). In Greece, the species is included in the national checklist of macrofungi, where it has been found under *Pinus nigra* (Zervakis et al. 1998). In North Macedonia, it has been reported from diverse habitats, including *Pinus nigra* plantations, oak forests and mixed coniferous stands consisting of *Pinus* sp., *Larix decidua* and *Cupressus arizonica* (Karadelev et al. 2018). Regarding its conservation status, R. torulosa is classified as Data Deficient (DD) in the Danish Red List (Læssøe 2018) and as Near Threatened (NT) in Alsace, France (Muller et al. 2014).

## Conclusions

This study represents the first effort to document the diversity and distribution of macrofungal species within the genus *Russula* across the territory of Kosovo. The findings offer crucial insights into the rich diversity of macrofungal species and their specific ecological roles within the forest communities where they are found. This research underscores the significant role of forest ecosystems in conserving macrofungal resources, which are vital to both ecological balance and biodiversity. Although this is an initial assessment, the data presented provides a foundation for understanding the ecological relationships of *Russula*. These results highlight the importance of sustained, long-term research to deepen our understanding of *Russula* diversity and its contribution to enriching the mycobiota of Kosovo, which is essential for future conservation efforts and ecological studies.

### Author contributions

Conceptualization, Q.R.; methodology, Q.R., and M.K.; formal analysis, M.K.; investigation, Q.R., and M.K.; writing—original draft preparation, Q.R.; writing—review and editing, Q.R.; supervision, M.K. All authors have read and agreed to the published version of the manuscript.

### Acknowledgements

This study was made possible with the valuable assistance of individuals who contributed to the research on Kosovo's mycobiota. We sincerely appreciate the support provided, particularly in fieldwork, by the following individuals (listed alphabetically): Destan Sopa, Granit Kabashaj, Katrerina Rusevska, Slavica Tofilovska, Pajtim Bytyqi, and Tome Jovanovski. Special thanks go to Valbon Bytyqi for his efforts in preparing the map of the surveyed areas.

### Conflict of Interest

The authors declare that there are no conflicts of interest related to this study.

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Original Research

# Chemical characterization and antifungal activity of extracts from the edible fungus *Laetiporus sulphureus* against *Fusarium oxysporum*, the causative agent of vascular fusariosis in Moroccan date palm

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## Abstract

This study examined the valorization of the edible and medicinal mushroom *Laetiporus sulphureus*, collected from northeastern Morocco, by analyzing its chemical composition and evaluating the antioxidant and antifungal activities of its methanolic, acetonic, ethanolic, hydromethanolic, hydroacetonic, and hydroethanolic extracts. Total phenolic content (TPC) and total flavonoid content (TFC) were determined using the Folin–Ciocalteu and aluminum chloride methods, respectively, while chemical characterization was performed using two chromatographic analyses: Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography coupled with Mass Spectrometry (HPLC-MS). Concerning biological activities, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method was used to evaluate antioxidant capacity, while the microdilution method was employed to assess antifungal activity against *Fusarium oxysporum f. sp. albedinis.* The results revealed significant phenolic compound contents, with TPC ranging from 19.24 to 30.47mg of gallic acid equivalent GAE/g and TFC from 11.09 to 17.98 mg of catechin equivalent CE/g of dry crude extract. GC-MS analysis identified 106 biologically active compounds across all samples, primarily classified as sugars (41%), fatty acids (18%), and organic acids (7%). HPLC-MS identified and quantified 25 polyphenols,

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**Citation:** Abbassi, K., Benoutman, A., Erbiai, E. H., Taheri, F. Z., Makrane, H., Saidi, R., Legssyer, M., Esteves da Silva, J. C. G., Maouni, A., (2025). Chemical characterization and antifungal activity of extracts from the

with catechin (28.3 µg/g dry weight), kaempferol (18.14 µg/g dry weight), and isorhamnetin (10.98 µg/g dry weight) as the major compounds. Among the tested extracts, the hydroethanolic extract demonstrated the highest antioxidant activity ($IC_{50}$ = 2.97 mg/mL), whereas the hydroacetic extract exhibited the lowest ($IC_{50}$ = 6 mg/mL). All extracts inhibited *F. oxysporum* growth, with minimum inhibitory concentration (MIC) ranging from 1.20 to 3.20 mg/mL and minimum fungicidal concentration (MFC) from 3.20 to 5.80 mg/mL. These findings highlight *L. sulphureus* as a promising source of bioactive compounds with antioxidant and antifungal properties, supporting its potential application in pharmaceutical and agricultural fields.

edible fungus *Laetiporus sulphureus* against *Fusarium oxysporum*, the causative agent of vascular fusariosis in Moroccan date palm. Acta Biologica Slovenica 68 (3)

**Received:** 21.03.2025 / **Accepted:** 28.06.2025 / **Published:** 30.06.2025

**https://doi.org/10.14720/abs.68.3.22194**



**Keywords**

*Laetiporus sulphureus*; phenolic compounds; antioxidant activity; antifungal activity; *Fusarium oxysporum* f. sp. *albedinis*

## Kemična karakterizacija in protiglivična aktivnost izvlečkov iz užitne gobe *Laetiporus sulphureus* proti *Fusarium oxysporum*, povzročitelju vaskularne fusarioze na maroški datljevi palmi

### Izvleček

V tej študiji je bila proučena uporabna vrednost užitnih in zdravilnih gob *Laetiporus sulphureus*, nabranih v severovzhodnem Maroku, z analizo njihove kemijske sestave in oceno antioksidativnih in protiglivičnih lastnosti njihovih izvlečkov z metanolom, acetonom, etanolom, hidrometanolom, hidroacetonom in hidroetanolnom. Skupna vsebnost fenolov (TPC) in skupna vsebnost flavonoidov (TFC) sta bili določeni z metodo Folin–Ciocalteu oziroma metodo aluminijevega klorida, kemijska karakterizacija pa je bila opravljena z dvema kromatografskima analizama: plinsko kromatografijo–masno spektrometrijo (GC-MS) in tekočinsko kromatografijo visoke ločljivosti v kombinaciji z masno spektrometrijo (HPLC-MS). Glede bioloških aktivnosti je bila za oceno antioksidativne sposobnosti uporabljena metoda lovljenja radikalov z 2,2-difenil-1-picrilhidrazil (DPPH), medtem ko je bila za oceno protiglivične aktivnosti proti *Fusarium oxysporum f. sp. albedinis* uporabljena metoda mikroredčitev. Rezultati so pokazali znatno vsebnost fenolnih spojin, s TPC v razponu od 19,24 do 30,47 mg ekvivalenta galne kisline GAE/g in TFC od 11,09 do 17,98 mg ekvivalenta katehin CE/g suhega surovega ekstrakta. Analiza GC-MS je v vseh vzorcih identificirala 106 biološko aktivnih spojin, ki so bile v glavnem razvrščene med sladkorje (41 %), maščobne kisline (18 %) in organske kisline (7 %). HPLC-MS je identificirala in količinsko opredelila 25 polifenolov, med katerimi so bili glavne sestavine katehin (28,3 µg/g suhe teže), kaempferol (18,14 µg/g suhe teže) in izorhamnetin (10,98 µg/g suhe teže). Med testiranimi ekstrakti je hidroetanolni ekstrakt pokazal najvišjo antioksidativno aktivnost ($IC_{50}$ = 2,97 mg/mL), medtem ko je hidroacetonski ekstrakt pokazal najnižjo ($IC_{50}$ = 6 mg/mL). Vsi ekstrakti so zavirali rast *F. oxysporum*, z minimalno inhibitorno koncentracijo (MIC) od 1,20 do 3,20 mg/ml in minimalno fungicidno koncentracijo (MFC) od 3,20 do 5,80 mg/ml. Ti rezultati potrjujejo *L. sulphureus* kot obetaven vir bioaktivnih spojin z antioksidativnimi in protiglivičnimi lastnostmi, kar podpira njegovo potencialno uporabo na farmacevtskem in kmetijskem področju.

### Ključne besede

*Laetiporus sulphureus*; fenolni spojini; antioksidativna aktivnost; protiglivična aktivnost; *Fusarium oxysporum* f. sp. *albedinis*

# Introduction

Fungi are essential in ecosystems as decomposers, pathogens, or symbionts and hold growing potential in biotechnology, medicine, and agriculture. *Laetiporus sulphureus* (Bull.) Murrill (1920) [syn. *Polyporus sulphureus* (Bull.) Fr.] is an edible and medicinal fungal species belonging to the Fomitopsidaceae family, though some classifications place it in Laetiporaceae, within the order Polyporales. Commonly known as "chicken of the woods," "chicken polypore," or "sulphur polypore," this basidiomycete is distinguished by its bright-colored fruiting bodies, which typically emerge directly from the trunks of trees. *Laetiporus sulphureus* is a fungus that acts both as a weak parasite and as a saprotroph. It mainly attacks living but weakened trees (carob trees) through wounds, causing wood decay and brown rot. Once the tree dies or becomes too damaged, the fungus switches to feeding on the dead wood as a saprotroph (Venturella et al., 2020; Lear et al., 2009; Luangharn et al., 2014). This species is widely distributed across various continents, including Europe, Asia, Africa, and the Americas (Batlle et al., 1997; Sillo et al., 2018). Although widely studied for its medicinal and culinary uses, research on its chemical composition and bioactive properties, particularly when parasitizing economically important plants like the carob tree (*Ceratonia siliqua* L.), remains limited.

The carob tree, native to the Mediterranean region and widely cultivated for its edible pods, is of significant economic and ecological value, especially in Morocco. A long-standing agricultural staple, its pods are used in food production, animal feed, and as a source of locust bean gum for the food and pharmaceutical industries (El Kahkahi et al., 2014). Ecologically, the carob tree thrives in arid and semi-arid regions, with its deep root system improving soil fertility and preventing erosion, making it a key species for sustainable land management (Thomas et al., 2024). Biotic stressors, including parasitic fungi, increasingly threaten the health and productivity of carob trees. Among these, *L. sulphureus*, a wood-decaying fungus, has been identified as a pathogen affecting carob trees. Known to infect various tree species, it damages wood and compromises tree vitality (Castello et al., 2023). Although *L. sulphureus* has been studied in relation to other tree species, its interaction with carob trees remains relatively underexplored, and understanding its biochemical and functional properties in this context could reveal crucial insights into its role in carob tree health.

*Laetiporus sulphureus* is a basidiomycete fungus with a dual function, oscillating between pathogenicity and protection during the parasitic phase. It is an opportunistic pathogen for carob trees, while generating bioactive compounds that can affect the tree's resilience (Schmidt, 2006). Its interaction with the host is complex and is based on the tree's physiological state as well as environmental conditions.

Numerous studies have explored the chemical compositions and some biological activities of *L. sulphureus*. However, to date, no research has been conducted on this mushroom collected from Morocco. Additionally, no studies specifically report the antifungal effects of biomolecules extracted from *L. sulphureus* against *F. oxysporum*, a pathogenic agent responsible for *Fusarium* wilt in date palm trees in Morocco. This study has a twofold purpose: first, to analyze the chemical composition of various crude extracts of *L. sulphureus* obtained from carob trees in northeastern Morocco; and second, to assess their antioxidant potential using the DPPH assay, as well as their antifungal efficacy against *F. oxysporum* f. sp. *albedinis*, the causative agent of Bayoud disease in date palms.

# Materials and Methods

## Mushroom Material

The fruiting bodies of *Laetiporus sulphureus* were collected in autumn (November 2020 from a carob tree in northeastern Morocco (34° 41′ 12″ N, 1° 54′ 41″ W). In Morocco, the carob tree is a thermophilous, lowland species typically found at altitudes of up to 500–600 meters and was observed at 450 meters in this region. Identification of the collected specimen was based on the examination of key macroscopic and microscopic characteristics, following the identification keys (Malençon and Bertault, 1970; Régis, 1994). The species was further confirmed as *L. sulphureus* through comparison with authenticated reference materials available in scientific literature and online databases, as well as by consulting previous studies on this species (Castello et al., 2023). After collection, *L. sulphureus* fruit bodies were cleaned, weighed, cut into small pieces, left in the shade for 10 days to dry, and then ground into powder.

## Preparation of Crude Extracts

The mushroom extracts were prepared using six different

solvents: methanol, hydro-methanol, acetone, hydro-acetone, ethanol, and hydro-ethanol. The extraction process was performed following the method described by Erbiai et al. (2021), with slight modifications. One g of *L. sulphureus* powder was mixed with 10 mL of the respective solvent. The mixture was agitated using a magnetic stirrer at room temperature for 24 hours, followed by centrifugation for 20 minutes and filtration through Whatman No. 4 filter paper. The remaining residue was re-extracted twice using the same procedure to maximize compound recovery. The obtained extracts were concentrated using a rotary vacuum evaporator at 40°C and subsequently stored in the dark at −25°C for further analysis. The extraction yield was determined using the following formula:

$$\text{Yield (\%)} = \frac{\text{Extract weight after solvent evaporation}}{\text{Dry weight of fruiting body}} \times 100$$

## Bioactive Compound Contents

The total phenolic content (TPC), total flavonoid content (TFC), and total carotenoid content, including β-carotene (TβCC) and lycopene (TLC), were determined for each crude extract of *L. sulphureus* using spectrophotometric methods (Erbiai et al., 2021).

The TPCs were determined using the Folin–Ciocalteu method, which involved mixing one mL of each crude extract solution (1 mg/mL) with 5 mL of diluted Folin–Ciocalteu reagent (1/10) and 4 mL of 7.5% sodium carbonate. The tube was vortexed for 15 seconds and then allowed to rest for 30 minutes at 40 °C in the dark. The absorbance of the mixture was then measured at 760 nm in comparison to a blank. Gallic acid was used as the standard to create the calibration curve, and the results of multiple samples were expressed in milligrams of gallic acid equivalents (GAE) per gram of dry crude extract (DE).

TFCs were estimated using the aluminium chloride colorimetric technique. In this case, 1100 µL of distilled water, 75 µL of $NaNO_2$ (5%), and 250 µL of each diluted plant extract (1mg/mL) were combined. 75 µL of a 10% $AlCl_3$ solution was added five minutes later, and it was left to stand for six more minutes. Using a vortex device, 1000 µL of 4% NaOH solution was introduced and swirled. After allowing the combination to stand for a further 15 minutes, the absorbance at 510 nm was measured in comparison to the blank using catechin's standard flavonoid calibration curve. The results were represented as mg of catechin equivalents (CE)/g of DE.

The TβC and TLC content were determined following the method previously described by the authors (Nagata and Yamashita, 1992). A sample of 0.25 g of mushroom powder was mixed with 5 mL of a solvent hexane-acetone (4:6) for 1 minute. Spectrophotometric readings were then taken at wavelengths of 663 nm, 645 nm, 505 nm, and 453 nm. From these values, the contents of β-carotene and lycopene were calculated according to the following equations:

$$\beta - \text{Carotene (mg/100 ml)} = 0.216\ A663 - 0.304\ A505 + 0.452\ A453$$

$$\text{Lycopene (mg/100 ml)} = 0.0458\ A663 + 0.372\ A505 - 0.0806\ A453$$

## HPLC-MS Analysis of Individual Phenolic Compounds

*Extraction of Phenolic Compounds*: The extraction of phenolic compounds was carried out following the procedure described by Erbiai et al. (2021), with minor modifications. One gram of *L. sulphureus* powder was extracted using 20 mL of hydro-methanol (methanol/water, 80:20, v/v) at −20°C for 2 hours. The mixture was then centrifuged at 3000 rpm for 10 minutes, and the supernatant was filtered through Whatman No. 4 paper. The residue was subjected to a second extraction under the same conditions. To remove the methanol, the combined extracts were evaporated at 40°C under reduced pressure. The aqueous phase was then subjected to liquid-liquid extraction twice with 20 mL of diethyl ether and 20 mL of ethyl acetate. Afterward, anhydrous sodium sulfate was added to the combined organic phase, which was filtered through Whatman No. 4 paper and evaporated at 40°C until completely dry. Five milligrams of the extracted material were dissolved in 1 mL of methanol: water (80:20, v/v) and then filtered through a 0.22 µm disposable LC filter disc before HPLC analysis.

*Analysis of Phenolic Compounds by HPLC-MS*: Using comparable conditions and HPLC apparatus, the identification and quantification of various phenolic compounds present in *L. sulphureus* were performed following the method previously described by the authors (Erbiai et al., 2021). High-performance liquid chromatography-mass spectrometry (HPLC-MS) was employed to characterize the polyphenolic extract. Chromatographic separation was carried out using an Acclaim™ 120 reverse phase C18 column (3 µm, 150 × 4.6 mm) maintained at 35°C, with 280 nm as the optimal wavelength for detecting phenolic compounds.

The mobile phase consisted of 1% acetic acid (A) and 100% acetonitrile (B). The identification of the phenolic compounds was based on their UV-VIS spectra, mass spectra, and retention times, which were compared to commercial standards. Quantification of the peaks was achieved by computing the areas of the peaks at 280 nm and comparing them to calibration curves derived from the standard of each compound. The final results were expressed in µg per gram of dry weight (DW).

## Biomolecules Analysis by GC–MS of *L. Sulphureus*

Before GC-MS analysis, 10 mg of crude extract was derivatized by adding 100 µL of BSTFA (N, O-bis(trimethylsilyl) trifluoroacetamide) and 100 µL of anhydrous pyridine. The mixture was heated at 80°C for 25 minutes and then diluted with 200 µL of chloroform. The chemical composition of the six extracts of *L. sulphureus* was analyzed using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific). The GC system was fitted with a TG5-MS capillary column (60 m × 0.25 mm i.d.; 0.25 µm film thickness), which uses a nonpolar stationary phase (5% phenyl 95% dimethylpolysiloxane). Helium was used as the carrier gas at a flow rate of 1.2 mL/min, and the column temperature was programmed to increase from 40°C to 200°C at a rate of 6°C/min. The components were identified by comparing spectral data with the National Institute of Standards and Technology (NIST) databases and Kovats retention indices (RI) in relation to a homologous series of alkanes (C8–C20 and C21–C40). Data processing and acquisition were carried out using the NIST MS Search 2.2 Library 2014 and Thermo Xcalibur™ 2.2 SP1.48 software, respectively (Erbiai et al., 2023).

## Determination of the Antioxidant Activity

The antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, following the procedure described by Erbiai et al. (2021) with slight modifications. A volume of 0.5 mL of the sample at each concentration (ranging from 8 to 0.5 mg/mL, prepared by serial twofold dilution) was mixed with 2.7 mL of a methanol solution of DPPH. The mixture was then incubated in the dark for 30 minutes. Ascorbic acid (commercial standard) was used as the positive control, and methanol was used as the negative control. After incubation, the absorbance was measured at 517 nm using a UV–Vis spectrophotometer. The inhibition of DPPH free radicals was calculated using the following equation:

$$\% \text{ of inhibition} = \left[ \frac{(A_{DPPH} - A_{SAMPLE})}{A_{DPPH}} \right] \times 100$$

Where $A_{sample}$ represents the absorbance of the test extract and $A_{DPPH}$ represents the absorbance of the DPPH solution. A graph of the percentage of radical scavenging activity (RSA) against extract/standard concentration was used to determine the extract/standard concentration ($IC_{50}$) that decreased the RSA by 50%.

## Assessment of Antifungal Activity

*Fungal Pathogen and Growth Conditions:* The *Fusarium oxysporum* f. sp. *albedinis* (Foa) strain used in this study exhibits the same characteristics as the one previously studied by the authors in our laboratory (Ettakifi et al., 2023; Rahmouni et al., 2019). The pathogen was isolated from soil around the root, and infected leaves were collected from the same Errachidia region. The identification protocol followed in this study was previously outlined by the same authors. Following the isolation and identification of *Fusarium f. sp. albedinis* based on key morphological and cultural traits, notably the shape and size of macroconidia, pigmentation, and growth rate on agar media, pathogenicity assays were performed on tomato leaves and young date palm seedlings. The strain used in these tests was selected for its high pathogenicity and virulence, making it a representative candidate for evaluating host susceptibility and disease progression.

*Antifungal Assays:* All *L. sulphureus* extracts were dissolved in dimethyl sulfoxide (DMSO) and RPMI 1640 broth to achieve the required concentrations, ensuring that the DMSO concentration did not exceed 3%. Then, 60 µL of each concentration was added to each well of a U-bottomed 96-well plate, along with 60 µL of a *Fusarium oxysporum* (*Foa*) spore suspension at a final concentration of $10^4$ spores/mL. The 96-well microplates were incubated for 48 h at 25 °C. The spore suspension was prepared by diluting a stock suspension in physiological water with a drop of Tween 20. Carbendazim 50% was used as a reference positive control fungicide, while only DMSO and RPMI 1640 broth were used as negative controls.

*Determination of MIC and MFC Values:* The minimal

inhibitory concentration (MIC) was defined as the average of the concentrations in the wells that exhibited no turbidity after incubation. The minimum fungicidal concentration (MFC) was determined by inoculating 10 µL from the wells that showed no turbidity during the MIC assessment onto a Petri dish containing potato dextrose agar (PDA) medium. The MFC was considered the lowest concentration at which no visible fungal growth occurred on the PDA medium after incubation.

# Results and Discussion

## Extraction Yield and Bioactive Compounds Contents

To study the bioactive compounds present in the fruiting body of *L. sulphureus*, six different solvents were used for extraction: *L. sulphureus* methanol (LsME), acetone (LsAE), ethanol (LsEE), hydromethanol (LsHME), hydroacetone (LsHAE), and hydroethanol (LsHEE). The extraction yield and the determination of bioactive compounds, specifically total phenolic content (TPC), total flavonoid content (TFC), and carotenoid contents, are summarized in Table 1.

The extraction yield results indicated that the highest yields were obtained for the hydro-methanolic (*LsHME*, 14.5%) and hydro-acetonic (*LsHAE*, 13.5%) extracts. In contrast, the lowest yields were observed for the ethanolic (*LsEE*, 5%) and hydro-ethanolic (*LsHEE*, 7%) extracts. The methanolic (*LsME*) and acetonic (*LsAE*) crude extracts exhibited identical yields of 8.5% each (Table 1). Our findings demonstrated significantly higher extraction yields compared to the study by Olennikov et al. (2011), where the reported yields were as follows: 3.77% for water, 1.74% for polysaccharide, 0.61% for hexane, 0.51% for butanol, 0.30% for chloroform, and 0.20% for ethyl acetate.

The *LsME* showed the highest TPC at 30.47 mg GAE/g DE, while the *LsHEE* had the lowest at 19.24 mg GAE/g DE. These values exceed those reported by Kolundzic et al. (2016) and Lin and Lee (2020), who found 14.10 and 7.8 mg GAE/g DW, respectively. Karaman et al. (2010) observed even lower TPC values in methanolic (7.25 ± 0.01 mg/g DW) and chloroform extracts (0.33 ± 0.05 mg/g DW). Similarly, Acharya et al. (2016) reported a TPC of 21.08 ± 0.27 µg GAE/mg, which remains lower than our findings. Petrović et al. (2014) also documented lower TPC values in aqueous methanolic, ethanolic, and water extracts. However, Gabriela et al. (2016) and Olennikov et al. (2011) reported higher TPC levels in *L. sulphureus* extracts. Phenolic compounds, as key secondary metabolites, play a crucial role in the antioxidant properties of medicinal mushrooms.

Regarding TFC, the methanolic extract (*LsME*) of *L. sulphureus* showed the highest value, measuring 17.98 mg CE/g DE, whereas the hydro-ethanolic extract (*LsHEE*) had the lowest flavonoid content at 11.09 mg CE/g DE (Table 2). Olennikov et al. (2011) reported a higher TFC in ethyl acetate (90.77 ± 2.72 mg/g) and butanol extracts (27.14 ± 0.81 mg/g) compared to our tested extracts. However, the flavonoid content in chloroform (14.82 ± 0.44 mg/g) and water fractions (9.22 ± 0.27 mg/g) was lower than the values obtained in this study (Table 2). Additionally, Acharya et al. (2016) and Turkoglu et al. (2007) reported lower flavonoid contents compared to our findings, with values of 1.93 µg/mg and 14.2 µg/mg quercetin equivalent, respectively.

**Table 1.** Extraction Yield (%) and bioactive compounds of *L. sulphureus* extracts.

**Tabela 1.** Izkoristek ekstrakta (%) in bioaktivne sestavine izvlečkov *L. sulphureus*.

| Bioactive compounds | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
|---|---|---|---|---|---|---|
| Extractive Yield (%) | 8.5 ± 0.02 | 14.5 ± 0.05 | 8.5 ± 0.05 | 13.5 ± 0.05 | 5 ± 0.03 | 7 ± 0.03 |
| Total phenolic contents (mg GAE/g of DE) | 30.47 ± 0.08 | 26.16 ± 0.17 | 28.57 ± 0.08 | 20.11 ± 0.5 | 20.67 ± 0.79 | 19.24 ± 0.75 |
| Total flavonoid contents (mg CE/g of DE) | 17.98 ± 0.19 | 14.88 ± 0.39 | 17.18 ± 0.19 | 13.85 ± 0.86 | 12.70 ± 0.19 | 11.09 ± 0.19 |
| | Solvent hexane-acetone (4:6) | | | | | |
| β-carotene content (µg/mg DW) | 0.107 ± 0.01 | | | | | |
| Lycopene content (µg/mg DW) | 0.093 ± 0.00 | | | | | |

Values are expressed as the mean ± SD for three replicates. LsME: Methanolic extract; LsHME: hydromethanolic extract; LsAE: acetonic extract; LsHAE: hydroacetonic extract; LsEE: ethanolic extract; and LsHEE: hydroethanolic extract.

Regarding carotenoids, the β-carotene and lycopene contents in our extract were 0.107 µg/mg and 0.093 µg/mg dry weight (DW), respectively. In comparison, Acharya et al. (2016) reported lower β-carotene levels (0.05 ± 0.003 µg/mg) but significantly higher lycopene content (14.03 ± 0.92 µg/g DW). Kopylchuk et al. (2023) found β-carotene concentrations in ethanol extracts of *L. sulphureus* and other fungi ranging from 2.1 to 3.1 µg/g DW. Additionally, Barros et al. (2008) analyzed carotenoid profiles in wild mushrooms, reporting the highest values in *Tricholoma acerbum* with 75.48 ± 2.01 µg/g of β-carotene and 39.65 ± 1.33 µg/g of lycopene. These comparisons highlight the variability in carotenoid content depending on species, geographical origin, and extraction method.

## Characterization of Phenolic Compounds by HPLC-MS

Mushrooms are widely recognized for their richness in phenolic compounds, which possess a variety of biological activities, particularly antioxidant, antimicrobial, and anticancer properties (Chu et al., 2023). In this study, the phenolic compounds in the Moroccan mushroom *L. sulphureus* were characterized for the first time using Liquid Chromatography-Mass Spectrometry (LC-MS), a powerful analytical technique that is commonly employed for the identification and quantification of such compounds (Chu et al., 2023). The results are summarized in Figure 1 and Table 2. Figure 1 displays the chromatographic peaks of the phenolic compounds detected in the sample, which were identified and quantified. The detailed information, including the name, content, retention time, molecular weight, and corresponding m/z values, is presented in Table 2.

As presented in Table 2, catechin (28.3 µg/g DW) exhibited the highest concentration among all the detected polyphenols, followed by kaempferol (18.14 µg/g), isorhamnetin (10.98 µg/g), protocatechuic acid (9.83 µg/g), naringin (8.43 µg/g), apigenin (8.19 µg/g), and rutin (7.82 µg/g). Salicylic acid, luteolin, and caffeic acid were quantified at relatively lower concentrations (<1 µg/g). However, six polyphenolic compounds were not detected in the Moroccan *L. sulphureus*, namely gallic acid, vanillic acid, syringic acid, vanillin, apigenin 7-glucoside, and methyl paraben.

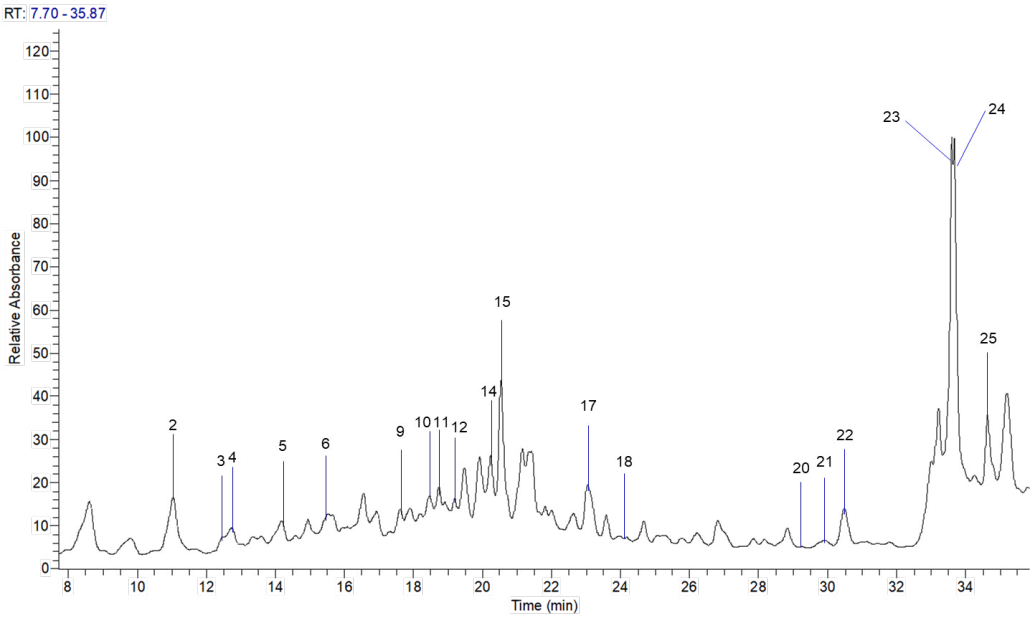


**Figure 1.** LC-MS chromatogram at 280 nm of individual polyphenols in *L. sulphureus* extract.

**Slika 1.** LC-MS kromatogram pri 280 nm posameznih polifenolov v ekstraktu *L. sulphureus*.

**Table 2.** Individual polyphenols in the Laetiporus sulphureus analysed by HPLC–MS in negative mode.

**Tabela 2.** Posamezni polifenoli v Laetiporus sulphureus, analizirani s HPLC–MS v negativnem načinu.

| Peak N°. | RT (min) | MW | Recorded m/z | Individual polyphenols | Content (µg/g dw) |
|---|---|---|---|---|---|
| 1 | - | - | - | Gallic acid | ND |
| 2 | 11.03 | 154.12 | 153.93 | Protocatechuic acid | 9.83 ± 0.02 |
| 3 | 12.48 | 354.31 | 353.05 | Chlorogenic acid | 4.68 ± 0.03 |
| 4 | 12.83 | 290.08 | 290.91 | Catechin | 28.3 ± 0.14 |
| 5 | 14.21 | 138.03 | 137.23 | p-Hydroxybenzoic acid | 4.63 ± 0.01 |
| 6 | 15.52 | 180.04 | 179.01 | Caffeic acid | 0.77 ± 0.01 |
| 7 | - | - | - | Vanillic acid | ND |
| 8 | - | - | - | Syringic acid | ND |
| 9 | 17.72 | 610.15 | 609.11 | Rutin | 7.82 ± 0.06 |
| 10 | 18.45 | 302.20 | 301.16 | Ellagic acid | 3.55 ± 0.03 |
| 11 | 18.74 | 448.37 | 447.05 | Luteolin 7-glucoside | 3.49 ± 0.04 |
| 12 | 19.19 | 164.05 | 164.17 | p-Coumaric acid | 0.81 ± 0.01 |
| 13 | - | - | - | Vanillin | ND |
| 14 | 19.89 | 194.19 | 192.38 | Ferulic acid | 3.11 ± 0.03 |
| 15 | 20.77 | 580.18 | 579.51 | Naringin | 8.43 ± 0.03 |
| 16 | - | - | - | Apigenin 7-glucoside | ND |
| 17 | 23.14 | 360.08 | 359.17 | Rosmarinic acid | 2.5 ± 0.02 |
| 18 | 24.14 | 138.03 | 137.16 | Salicylic acid | 0.54 ± 0.01 |
| 19 | - | - | - | Methyl paraben | ND |
| 20 | 29.07 | 286.23 | 285.79 | Luteolin | 0.47 ± 0.01 |
| 21 | 29.9 | 302.04 | 301.62 | Quercetin | 3.33 ± 0.03 |
| 22 | 30.49 | 148.05 | 147.55 | Cinnamic acid | 0.83 ± 0.01 |
| 23 | 33.5 | 270.05 | 269.29 | Apigenin | 8.19 ± 0.04 |
| 24 | 33.7 | 286.05 | 285.85 | Kaempferol | 18.14 ± 0.12 |
| 25 | 34.63 | 316.27 | 315.63 | Isorhamnetin | 10.98 ± 0.08 |

Each value is expressed as means ± SD of three independent measurements. Rt—retention time; MW — Molecular weight; ND—not detected; dw—dry weight.

The characterization of individual polyphenols of *L. sulphureus* has been the subject of several studies, revealing both consistencies and variations in compound presence and concentration (Nowacka et al., 2014; Jovanović et al., 2023; Olennikov et al., 2011; Çayan et al., 2020; Sulkowska-Ziaja et al., 2012; Woldegiorgis et al., 2014). The main compound detected in our mushroom, catechin, is a well-known flavonoid with strong antioxidant and antimicrobial properties (Latos et al., 2020). It has been previously identified in the study in a Russian sample with a concentration of 14.04 mg/g of ethyl acetate extract (Olennikov et al., 2011; Sułkowska-Ziaja et al., 2018) and was also found under hydrate form as the major compounds in the Turkish mushroom with a value of 4.01 µg/g (Çayan et al., 2020). Olennikov et al. (2011) determined seven polyphenols in the Russian mushroom, gallic acid being the most identified compound, which was not detected in our sample; this might be due to metabolic or ecological adaptations in *L. sulphureus*. In addition, the study from Serbia quantified seven phenols, namely vanillic acid, epicatechin, naringenin, ferulic acid, rosmarinic acid, p-hydroxybenzoic acid, and luteolin (Jovanović et al., 2023). Generally, the findings align with existing literature, as several individual phenolic compounds were detected and quantified in *L. sulphureus*. However, variations in the presence and concentrations of these compounds may be influenced by methodological, geographical, and environmental factors. This study highlights that Moroccan *L. sulphureus* is rich in phenolic compounds and exhibits significant diversity, positioning it as a promising and sustainable resource for the pharmaceutical industry.

## Chemical Composition by GC-MS

The chemical compositions of the six extracts of *L. sulphureus* were analyzed using GC-MS, and the results are presented in Tables 3-8. A total of 105 components were tentatively identified in all extracts, which were classified into five main groups for each extract: sugars (Table 3), fatty acids (Table 4), steroids (Table 5), organic acids (Table 6), alcohols (Table 7), and other compounds (Table 8). Generally, sugars were found to be the predominant chemical group in all the extracts, followed by fatty acids and steroids.

The methanolic extract of *L. sulphureus* contained 51 compounds (80.69% of the total extract), with the major components being adonitol (13.04%), 1,5-anhydrohexitol (12.47%), and silanol (11.3%). The hydromethanolic extract comprised 33 compounds (66.46%), dominated by ergosterol (10.45%) and glycerol (9.37%). The acetonic extract included 38 compounds (75.82%), with ergosterol (13.99%) and 11-octadecenoic acid (10.17%) as the most abundant. The hydroacetonic extract consisted of 51 compounds (100%), primarily xylitol (11.36%) and 1,5-anhydrohexitol (11.34%). The ethanolic extract contained 50 compounds (98.85%), with xylitol (12.86%) and 1,5-anhydrohexitol (10.56%) as the dominant components. The hydroethanolic extract identified 43 compounds (91.33%), with major compounds including 1-(p-methoxyphenyl)-2-phenylbenzimidazole (13.74%), xylitol (13.20%), 1,5-anhydrohexitol (7.59%), D-pinitol (6.15%), D-sorbitol (4.95%), and erythritol (3.51%) (Table 3-8).

Table 3 presents the sugar composition of the six extracts, highlighting their major biomolecular constituents. LaME contained 23 biomolecules, accounting for 58.32% of the extract, with 1,5-anhydrohexitol (12.47%) and adonitol (13.04%) as the predominant compounds. LsHME comprised 11 biomolecules (22.25%), primarily glycerol (9.37%) and erythritol (3.19%). LsAE included 15 sugars, with xylitol (9.05%) and glycerol (7.38%) as the dominant components. LsHAE was composed of 26 biomolecules, making up 56.96% of the extract, with xylitol (11.35%) and 1,5-anhydrohexitol (10.56%) as the major sugars. LsEE and LsHEE contained 21 (58.49%) and 25 (58.56%) biomolecules, respectively, with xylitol (12.86% and 11.35%) and 1,5-anhydrohexitol (7.59% and 10.56%) as the most abundant sugars.

The second most predominant group, fatty acids, is summarized in Table 4, with the highest percentages detected in LsAE (17.46%), LsHME (13.93%), LsHAE (13.57%), and LsEE (11.26%). Vaccenic acid was the primary fatty acid in five of the solvent extracts, including LsME (3.21%), LsHME (5.61%), LsAE (10.17%), LsEE (6.69%), and LsHEE (1.93%). In contrast, LsHAE was primarily composed of linoelaidic acid (10.30%). The prevalence of vaccenic acid in most extracts suggests its potential role in the bioactivity of these fractions, given its known biological properties (Fan et al., 2023; Lim et al., 2014).

Ergosterol was identified as the predominant steroid in all solvent extracts, with acetone (LsAE, 13.99%) and hydromethanol (LsHME, 10.45%) being the most effective solvents for its extraction (Table 5). These results demonstrate that *L. sulphureus* is a rich source of ergosterol, a vital sterol in fungal cell membranes known for its antifungal properties (Eliaš et al., 2024).

As shown in Table 6, organic acids were detected in significant percentages, with the hydroacetonic extract (LsHAE) containing the highest proportion (7.14%), while the hydroethanolic extract (LsHEE) had a significantly lower concentration (0.19%). Malic acid was the predominant organic acid in *L. sulphureus*, particularly in the LsAE (3.57%) and LsHAE (3.75%) extracts. This acid is known as a key metabolite involved in cellular energy production and may have biological activities, including antioxidant and antimicrobial activities (Wu et al., 2024; Rosa et al., 2009).

The alcohol group was detected in varying quantities, ranging from 0.31% to 14.31% (Table 7). Silanol (11.30%) was the predominant alcohol in LsME, while 1,2,3-butanetriol (6.82%) was the major alcohol in LsHME.

Table 8 lists the rest of the compound groups, which varied from 4.56% to 25.63%.

*L. sulphureus* extracts were rich in sugars, with hydroethanolic (58.56%), ethanolic (58.49%), and methanolic (58.32%) extracts showing the highest concentrations. Key sugars included 1,5-anhydrohexitol, xylitol, and glycerol. Wu et al. (2005) identified 40 volatile compounds, including acids, esters, alcohols, hydrocarbons, aldehydes, and ketones, with 9,12-octadecadienoic acid being highly abundant (1000–5000 µg/kg). In contrast, our study found this compound in low concentrations in hydromethanolic (0.65%), ethanolic (0.66%), and hydroethanolic (0.57%) extracts. Furthermore, Wu et al. (2005) did not detect 1,5-anhydrohexitol, xylitol, glycerol, ergosterol, silanol, or 11-octadecenoic acid, which were prominent in our study. Notably, silanol (11.3%) was found only in the methanolic extract and may enhance its antimicrobial activity (Togashi et al., 2007). Petrović et al. (2013) reported high concentrations of hydrocarbons, ketones, alcohols, and fatty acids in *L. sulphureus* extracts.

**Table 3.** Sugar compositions of derivatized extracts of *L. sulphureus* by GC-MS analysis.

**Tabela 3.** Sestava sladkorjev v derivatiziranih izvlečkih *L. sulphureus*, analizirana z GC-MS.

| Biomolecules name | Retention Index | Percentage of compositions in each extract of *L. sulphureus* | | | | | |
|---|---|---|---|---|---|---|---|
| | | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
| Glyceric acid | 1185.09 | 0.33 | - | 0.32 | 0.49 | 0.33 | 0.43 |
| Glycerol | 1136.02 | - | 9.37 | 7.38 | 7.55 | 7.32 | - |
| 2-Deoxypentofuranose | 1451.07 | - | 1.03 | - | - | - | - |
| 2-Deoxyribose | 1339.77 | 0.54 | - | - | 0.12 | 0.94 | - |
| Deoxyribopyranose | 1433.87 | 0.35 | 2.18 | 0.52 | 0.28 | 0.54 | 0.4 |
| D-Arabinopyranose | 1515.3 | 0.25 | - | - | - | 2.73 | - |
| D-Erythrofuranose | 1543.87 | 0.46 | 0.83 | 0.43 | - | 0.49 | - |
| Glucose | 1647.76 | 1.06 | - | 0.19 | - | - | - |
| L-Arabitol | 1550 | 1.97 | 0.93 | - | 1.04 | - | 3.29 |
| Xylitol | 1553.06 | 3.01 | - | 9.05 | 11.36 | 12.86 | 13.20 |
| Adonitol | 1567.34 | 13.04 | - | - | - | - | - |
| 1,4-Dioxane | 1544.38 | - | 1.12 | 0.36 | 0.73 | - | 0.52 |
| L-Threose | 1748.52 | - | - | - | 0.87 | - | - |
| Lactose | 2180.45 | 0.24 | - | - | - | - | 0.06 |
| D-Xylopyranose | 2405.45 | 0.41 | - | - | - | - | - |
| D-Allofuranose | 1818.4 | 0.55 | | | 0.92 | 0.89 | 0.56 |
| Galactopyranose | 1656.21 | - | - | - | 0.84 | - | - |
| Galactofuranose | 1655.72 | - | - | 0.53 | - | - | - |
| D-Galactopyranoside | 1672.63 | - | 0.56 | - | - | - | - |
| N-Acetyl-D-galactosamine | 1911.11 | 0.61 | - | - | 0.25 | 0.55 | 0.17 |
| D-Talofuranose | 1709.8 | 3.13 | - | - | 0.32 | - | 0.39 |
| D-Talopyranose | 1736.27 | - | - | 0.67 | 1.31 | - | 0.35 |
| D-Glucopyranose | 1810.44 | 3.12 | - | 2.02 | 1.61 | 1.93 | 3.37 |
| D-Ribofuranose | 1635.32 | - | - | 0.79 | 3.47 | - | 2.35 |
| D-Turanose | 1709.31 | - | - | - | - | - | 3.19 |
| D-Allopyranose | 1727.45 | - | - | - | - | 1.48 | 1.41 |
| L-Threitol | 1353.97 | 3.03 | 1.51 | - | 0.53 | 2.58 | 0.38 |
| 1,5-Anhydrohexitol | 1761.76 | 12.47 | 0.52 | 0.95 | 11.34 | 10.56 | 7.59 |
| D-Sorbitol | 1771.56 | 1.06 | - | - | 0.43 | - | 4.95 |
| Methyl-glucofuranoside | 1680.59 | 1.92 | - | - | 3.11 | 1.94 | 2.35 |
| Levoglucosan | 1736.27 | 1.58 | - | - | 2.13 | - | 0.26 |
| Myo-Inositol | 1786.27 | 2.39 | - | - | 2.08 | 2.66 | 2.28 |
| D-Lyxopyranose | 2183.5 | - | - | - | 0.18 | 0.36 | 0.23 |
| D-Lyxofuranose | 1371.02 | - | - | - | - | 0.35 | - |
| D-Mannose | 1648.25 | - | - | - | 0.69 | - | - |
| D-Mannitol | 1764.21 | - | 0.67 | 2.33 | - | 0.94 | 1 |
| 2-Mannobiose | 2476.76 | 0.44 | - | - | - | - | - |
| D-Pinitol | 1672.13 | 6.36 | - | 1.95 | 2.52 | 5.2 | 6.15 |
| Erythritol | 1556.63 | - | 3.79 | 1.74 | 2.66 | 2.67 | 3.51 |
| D-Galactose oxime | 1959.09 | - | - | - | - | - | 0.17 |
| Methyl galactoside | 1736.76 | - | - | - | 0.13 | 1.17 | - |
| Total | | 58.32 | 22.51 | 29.23 | 56.96 | 58.49 | 58.56 |

LsME: Methanolic extract; LsHME: hydromethanolic extract; LsAE: acetonic extract; LsHAE: hydroacetonic extract; LsEE: ethanolic extract, and LsHEE: hydroethanolic extract.

**Table 4.** Fatty acids of derivatized extracts of *L. sulphureus* by GC-MS analysis.

**Tabela 4.** Maščobne kisline derivatiziranih ekstraktov *L. sulphureus* z analizo GC-MS.

| Biomolecules name | Retention Index | Percentage of compositions in each extract of *L. sulphureus* | | | | | |
|---|---|---|---|---|---|---|---|
| | | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
| 2-Butenedioic acid | 1195.65 | 0.28 | - | - | 0.39 | 0.4 | 0.4 |
| Butanedioic acid | 1165.83 | - | - | 0.81 | 0.6 | 0.66 | - |
| Butanoic acid | 1217.04 | 0.11 | - | - | - | - | 0.05 |
| Stearic acid | 1986.78 | 0.32 | 0.78 | 0.47 | 0.3 | - | - |
| 9,12-Octadecadienoic acid | 1846.2 | - | 0.65 | - | - | 0.66 | 0.57 |
| Vaccenic acid | 1919.19 | 3.21 | 5.61 | 10.17 | - | 6.69 | 1.93 |
| Cis-13-Octadecenoic acid | 1919.69 | - | 4.66 | - | - | 1.56 | - |
| Stigmastanol | 2993.1 | 0.11 | 0.25 | 0.27 | 0.17 | 0.17 | - |
| Palmitic Acid | 1836.31 | 0.77 | 1.98 | 1.99 | 1.81 | 1.12 | - |
| Oleic Acid | 1919.69 | - | - | 2.73 | - | - | - |
| Oleanolic acid | 2677.63 | - | - | - | - | - | 0.11 |
| Linoelaidic acid | 1915.15 | - | - | 1.02 | 10.3 | - | 0.5 |
| Eicosanoic acid | 1963.63 | - | - | - | - | - | 0.1 |
| Petroselinic acid | 1983.83 | - | - | - | - | - | 0.81 |
| Dodecanoic acid | 3100 | - | - | - | - | - | 0.06 |
| Phosphoric acid | 1601.99 | - | - | - | 0.57 | - | - |
| Docosanoic acid | 3037.02 | - | - | - | 0.16 | - | - |
| Total | | 4.8 | 13.93 | 17.46 | 13.57 | 11.26 | 4.53 |

**Table 5.** Steroids of derivatized extracts of *L. sulphureus* by GC-MS analysis.

**Tabela 5.** Steroidi derivatiziranih izvlečkov *L. sulphureus* z analizo GC-MS.

| Biomolecules name | Retention Index | Percentage of compositions in each extract of *L. sulphureus* | | | | | |
|---|---|---|---|---|---|---|---|
| | | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
| Ergosterol | 2881.25 | 4.59 | 10.45 | 13.99 | 5.52 | 5.23 | 1.56 |
| Ergosta-7,22-dien-3-ol | 2897.53 | 0.67 | 1.42 | 1.64 | 0.99 | 0.93 | - |
| Ergosta-5,7,9,22-tetraen-3-ol | 2636.84 | - | 0.93 | 0.37 | 0.75 | 1.13 | 0.4 |
| Desmosterol | 2860.22 | 0.33 | - | - | - | - | - |
| Total | | 5.59 | 12.8 | 16 | 7.26 | 7.29 | 1.96 |

**Table 6.** Organic acids of derivatized extracts of *L. sulphureus* by GC-MS analysis.

**Tabela 6.** Organske kisline derivatiziranih ekstraktov *L. sulphureus* z analizo GC-MS.

| Biomolecules name | Retention Index | Percentage of compositions in each extract of *L. sulphureus* | | | | | |
|---|---|---|---|---|---|---|---|
| | | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
| Malic acid | 1331.81 | 0.21 | - | 3.57 | 3.75 | 1.09 | - |
| Gluconolactone | 1635.32 | 2.62 | - | - | 0.68 | - | - |
| Phthalic acid | 2270.11 | - | 0.49 | 0.22 | 1.44 | 0.55 | 0.19 |
| Fumaric acid | 1193.78 | - | - | 0.25 | - | 0.17 | - |
| Methylsuccinic acid | 1117.01 | - | - | - | 0.17 | 0.13 | - |
| L-Threonic acid | 1317.04 | - | - | - | 0.18 | 0.22 | - |
| Tartaric acid | 1339.77 | - | - | 0.24 | 0.92 | - | - |
| Total | | 2.83 | 0.49 | 4.28 | 7.14 | 2.16 | 0.19 |

**Table 7.** Alcohols of derivatized extracts of *L. sulphureus* by GC-MS analysis.

**Tabela 7.** Alkoholi derivatiziranih ekstraktov *L. sulphureus* z analizo GC-MS.

| Biomolecules name | Retention Index | Percentage of compositions in each extract of *L. sulphureus* | | | | | |
|---|---|---|---|---|---|---|---|
| | | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
| 1,3-Butanediol | 1050 | 0.43 | - | - | - | - | - |
| 1,2,3-Butanetriol | 1063.92 | 1.43 | 6.82 | 0.83 | 0.25 | 0.46 | 0.46 |
| 1,2,4-Butanetriol | 1212.65 | - | 0.18 | - | 0.06 | - | - |
| Diethylene glycol | 1169.56 | 0.2 | - | - | - | - | - |
| 10-Chloro-1-decanol | 1933.83 | 0.95 | - | - | - | - | - |
| Dulcitol | 1744.6 | - | - | 0.36 | - | - | - |
| L-Fucitol | 1760.29 | - | - | 3.1 | - | 1.39 | - |
| Silanol | 1138.5 | 11.3 | - | - | - | - | - |
| Total | | 14.31 | 7 | 4.29 | 0.31 | 1.85 | 0.46 |

**Table 8.** Other groups of derivatized extracts of *L. sulphureus* by GC-MS analysis.

**Tabela 8.** Druge skupine derivatiziranih izvlečkov *L. sulphureus* z analizo GC-MS.

| Biomolecules name | Retention Index | Percentage of compositions in each extract of *L. sulphureus* | | | | | |
|---|---|---|---|---|---|---|---|
| | | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE |
| 2-Monostearin | 2450.9 | 0.23 | - | 0.55 | - | - | - |
| 5-Methyluridine | 2461.21 | 0.17 | - | - | - | - | - |
| Palladium | 3239.14 | 0.11 | - | - | - | - | - |
| Trisiloxane | 1017.08 | 0.06 | 0.46 | - | - | - | - |
| 1-(Trimethylsilylmethyl) dimethylsilyl oxyhexane | 1190.06 | 0.18 | - | - | - | - | - |
| Silane | 1656.71 | - | - | - | - | 1.69 | - |
| 2,6-Bis(tert-butyl)phenol | 1378.4 | 0.42 | - | - | - | - | - |
| 1-Deoxypentitol | 1382.95 | 0.27 | - | - | 0.23 | 0.39 | 0.26 |
| D-Erythro-Pentitol | 1443.01 | 0.68 | - | 0.47 | 0.63 | 0.9 | 0.85 |
| Diglycerol | 1747.54 | 1.36 | - | - | - | - | - |
| 1,3-Propanediol | 1655.72 | 1.76 | - | - | - | - | - |
| Campesterol | 2952.21 | 0.67 | 0.58 | - | - | - | - |
| Lycophyl | 3071.36 | 0.23 | - | - | - | - | - |
| Pentasiloxane | 1047.46 | - | 1.2 | - | - | - | - |
| Phosphonic acid | 1055.06 | - | 0.56 | - | - | 0.88 | 2.53 |
| Uridine | 2201.72 | - | 0.09 | 0.12 | 0.16 | 0.5 | - |
| Benzenepropanoic acid | 3387.18 | - | | 1.63 | 1.32 | 0.09 | - |
| Glycerol monostearate | 2461.81 | - | 0.4 | 1.59 | 0.26 | 0.51 | - |
| 1-(p-Methoxyphenyl)-2-phenylbenzimidazole | 1138.5 | - | - | - | - | - | 13.74 |
| 2,2-Dimethyl-5-dioxolane-4-carboxaldehyd | 1688.55 | | 1.4 | - | - | - | - |
| Eicosane | 1969.69 | - | - | - | - | 0.38 | - |
| Octadecane | 2133.9 | - | - | - | - | 0.15 | - |
| Heptadecane | 3337.52 | - | - | 0.2 | - | 0.18 | - |
| Cholestane | 2438.78 | - | 0.16 | - | - | - | - |
| Deoxycholic acid | 3239.42 | - | - | - | 0.82 | 0.94 | - |
| Acetin | 1050.63 | - | 2.98 | - | - | 0.63 | 0.66 |
| 2-Oxiranemethanol | 1591.32 | - | 1.9 | - | - | - | - |
| Totals | | 6.47 | 9.73 | 4.56 | 14.76 | 17.8 | 25.63 |

Rapior et al. (2000) identified 26 volatile compounds in L. sulphureus, with major constituents being (Z)-3-methylcinnamaldehyde (27.5%), ethyl pentadecanoate (18.0%), 2-phenylethanol (6.4%), and benzaldehyde (4.0%). Comparisons suggest that L. sulphureus' chemical composition varies due to factors like species diversity, growth conditions, host, location, and age. Specific volatile compounds may also serve as chemotaxonomic markers for this species (Wu et al., 2005).

## Antioxidant Activity

The antioxidant activity of the different crude extracts of *L. sulphureus* was evaluated using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay. Table 9 presents the measured antioxidant activity of the six extracts. The $IC_{50}$ values of the samples were determined graphically based on their radical-scavenging activity inhibition (%) to provide the concentration required for 50% inhibition. A lower $IC_{50}$ value indicates a higher antiradical potential.

The antioxidant activity of *L. sulphureus* was evaluated, with the hydroethanolic extract showing the highest capacity ($IC_{50}$ = 2.97 ± 0.05 mg/mL), while the hydroacetonic extract demonstrated the lowest activity ($IC_{50}$ = 6 ± 0.04 mg/mL). Ascorbic acid, used as a positive control, exhibited stronger activity ($IC_{50}$ = 0.036 mg/mL). Petrović et al. (2013) reported an $IC_{50}$ of 6.80 ± 0.18 mg/mL for a methanolic extract, which is higher than the $IC_{50}$ observed for our methanolic extract ($IC_{50}$ = 4.16 ± 0.04 mg/mL) (Petrović et al., 2013). Kolundzic et al. (2016) and Acharya et al. (2016) found lower $IC_{50}$ values (0.66 ± 0.15 mg/mL and 0.11 ± 0.01 mg/mL, respectively). Petrović et al. (2014) reported higher $IC_{50}$ values for ethanolic extracts (107 ± 3.4 mg/mL and 77.6 ± 1.4 mg/mL) compared to our findings ($IC_{50}$ = 3.71 ± 0.06 mg/mL). Klaus et al. (2013) noted strong DPPH scavenging activity ($EC_{50}$ = 0.5 ± 0.2 mg/mL) for polysaccharides, while Olennikov et al. (2011) reported significant antioxidant capacity for ethyl acetate extracts (74.98 ± 1.49 µg/mL). Turkoglu et al. (2007) observed dose-dependent DPPH scavenging (14-86% inhibition at 100-800 µg/mL). Acharya et al. (2016) also reported strong DPPH activity ($EC_{50}$ = 0.11 ± 0.01 mg/mL) for a methanol extract. These results highlight the variability in antioxidant activity influenced by extraction methods and sources.

## Antifungal Activity

The results presented in Table 10 show the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the *L. sulphureus* crude extracts regarding antifungal activity. These values are compared with those of the synthetic fungicide Carbendazim 50%, and they are used as a reference. The hydroacetonic extract (LsHAE) exhibited the lowest MIC of 1.20 ± 0.40 mg/mL, making it the most effective among the extracts tested in inhibiting fungal growth. In comparison, the synthetic fungicide Carbendazim had a MIC of 2.00 mg/mL, demonstrating that the hydroacetonic extract is more effective than the fungicide at the specified concentration. The LsHAE extract also showed the lowest MFC (3.20 ± 0.40 mg/mL), suggesting that it is not only effective at inhibiting fungal growth but also at eliminating fungi at relatively low concentrations. In comparison, Carbendazim exhibited an MFC of 4.00 mg/mL, indicating that the LsHAE extract is slightly more potent in eliminating fungi. Furthermore, the LsHEE extract exhibited significant antifungal activity, with a MIC of 1.40 ± 0.49 mg/mL and an MFC of 5.80 ± 0.40 mg/mL. The LsEE, LsAE, LsME, and LsHME extracts also inhibited the growth of *Fusarium oxysporum*, but the MIC and MFC values were lower for LsEE and LsAE (MIC 2.00–2.20 mg/mL and MFC 4.60–4.20 mg/mL) compared to LsME and LsHME (MIC 2.60–3.20 mg/mL and MFC 3.40–4.00 mg/mL) (Table 10).

Pârvu et al. (2010) demonstrated that the hydroalcoholic extract exhibited antifungal activity against *Fusarium oxysporum*, with a MIC value of 160 µL/mL, observed within the concentration range of 40 to 160 µL/mL. Other studies have shown that ethyl acetate extracts exhibited moderate activity against *Fusarium roseum*, with an inhibition zone diameter of less than 10 mm. However, diethyl ether and

**Table 9.** IC50 (mg/mL) values of antioxidant activity of *L. sulphureus* extracts.

**Tabela 9.** Vrednosti IC50 (mg/mL) antioksidativne aktivnosti izvlečkov *L. sulphureus*.

| *L. sulphureus* extracts | LsME | LsHME | LsAE | LsHAE | LsEE | LsHEE | Ascorbic acid |
|---|---|---|---|---|---|---|---|
| DPPH assay: IC50 Values (mg/mL) | 4.16 ± 0.04 | 3.53 ± 0.06 | 5.82 ± 0.03 | 6.00 ± 0.04 | 3.71 ± 0.06 | 2.97 ± 0.05 | 0.036 ± 0.002 |

Results are expressed as the mean ± SD for three replicates. Ascorbic acid (commercial standard) was used as a positive control

**Table 10.** MIC and MFC values of the antifungal activity of *L. sulphureus* crude extracts and reference fungicide against *F. oxysporum* f. sp. *albedinis*.
**Tabela 10.** Vrednosti MIC in MFC protiglivične aktivnosti surovih izvlečkov *L. sulphureus* in referenčnega fungicida proti *F. oxysporum* f. sp. *albedinis*.

| ***L. sulphureus* extracts** | **LsME** | **LsHME** | **LsAE** | **LsHAE** | **LsEE** | **LsHEE** | **Carbendazim 50%** |
|---|---|---|---|---|---|---|---|
| MIC (mg/mL) | 2.60 ± 0.49 | 3.20 ± 0.40 | 2.20 ± 0.98 | 1.20 ± 0.40 | 2.00 ± 00 | 1.40 ± 0.49 | 2.00 ± 00 |
| MFC (mg/mL) | 3.40 ± 049 | 4.00 ± 0.63 | 4.20 ± 0.40 | 3.20 ± 0.40 | 4.60 ± 0.80 | 5.80 ± 0.40 | 4.00 ± 00 |

Results are expressed as the mean ± SD (n = 6). MIC: minimum inhibitory concentration and MFC: minimum fungicidal concentration. Carbendazim 50% is a fungicide that was used as a reference control.

dichloromethane extracts were ineffective in inhibiting the growth of all strains of *Fusarium oxysporum* and *Fusarium roseum* (Srivastava and Sharma, 2011).

Parroni et al. (2019) found that the culture filtrate of *Trametes versicolor* inhibited the growth of *Fusarium langsethiae* by 53.8% at a concentration of 0.04% w/v and by 61.4% at 0.08% w/v after 5 days. Sangeetha et al. (2018) reported that Ophiocordyceps sinensis volatile compounds inhibited *F. oxysporum* strains by 48-52%.

Plant extracts have also shown antifungal potential. Clémentine et al. (2019) found methanolic and dichloromethane extracts from five plants inhibited *F. oxysporum* at 10 mg/ mL. Soro et al. (2012) noted *Nicotiana tabacum* and *Chromolaena odorata* extracts inhibited fungal growth by 100% and 80%, respectively, at 60 g/L. *Erythrina senegalensis* and *Zanthoxylum zanthoxyloides* were effective against *F. oxysporum* forms. Bougandoura and Bendimerad. (2012) highlighted *Satureja calamintha* methanolic extract's efficacy due to its phenolic content.

Fungal antagonists like *Trichoderma* and endophytic fungi also show promise against *F. oxysporum* (Benhammouda et al., 2021; Wen et al., 2022). Thangaraj et al. (2021) found that *Coprinus cinereus* and *Ganoderma lucidum* VOCs inhibited *F. oxysporum* by 70% and 60.28%, respectively, with key compounds like Alfa copaene and trichloromethane identified. These findings underscore the potential of fungal and plant-derived compounds as antifungal agents.

Overall, the significant antifungal activity observed in this study can likely be attributed to the presence of phenolic compounds, fatty acids, organic acids, steroids, and other bioactive molecules. These findings suggest that *Laetiporus sulphureus* could be a promising source of natural antifungal compounds. However, further investigations into the detailed chemical composition of these extracts, along with studies on their mechanisms of action, are required to confirm these properties and evaluate their potential for clinical applications.

# Conclusions

This study demonstrated that the edible and medicinal mushroom *Laetiporus sulphureus*, collected from Morocco, is a rich source of biologically active compounds, including phenolic compounds, sugars, fatty acids, organic acids, amino acids, and terpenoids. These bioactive constituents exhibited significant antioxidant properties and effectively inhibited the growth of *Fusarium oxysporum*, the causal agent of palm tree disease. The chemical composition of *L. sulphureus* varied depending on the extraction solvent, influencing its biological activities. The hydroethanolic extract exhibited the highest antioxidant potential, while the hydroacetonic extract demonstrated the strongest antifungal activity. These findings highlight the potential of *L. sulphureus* as a valuable natural resource with application in food, medicinal, and agricultural sectors, warranting further investigation into its functional properties in nutrition, therapeutic efficacy, and biocontrol capabilities.

In the conclusion, the application of biomolecules against *Fusarium oxysporum* can be carried out directly on infected plants, by injection into irrigation water, or by considering the possibility of cultivating this parasitic/saprophytic fungus in date palm growing areas.

## Author Contributions

Conceptualization, A.M. and K.A.; methodology, K.A., E.H.E., A.B. and A.M.; software, E.H.E.; validation, R.S., H.M., M.E. and A.M.; formal analysis, K.A., T.F.Z., E.H.E. and J.C.G.E.d.S; investigation, K.A., T.F.Z.; resources, A.M.; data curation, E.H.E.; writing—original draft preparation, K.A., E.H.E., A.B. and A.M.; writing—review and editing, K.A., E.H.E., A.B. and A.M; visualization, R.S., H.M., M.E. and E.H.E; supervision, A.M., E.H.E., J.C.G.E.d.S. and A.M.; project administration, A.M.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

## Acknowledgement

We express our gratitude to Professor Joaquim C. G. Esteves da Silva for providing us with the opportunity to conduct the GC-MS and HPLC-MS analyses at the Faculty of Sciences, University of Porto, Portugal.

## Funding

Our research work was carried out in the Laboratory "Biology, Environment and Sustainable Development" at Abdelmalek Essaadi University-Tetouan within the Moroccan project supported by MESRSI and CNRST (PPR2–35/2016).

## Conflicts of Interest

The authors declare no conflicts of interest.

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ABS

Original Research

# Natural Biocidal Potential of *Thymus vulgaris* Essential Oil from the Souk Ahras Region, Algeria: Chemical Composition and Larvicidal Activity

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## Abstract

The aim of the present study was to determine the chemical composition of the essential oil (EO) extracted from the aerial parts of *Thymus vulgaris* collected in the Souk Ahras region of northeastern Algeria to evaluate their insecticidal activity against *Culex pipiens* larvae. The EO was obtained by hydrodistillation, and its chemical composition was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). Thirty-eight (38) components have been identified in the *T. vulgaris* EO, corresponding to 99.98% of the total composition, where p-cymene (21.32%), carvacrol (19.85%), γ-terpinene (19.12%) and thymol (18.78%) are the main compounds identified. The larvicidal activity was assessed against *Culex pipiens* fourth instar larvae following World Health Organization (WHO) guidelines, with $LC_{50}$ (lethal concentration for 50% mortality) and $LC_{90}$ (lethal concentration for 90% mortality) values determined at different exposure times. The EO showed strong larvicidal activity, with $LC_{50}$ values of 3.01 ppm, 2.48 ppm, and 2.32 ppm at 24, 48, and 72 hours, respectively. Additionally, the EO significantly inhibited acetylcholinesterase (AChE) activity and enhanced glutathione S-transferase (GST) activity, indicating involvement of neurotoxic and detoxification pathways. These findings underscore the effectiveness of *Thymus vulgaris* EO as a potent natural biocide against mosquito larvae and highlight its potential as a sustainable, eco-friendly alternative to synthetic insecticides.

## Keywords

*Thymus vulgaris* essential oils; Souk Ahras; larvicidal activity; *Culex pipiens*

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**Citation:** Radia, D., Assia, A., Ali, B., Louisa, K., Asma, N., Nadia, M., (2025). Natural Biocidal Potential of *Thymus vulgaris* Essential Oil from the Souk Ahras Region, Algeria: Chemical Composition and Larvicidal Activity. Acta Biologica Slovenica 68 (3)

**Received:** 08.11.2024 / **Accepted:** 30.05.2025 / **Published:** 20.06.2025

**https://doi.org/10.14720/abs.68.3.20144**

## Naravni biocidni potencial eteričnega olja pridobljenega iz vrste *Thymus vulgaris* iz regije Souk Ahras v Alžiriji: kemijska sestava in larvicidna aktivnost

**Izvleček**

Namen te študije je bil določitev kemijske sestave eteričnega olja (EO), pridobljenega iz nadzemnih delov rastline *Thymus vulgaris*, nabrane v regiji Souk Ahras v severovzhodni Alžiriji, ter ovrednotiti njegov insekticidni učinek na ličinke komarja *Culex pipiens*. Eterično olje je bilo pridobljeno s hidrodestilacijo, njegova kemijska sestava pa analizirana z uporabo plinske kromatografije z masno spektrometrijo (GC-MS). V EO *T. vulgaris* je bilo identificiranih osemintrideset (38) sestavin, kar predstavlja 99,98 % celotne sestave. Glavne spojine so bile p-cimen (21,32 %), karvakrol (19,85 %), γ-terpinen (19,12 %) in timol (18,78 %). Larvicidna aktivnost je bila ocenjena na ličinkah četrtega stadija komarja *Cx. pipiens* v skladu s smernicami Svetovne zdravstvene organizacije (WHO). Določene so bile vrednosti $LC_{50}$ (letalna koncentracija za 50 % smrtnosti) in $LC_{90}$ (letalna koncentracija za 90 % smrtnosti) za različne čase izpostavljenosti. EO je pokazalo močno larvicidno delovanje z vrednostmi $LC_{50}$ 3,01 ppm, 2,48 ppm in 2,32 ppm po 24, 48 in 72 urah. Poleg tega je EO bistveno zavrlo aktivnost acetilholinesteraze (AChE) in povečalo aktivnost glutation-S-transferaze (GST), kar nakazuje na vključevanje nevrotoksičnih in razstrupljevalnih poti. Ti rezultati izpostavljajo učinkovitost eteričnega olja *Thymus vulgaris* kot močnega naravnega biocida proti ličinkam komarjev ter njegov potencial kot trajnostne in okolju prijazne alternative sintetičnim insekticidom.

**Ključne besede**

Eterična olja *Thymus vulgaris*; Souk Ahras; larvicidna aktivnost; *Culex pipiens*

# Introduction

Annually, millions of people are affected by diseases transmitted by insects, such as dengue fever, malaria, filariasis, chikungunya, and West Nile virus, which continue to be critical global public health challenges (da Silva Ramos et al., 2019; Dahmana and Mediannikov, 2020). Particularly, *Culex pipiens* is a common mosquito species that spreads several types of diseases and causes serious health problems in humans as well as animals (Halimi et al., 2022). Traditional chemical control methods, especially synthetic larvicides, have led to the development of insecticide resistance, rising treatment costs, and increasing environmental contamination (Bharathithasan et al., 2021).

The urgent need to develop sustainable and eco-friendly mosquito control strategies has increased because of these challenges. In recent years, plant-derived compounds, particularly essential oils (EOs), have shown tremendous potential as natural larvicides due to their complex combinations of bioactive chemicals, safety, and biodegradability (Govindarajan et al., 2016; Giunti et al., 2023). Several essential oils from plants such as *Origanum vulgare, Cymbopogon citratus*, and Mentha species have shown significant larvicidal activity against mosquito vectors (de Oliveira et al., 2021; Badawy et al., 2018). Among them, *Thymus vulgaris L.* (common thyme or locally referred to as Zaatar), a perennial shrub belonging to the Lamiaceae family, is particularly noteworthy for its rich phytochemical profile and broad range of biological activities (Prasanth et al., 2014).

Native to the Mediterranean basin and widely distributed in northeastern Algeria, including the Souk Ahras region, *T. vulgaris* has been traditionally used for its medicinal, culinary, and aromatic properties. The essential oil extracted from its aerial parts is known to possess antimicrobial, anti-inflammatory, antioxidant, and insecticidal properties, attributed mainly to its high content of monoterpenes such as thymol and carvacrol (Amiri, 2012; Boukhatem et al., 2020). Despite the recognized bioactivity of *T. vulgaris EO*, few studies have thoroughly explored its chemical composition and larvicidal potential against *Culex pipiens* populations from Algeria under standardized conditions (Tine-Djebbar et al., 2016; Bouguerra et al., 2017).

Because essential oils provide a natural substitute for chemical insecticides that have fewer environmental risks, and because of their established bioactive qualities, particularly their insecticidal potential against various mosquito

species, the essential oil was selected for this investigation. According to Tongnuanchan and Benjakul (2014), the volatile bioactive components were preserved by using hydro distillation, a widely utilized method for extracting plant essential oils without the use of chemical solvents.

Previous studies have emphasized the importance of chemical composition in determining the larvicidal efficacy of essential oils (Sarma et al., 2019; Senthil-Nathan, 2020). The bioactivity of essential oils is greatly influenced by variables such as plant chemotype, place of origin, and extraction technique (Lalami et al., 2016). However, few comparative studies have focused on *T. vulgaris* from Algeria, where environmental conditions may lead to a unique chemical profile affecting its efficacy as a larvicide (Amiri, 2012; Boukhatem et al., 2020).

Therefore, the aim of this study is to characterize the chemical composition of the essential oil extracted from *Thymus vulgaris* collected in the Souk Ahras region and to evaluate its larvicidal activity against *Culex pipiens* larvae. Furthermore, this work investigates the biochemical mechanisms underlying its insecticidal effect by assessing the modulation of key enzymes such as acetylcholinesterase (AChE) and glutathione S-transferase (GST). The findings aim to contribute to the development of sustainable, plant-based vector control strategies.

# Materials and Methods

## Plant Collection

Fresh aerial parts of *Thymus vulgaris* were collected during the flowering period in June 2023 from the Ouled Moumen region in Souk Ahras (Northeastern Algeria), with a total weight of 1.5 kg. The plant was identified by Dr. Ketfi Louiza, a botanist at the University of Souk Ahras, Algeria. A voucher specimen (V. THv-5) was deposited in the herbarium of the Department of Botany at the Faculty of Science, Mohamed Cherif Messaadia University, Souk Ahras, Algeria.

## Extraction of Essential Oil

The fresh aerial parts of *Thymus vulgaris* were first thoroughly washed with water to remove any residues, and any damaged or unhealthy portions were discarded. The plant material was then laid out on paper and left to dry in the open air in the shade for 7 days. After drying, 200 g of the plant material was subjected to hydrodistillation. One litre of distilled water was added to the plant material, and the extraction process was carried out for 4 hours using a Clevenger-type apparatus. Following distillation, the essential oil was separated from the aqueous phase using a separating funnel. To remove any remaining water, the oil was dried with sodium sulphate (Sigma-Aldrich). Finally, the oil was collected and stored in an amber bottle at 4°C until further analysis for biological activity.

The essential oil yield was calculated by the ratio of the weight of the extracted oil to the dry weight of the plant material, expressed as a percentage. The yield (R) was calculated using the following formula:

$$R = \frac{PB}{PA} \cdot 100$$

R: Oil yield in %.
PA: Weight of the dry matter of the plant in g.
PB: Weight of the oil in g

## GC-MS Analysis

The components of the extracted essential oil were analyzed using a Shimadzu Gas Chromatography-Mass Spectrometry (GC-MS-QP2020) system. The system was equipped with a Rxi®-5ms fused capillary column (Phase: Crossbond® 5% diphenyl / 95% dimethyl polysiloxane), with dimensions of 30 m × 0.25 mm and a 0.25 µm film thickness. This column is equivalent to several others, including HP-1ms, HP-1msUI, DB-1ms, DB-5ms, and others, and is considered equivalent to USP G1, G2, and G38 phases.

For analysis, 0.5 µL of the sample was injected in split mode (1:80). The injector and detector were maintained at 250°C and 310°C, respectively. The column temperature program was as follows: it started at 50°C (held for 2 minutes), then increased to 310°C at a rate of 3°C/min, and was held at 310°C for 2 minutes. Helium (99.995% purity) was used as the carrier gas at a flow rate of 1 mL/min. The mass spectrometer was set to operate at an ionization voltage of 70 eV, with an ion source temperature of 200°C. Mass spectra were acquired in the range of 45-600 m/z.

## Identification of Essential Oil Components

The identification of the essential oil components was carried out by calculating the linear retention indices (LRI)

of each compound relative to a homologous series of n-alkanes (n-C8-C33). The calculated LRIs were compared with values from the literature (Adams, 2007; Babushok et al., 2011). Additionally, the mass spectra of the components were matched with spectra in the NIST (National Institute of Standards and Technology) and Wiley libraries (NIST17.lib, W11N17MAI, and FFNSC1.2.lib) for confirmation.

### Rearing of mosquito larvae of *Culex pipiens*

Toxicological assays were performed in the laboratory according to the standard larval susceptibility testing protocols established by the World Health Organization (WHO, 2005). Mass rearing was carried out in the laboratory using *Culex pipiens* eggs collected in the Annaba region, where it is widespread. After hatching, the larvae were reared in containers containing 200 mL of dechlorinated water maintained at a temperature of 25°C and a photoperiod of 14 hours of light. A diet consisting of a mixture of biscuit (75%) and dry yeast (25%) was provided daily. This rearing protocol was adapted from Boulares et al. (2023) with slight modifications.

### Larvicidal bioassays

The larvicidal activity was analysed according to the recommendations of the World Health Organisation (2005). The EO was dissolved in 1 ml of ethanol (Sigma-Aldrich) and then diluted in distilled water to obtain the desired concentrations. Control and treatment series with the different concentrations (1.25, 2.5 and 5 ppm) of EO were carried out in three replicates for each concentration in containers, each containing 25 newly exuviated larvae. After 24, 48 and 72 hours of exposure to different EO concentrations, mortality was recorded. Lethal concentrations (LC50 and LC90) and 95% confidence limits (95% CL) were calculated.

### Biomarker assay

The $LC_{50}$ and $LC_{90}$ concentrations determined at 24 hours were applied to fourth instar larvae, and their effects on acetylcholinesterase (AChE) activity were evaluated at specific time points: 24, 48, and 72 hours post-treatment. AChE activity was measured using acetylthiocholine as a substrate, according to the method described by Ellman et al. (1961). Briefly, larvae heads were homogenized in a detergent solution, and AChE activity was determined by monitoring the absorbance change at 412 nm over 20 minutes using UV spectrophotometry.

Glutathione S-transferase (GST) activity was assessed in the soluble fraction of the homogenate as the enzyme source, also at 24, 48, and 72 hours after treatment. The enzyme activity was measured using 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich) as a substrate, following the method of Habig et al. (1974). Absorbance changes were recorded at 340 nm, and GST activity was expressed as µM/min/mg of protein. Total protein content was quantified using the Bradford method (Bradford, 1976).

### Data analysis

For all experiments, the analyses were performed in triplicate, and the values are reported as the mean ± standard deviation (SD). ANOVA enabled us to assess whether the variations observed in mortality rates were statistically significant, with a p-value of less than 0.05, suggesting strong evidence against the null hypothesis. The results were analysed via the Student's test, with $\alpha = 0.05$. The analyses were carried out in IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, New York, USA).

## Results

Before presenting the specific findings, the results section provides a comprehensive overview of the chemical composition of *Thymus vulgaris* essential oil (EO), its larvicidal activity against *Culex pipiens* larvae, and the effects on key biochemical markers, including acetylcholinesterase (AChE) and glutathione S-transferase (GST) activities. The chemical analysis aims to characterize the EO constituents responsible for bioactivity, while the biological assays evaluate the larvicidal efficacy and elucidate possible mechanisms of action. This structured approach enables a thorough understanding of the potential of *T. vulgaris* EO as a natural larvicide.

### Chemical composition and yield

The yield of the essential oil of *T. vulgaris* extracted in our study was 1.18%, and GC/MS analysis of essential oil revealed the presence of thirty-eight (38) volatile components, which represent 99.98% of the overall chemical composition (Fig. 1, Table 1). p-cymene (21.32%), carvacrol

(19.85%), γ-terpinene (19.12%) and thymol (18.78%) were the main compounds identified in the essential oils of *Thymus*.

In our study, monoterpenes constituted a predominant proportion of this oil (93.75%) (Table 2), with the most abundant compounds belonging to the monoterpenes hydrocarbon fraction, of which 50.82% of the essential oils were represented by p-cymene (21.32%), γ-terpinene (19.12%) and α-terpinene (3.47 %), myrcene (1.64 %), α-thujene (1.46 %) and α-pinene (1.02 %) as the main constituents. The fraction of monoterpenes oxygenates (36.97%), in which carvacrol (19.85%) and thymol (18.78%) were the main components, had a low content of linalool (1.18%). On the other hand, the sesquiterpene fraction was lower (2.14%) with caryophyllene (2.11%) and β-sesquiphellandrene (2.60%) accompanied by other constituents at relatively low concentrations, such as 1-octen-3-ol (0.46%) and ketone (0.30%).

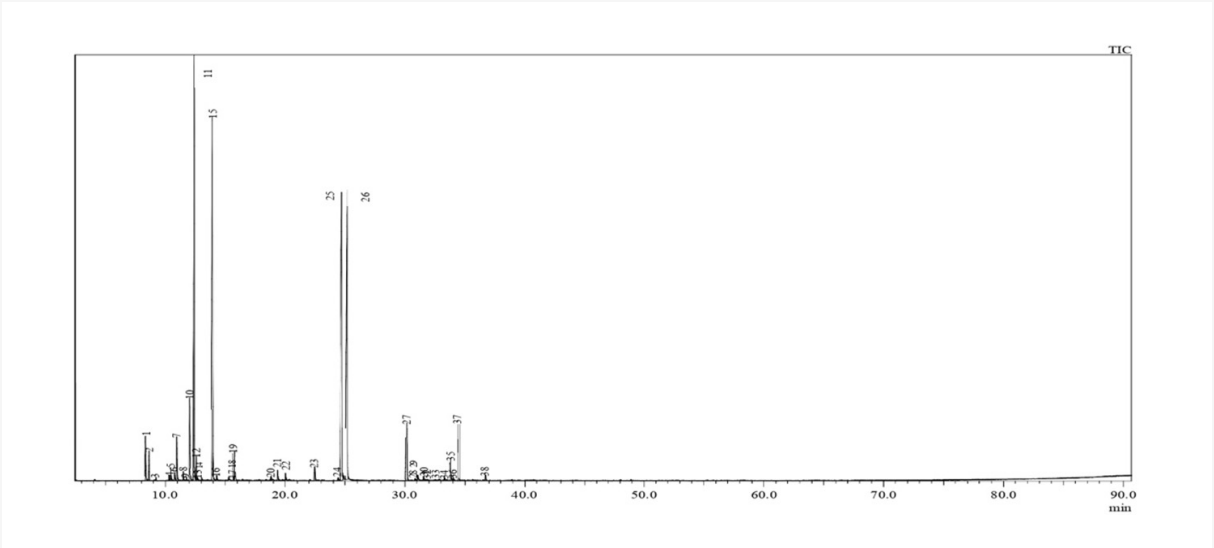


**Figure 1.** Chromatograms after GC–MS characterization of *Thymus vulgaris* essential oil.

**Slika 1.** Kromatogrami GC–MS analize eteričnega olja pridobljenega iz vrste *Thymus vulgaris*.

**Table 1.** Chemical composition of *Thymus vulgaris* essential oil from Souk Ahras, Algeria.

**Tabela 1.** Kemijska sestava eteričnega olja pridobljenega iz vrste *Thymus vulgaris* iz regije Souk Ahras, Alžirija.

| Peak | Retention Time (min) | Area% | Similarity | Index | Compounds |
|---|---|---|---|---|---|
| 1 | 8.389 | 1.46 | 97 | 921 | α-Thujene |
| 2 | 8.637 | 1.02 | 97 | 927 | α-Pinene |
| 3 | 9.204 | 0.13 | 97 | 941 | Camphene |
| 4 | 10.327 | 0.19 | 97 | 970 | β-Pinene |
| 5 | 10.449 | 0.46 | 97 | 973 | 1-Octen-3-ol |
| 6 | 10.757 | 0.30 | 96 | 981 | 3-Octanone |
| 7 | 10.953 | 1.64 | 96 | 985 | Myrcene |
| 8 | 11.501 | 0.34 | 95 | 999 | α-Phellandrene |
| 9 | 11.756 | 0.12 | 96 | 1005 | Δ-3-Carene |
| 10 | 12.027 | 3.47 | 97 | 1011 | α-Terpinene |

| | | | | | |
|---|---|---|---|---|---|
| 11 | 12.406 | 21.32 | 96 | 1019 | *p*-Cymene |
| 12 | 12.570 | 0.89 | 92 | 1023 | Limonene |
| 13 | 12.666 | 0.12 | 85 | 1025 | Eucalyptol |
| 14 | 13.026 | 0.10 | 97 | 1033 | β-(Z) Ocimene |
| 15 | 13.956 | 19.12 | 97 | 1054 | γ-Terpinene |
| 16 | 14.274 | 0.16 | 96 | 1061 | Sabinene hydrate <cis-> |
| 17 | 15.273 | 0.10 | 95 | 1083 | Terpinolene |
| 18 | 15.688 | 0.15 | 94 | 1092 | Sabinene hydrate <trans-> |
| 19 | 15.783 | 1.18 | 95 | 1094 | Linalool |
| 20 | 18.808 | 0.19 | 96 | 1160 | Borneol |
| 21 | 19.364 | 0.47 | 94 | 1172 | Terpinen-4-ol |
| 22 | 20.011 | 0.34 | 96 | 1185 | α-Terpineol |
| 23 | 22.456 | 0.61 | 96 | 1239 | Carvacrol methyl ether |
| 24 | 24.412 | 0.11 | 95 | 1282 | *p*-Cymen-7-ol |
| 25 | 24.733 | 18.78 | 97 | 1289 | Thymol |
| 26 | 25.160 | 19.85 | 96 | 1299 | Carvacrol |
| 27 | 30.156 | 2.74 | 96 | 1414 | Caryophyllene |
| 28 | 30.842 | 0.09 | 96 | 1431 | trans-α-Bergamotene |
| 29 | 31.057 | 0.25 | 88 | 1436 | Guaiacol |
| 30 | 31.567 | 0.14 | 97 | 1448 | α-Humulene |
| 31 | 31.725 | 0.06 | 92 | 1452 | β-(E)-Farnesene |
| 32 | 31.874 | 0.03 | 74 | 1456 | Alloaromadendrene |
| 33 | 32.528 | 0.04 | 94 | 1471 | γ-Muurolene |
| 34 | 33.295 | 0.10 | 92 | 1490 | Viridiflorene |
| 35 | 33.819 | 0.92 | 94 | 1503 | β-Bisabolene |
| 36 | 34.035 | 0.14 | 93 | 1508 | γ-Cadinene |
| 37 | 34.418 | 2.60 | 95 | 1518 | β-Sesquiphellandrene |
| 38 | 36.715 | 0.25 | 94 | 1577 | Caryophyllene oxide |

**Table 2.** Chemical classes of *Thymus vulgaris* essential oil from Souk Ahras, Algeria.

**Tabela 2.** Kemijska razvrstitev eteričnega olja pridobljenega iz vrste *Thymus vulgaris* iz regije Souk Ahras, Alžirija.

| Chemical class | Percentage (%) |
|---|---|
| Monoterpene hydrocarbons | 50.82 |
| Oxygenated monoterpenes | 42.93 |
| Sesquiterpene hydrocarbons | 4.77 |
| Oxygenated sesquiterpenes | 0.25 |
| Others | 0.25 |

## Larvicidal activity

The effects of *T. vulgaris* EO on *Cx. pipiens* L4 larvae were determined in terms of mortality levels observed during the exposure periods (24, 48 and 72 h). Mortality rates after 24 h exposure to *T. vulgaris* EO at concentrations of 1.25 ppm and five ppm ranged from 11% to 71%, respectively. Mortality rates of 79% and 83% were respectively recorded after 48 and 72 hours of exposure for the highest concentration (5 ppm). The analysis of variance of the data showed significant insecticidal activity ($p<0.001$) with a dose-response relationship (Fig. 2).

The estimated lethal concentrations from the equation of the regression line indicate variability in relation to the period of treatment: 24 hours (LC50: 3.01 and LC90: 8.16 ppm), 48 hours (LC50: 2.48 µg/let LC90: 6.38 ppm) and 72 hours (LC50: 2.32 and LC90: 5.91 ppm) (Table 3).

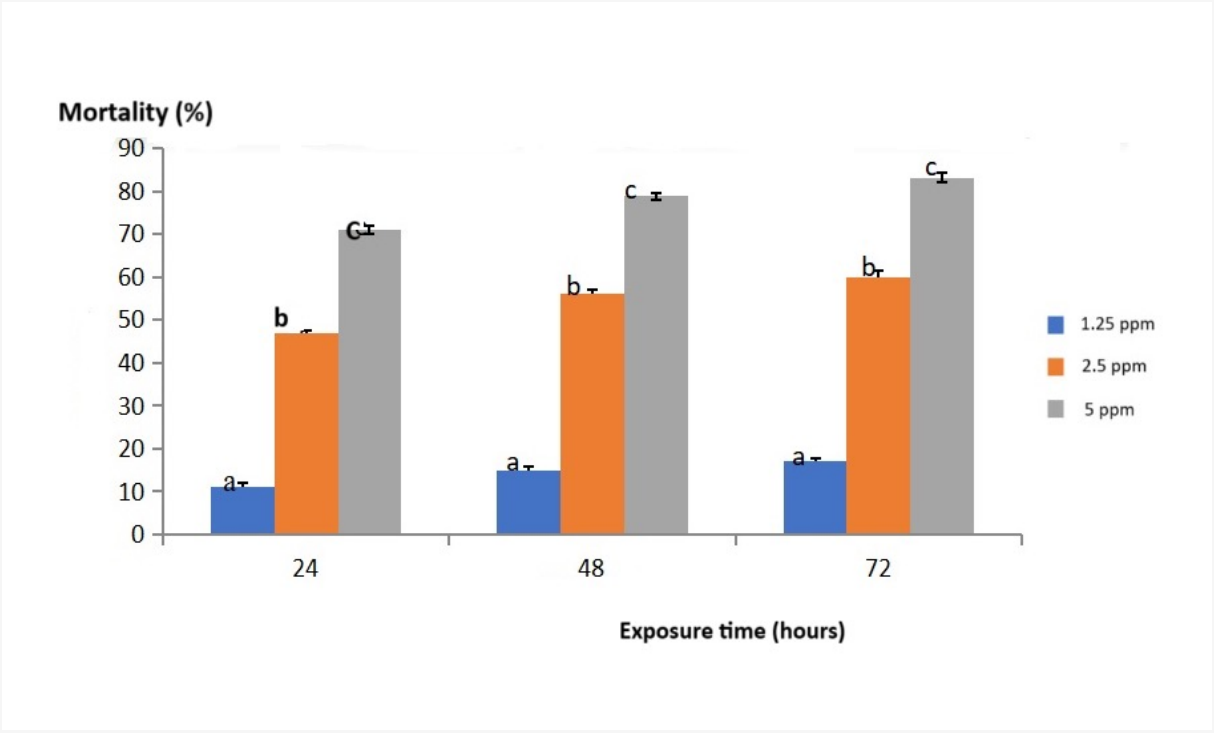


**Figure 2.** Effectiveness of *T. vulgaris* EO on fourth instar Cx. pipiens larvae: corrected mortality (m ± SD, n = 3 replicates each containing 25 larvae). For each line, values followed by different minuscule letters (a, b, c) are significantly different at $p < 0.001$.

**Slika 2.** Učinkovitost eteričnega olja pridobljenega iz vrste *T. vulgaris* proti ličinkam četrtega stadija vrste Cx. pipiens: smrtnost (m ± SD, n = 3 ponovitve, vsaka s 25 ličinkami). Za vsak stolpec so vrednosti, označene z različnimi malimi črkami (a, b, c), statistično značilno različne pri $p < 0,001$.

**Table 3.** Lethal concentrations (ppm, FL) of *T. vulgaris* essential oil against fourth instar larvae of *Cx. pipiens*.

**Tabela 3.** Letalna koncentracija (ppm, FL) eteričnega olja pridobljenega iz vrste *T. vulgaris* za ličinke četrtega stadija vrste *Cx. Pipiens*.

| Times (hours) | LC50 µg/l 95% FL | LC90 µg/l 95% FL | R2 | Regression equation |
|---|---|---|---|---|
| 24 | 3.01 | 8.16 | 0.97 | y = 3.1667x + 3.8483 |
| 48 | 2.48 | 6.38 | 0.93 | y = 2.3384x + 3.0187 |
| 72 | 2.32 | 5.91 | 0.93 | y = 2.3877x + 3.1879 |

### Specific acetylcholinesterase (AChE) activity

AChE activity was determined in the control and treated series. The comparison of means between the control and treated series at LC50 and LC90 showed that *T. vulgaris* EO induced a highly significant decrease (p=0.000) in the specific activity of AChE (µM/min/mg protein) at different periods (1, 2 and 3 days after treatment) as compared with the control series. The highest decrease in AChE activity was observed on day 3 in fourth instar larvae treated with CL90 and CL50. Two-way ANOVA showed significant effects of time ($F = 9.70$; $p < 0.0001$), treatment ($F = 59.22$; $p < 0.0001$) and time-treatment interaction ($F = 9.63$; $p < 0.0001$) (Table 4).

### Quantitation of the specific activity of glutathione S-transferase (GST)

The quantitative results of GST are presented in Table 5. EO of *T. vulgaris* induced a significant increase in GST-specific activity, and a comparison between the control series and those treated with LC50 and LC90 revealed a significant increase in GST-specific activity induced by EO of *T. vulgaris* after 24, 48 and 72 h of treatment. Two-way ANOVA indicated a highly significant effect of time ($F = 4.14$; $p<0.030$), treatment ($F = 145.03$; $p<0.0001$) and time-treatment interaction ($F = 32.45$; $p<0.001$) (Table 5).

## Discussion

The goal of this study was to characterize the chemical composition of an essential oil extracted from the common thyme plant collected in the Souk Ahras region of Algeria and to evaluate its larvicidal activity against *Culex pipiens* larvae. For this purpose, thyme oil was extracted by hydrodistillation and analyzed by GC-MS, and its bioactivity was evaluated by standardized larvicidal bioassays and enzymatic biomarker analyses. The essential oil yield

**Table 4.** Impact of *T. vulgaris* EO (LC50 and LC90) on specific AChE activity (µM/min/mg protein) in the whole body of fourth instar *Cx. pipiens*. Mean SD, three replicates, each containing 15 individuals. For each line, values followed by different minuscule letters are significantly different at $p < 0.05$, while for each series, values followed by different majuscule letters are significantly different at $p < 0.05$.

**Tabela 4.** Vpliv eteričnega olja pridobljenega iz vrste *T. vulgaris* (LC50 in LC90) na specifično aktivnost AChE (µM/min/mg beljakovin) v celotnem telesu četrtega stadija ličinke vrste *Cx. pipiens*. Srednje vrednosti SD, tri ponovitve, od katerih je vsaka vsebovala 15 osebkov. Vrednosti, ki si sledijo z različnimi malimi črkami, se pri vsaki seriji statistično značilno razlikujejo pri $p < 0,05$. Prav tako se vrednosti, ki si sledijo z različnimi velikimi črkami, pri vsaki seriji statistično značilno razlikujejo pri $p < 0,05$.

| | specific AChE activity (µM/min/mg protein) | | |
|---|---|---|---|
| **Exposure time (h)** | **Control** | **LC50** | **LC90** |
| 24 | $36.33\pm3.21^{a\,A}$ | $34.66\pm1.15^{a\,A}$ | $32.33\pm2.08^{a\,A}$ |
| 48 | $37.33\pm3.78^{a\,A}$ | $33.33\pm4.50^{a\,A}$ | $24.33\pm0.57^{b\,B}$ |
| 72 | $40.33\pm0.57^{a\,B}$ | $28.66\pm2.30^{b\,B}$ | $18.66\pm0.57^{c\,C}$ |

**Table 5.** Effect of *T. vulgaris* OE (LC50 and LC90) on the specific activity of GST (µM/min/mg of proteins) in the whole body of *Cx. pipiens* fourth instar larvae. Mean SD, three repetitions, each containing 15 individuals. For each line, values followed by different minuscule letters are significantly different at $p < 0.05$, while for each series, values followed by different majuscule letters are significantly different at $p < 0.05$.

**Tabela 5.** Vpliv eteričnega olja pridobljenega iz vrste *T. vulgaris* (LC50 in LC90) na specifično aktivnost GST (µM/min/mg beljakovin) v celotnem telesu četrtega stadija ličinke vrste *Cx. pipiens*. Srednje vrednosti SD, tri ponovitve, od katerih je vsaka vsebovala 15 osebkov. Vrednosti, ki si sledijo z različnimi malimi črkami, se pri vsaki seriji statistično značilno razlikujejo pri $p < 0,05$. Prav tako se vrednosti, ki si sledijo z različnimi velikimi črkami, pri vsaki seriji statistično značilno razlikujejo pri $p < 0,05$.

| | Specific GST activity (µM/min/mg protein) | | |
|---|---|---|---|
| **Exposure time (h)** | **Control** | **LC50** | **LC90** |
| 24 | 61.66±7.63 a A | 82.66±4.040a A | 133.33±11.54 b A |
| 48 | 64.66±17.89a A | 85.33±1.52b A | 156.66±20.18c B |
| 72 | 68.33±12.58a A | 110.66±7.63b | 160.66±9.23c B |

obtained (1.18%) is comparable to that reported by Hassani et al. (2017) for *T. vulgaris* from Blida, Algeria, using the same extraction technique. However, it is lower than the yields reported by Abdelli et al. (2017) in Tlemcen (2.2%) and Mostaganem (4.2%). These differences are likely due to a combination of biotic and abiotic factors, including environmental conditions, harvest time, plant chemotype, and variations in distillation parameters (Lalami et al., 2013). The chemical composition of the EO showed the dominance of four major monoterpenes: p-cymene, carvacrol, γ-terpinene, and thymol. While this profile aligns with common T. vulgaris chemotypes, quantitative differences are evident when compared with other studies. For instance, Boukhatem et al. (2020), analyzing a commercial oil from Bouira (Algeria), found carvacrol to be the overwhelmingly dominant compound (56.8%). In contrast, our sample showed more balanced proportions of p-cymene (21.32%) and carvacrol (19.85%), suggesting differences in plant origin (wild vs. cultivated), geographic source, and extraction methods.

Park et al. (2017) reported a chemotype from Korea dominated by thymol (40.04%) and p-cymene (29.97%), while Punya et al. (2019) described a γ-terpinene chemotype (32.60%) in India. Such variability in EO profiles is typically attributed to genetic differences, local adaptation, and environmental conditions (Amiri, 2012).

The predominance of monoterpenes in our oil (93.75%) is consistent with the chemotypic classifications proposed by Wesolowska and Jadczak (2019), which distinguish between phenolic (e.g., thymol, carvacrol) and non-phenolic (e.g., linalool, geraniol) chemotypes. The relatively high levels of p-cymene and γ-terpinene in our oil may reflect the specific pedoclimatic characteristics of Souk Ahras, such as soil composition, altitude, and microclimate. These factors likely influence the biosynthetic pathways responsible for secondary metabolite production.

This highlights the importance of thorough chemical characterization prior to biological evaluation, as EO composition directly affects bioactivity. Understanding regional chemotype expression is essential for optimizing the use of *T. vulgaris* EO in applications such as larvicide development, ensuring both efficacy and reproducibility. Researchers are continuously exploring innovative and environmentally sustainable methods for vector control (Bouaziz et al., 2025). In this context, understanding the insecticidal mechanisms of botanical extracts against mosquitoes is critical for the development of effective and ecologically friendly insect management strategies. According to Lalami et al. (2016), the insecticidal efficacy of plant-derived essential oils (EOs) is influenced by several factors, including the plant species, the age and specific parts of the plant used, as well as the physicochemical properties and chemical composition of the oils. These parameters play a key role in determining the bioactivity and overall potential of botanical insecticides.

The essential oil of *Thymus vulgaris* was specifically chosen for this study due to its superior insecticidal efficacy compared to other EOs. It has been recognized as a promising active ingredient for both existing and potential biopesticides (Pavela and Sedlák, 2018). In our study, we observed LC50 concentrations of 3.01 ppm at 24 hours, slightly decreasing to 2.48 ppm at 48 hours and 2.32 ppm at 72 hours. Similarly, LC90 values were 8.16 ppm at 24 hours, 6.38 ppm at 48 hours, and 5.91 ppm at 72 hours, suggesting that the insecticidal effect improved over time, with lower concentrations leading to 50% and 90% mortality as exposure duration increased.

In contrast, *T. vulgaris* essential oil from Blida (North Algeria), as reported by Bouguerra et al. (2017), showed significantly higher LC50 and LC90 values, indicating a less effective impact. Their study recorded LC50 values of 72.04 ppm at 24 hours, 68.61 ppm at 48 hours, and 62.12 ppm at 72 hours. LC90 values also increased, with 207.01 ppm at 24 hours, 190.54 ppm at 48 hours, and 169.82 ppm at 72 hours. Despite the improved efficacy over time, the concentrations required for substantial mortality were much higher than those in our study. These differences may be attributed to the chemical composition of the essential oil, which was not identical between studies. Although both studies used the same mosquito species (Culex pipiens), the oils differed in their major constituents, likely due to differences in plant origin, chemotype, or environmental growing conditions.

Supporting this notion, Baz et al. (2022) also reported strong larvicidal activity of *T. vulgaris* EO against *Cx. pipiens*, which they attributed to its rich monoterpene content. Beyond *Cx. pipiens*, the larvicidal potential of *T. vulgaris* EO, has been demonstrated against other mosquito species. For instance, de Oliveira et al. (2021) observed significant activity against *Aedes aegypti* larvae, with $LC_{50}$ values of 38.9 µg/mL and 37.7 µg/mL after 24 and 48 hours, respectively. Pavela (2009) reported an $LC_{50}$ of 33 µg/mL against *Cx. quinquefasciatus*. In addition, Huang et al. (2019) highlighted the potent larvicidal properties of individual EO

constituents, such as p-cymene and γ-terpinene, with $LC_{50}$ values of 17.8 µg/mL and 10.8 µg/mL, respectively, against *Ae. aegypti* larvae.

Taken together, these findings support the hypothesis that the larvicidal activity of *T. vulgaris* essential oil, particularly against *Culex pipiens*, is largely mediated by its major monoterpenic constituents, notably p-cymene, carvacrol, γ-terpinene, and thymol (Sheng et al., 2020). This chemical profile is consistent with the composition of the essential oil analyzed in our study, in which these four compounds were also identified as the predominant constituents.

Glutathione S-transferases (GSTs) are multifunctional enzymes involved in the detoxification of endogenous and xenobiotic substances. They also play roles in intracellular transport, hormone biosynthesis, and protection against oxidative stress (Enayati et al., 2005). In this study, we found that treating fourth instar *Cx. pipiens* larvae with *T. vulgaris* essential oil led to a significant increase in GST activity at different exposure times. This finding is similar to that of Bouguerra et al. (2018), who also observed activation of the detoxification system, marked by increased GST activity and decreased glutathione (GSH) levels. A similar increase in GST activity has been reported after treatment with essential oils of citrus fruits (Badawy et al., 2018) and *Mentha rotundifolia* (Kharoubi et al., 2020).

Acetylcholinesterase (AChE), α-carboxylesterase, and β-carboxylesterase are key enzymes in insects that detoxify xenobiotics. Resistance in insect populations is often associated with increased detoxification enzyme activity, which results from gene amplification or up-regulation after exposure to synthetic pesticides (Sengül Demirak and Canpolat, 2022). In our study, the enzymatic activity of AChE in fourth instar *Cx. pipiens* larvae treated with essential oil at two lethal concentrations (LC50 and LC90) revealed a significant decrease in AChE activity compared to the control. In contrast, Bouguerra et al. (2018) reported no impact on AChE activity in larvae treated with lower concentrations (LC25 and LC50). AChE is critical for the hydrolysis of acetylcholine, and its inhibition by certain pesticides can lead to pest mortality (Bhavya et al., 2018).

# Conclusions

The chemical composition of *Thymus vulgaris* essential oil, particularly its high content of monoterpenes such as p-cymene, carvacrol, γ-terpinene, and thymol, plays a significant role in its potent larvicidal activity. The essential oil demonstrated strong insecticidal effects, with low LC50 and LC90 values indicating its effectiveness in controlling mosquito larvae. These results highlight the potential of *T. vulgaris* essential oil as a promising natural larvicidal agent, with its chemical profile suggesting that its efficacy is closely linked to its specific monoterpene composition. This underscores the importance of the chemical composition in determining the biological activity of botanical extracts, making *T. vulgaris* a valuable candidate for further development in mosquito control strategies.

However, the study acknowledges potential limitations, such as variations in the EO's chemical composition due to environmental factors in the Souk-Ahras region, which may affect the consistency of its bioactivity. Further validation of the larvicidal activity under field conditions is also necessary to confirm the practical applicability of the EO as a mosquito control agent.

## Author Contributions

Methodology, D.R., B.A., and K.L.; validation, D.R., Y.Y., N.A. and Z.Z.; formal analysis, D.R., B.A., M.N., and K.L.; investigation, B.A.; resources, X.X.; data curation, D.R., B.A., and N.A.; writing—original draft preparation, D.R.; writing—review and editing, A.A., B.A.; visualization, D.R., B.A., and N.A. All authors have read and agreed to the published version of the manuscript.

## Acknowledgement

The authors acknowledged the Ministry of Research and Higher Education, the Republic of Algeria, the University of Mohamed Cherif Messaadia and PRFU (D00L02UN410120230004) for their generous provision of research facilities.

## Funding

This research received no external funding.

## Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Original Research

# Melatonin stimulates salt tolerance of soybean plants by modulating photosynthetic performance, osmoregulation, and the enzymatic antioxidant defence system

Mohamed El-sayed El-Awadi [1], Mervat Shamoon Sadak [1], Mahmoud Ahmed Khater [1], Mona Gergis Dawood [1]

## Abstract

Abiotic stress and climatic changes pose a serious threat to the agricultural sector. So, a pot experiment was performed at the National Research Centre greenhouse, Dokki, Cairo, Egypt, to investigate the influence of foliar spraying of melatonin (1.25, 2.50, 5.0 mM) on soybean plants exposed to irrigation with saline solution (6.25 dS/m). Results show that salinity stress (S1: 6.25 dS/m) significantly decreased vegetative growth parameters, photosynthetic pigments, indole acetic acid (IAA), membrane stability index (MSI), seed yield quantity and quality, accompanied by significant increases in carotenoid, phenolic content, osmolytes, and antioxidant enzymes activity. On the other hand, melatonin treatments at all levels showed the opposite trend of salinity stress. Melatonin treatments significantly improved the growth and productivity of soybean plants, either irrigated with tap water or saline solution (6.25 dS/m), relative to corresponding controls. The optimum treatment was 2.50 mM melatonin. Since, it increased plant dry weight by 2.04 times in plant irrigated with tap water and by 2.79 times in plant irrigated with saline solution (6.25 dS/m) as well as increased seed yield /plant by 116.13% in plant irrigated with tap water (0.23 dS/m) and by 74.64% in plant irrigated with saline solution (6.25 dS/m) relative to corresponding controls. In addition, melatonin treatments increased salinity tolerance of soybean plants via increasing total photosynthetic pigments, IAA, phenolic content, osmolytes contents (free amino acids, proline, total soluble sugar), membrane stability index, activity of antioxidant enzymes (superoxide dismutase, catalase, peroxide), quality of seed (oil, protein, and carbohydrate contents) and decreasing hydrogen peroxide, malondialdehyde, membrane leakage (ML) either in soybean plants irrigated with tap water (0.23 dS/m) or saline solution (6.25 dS/m). It could be recommended that melatonin treatments ameliorate the harmful effects of salinity on soybeans.

## Keywords

*Glycine max L.*, abiotic stress, salinity tolerance, N-acetyl-5-methoxytryptamine

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**Citation:** El-Awadi, M. E., Sadak, M. S., Khater, M. A., Dawood, M. G., (2025). Melatonin stimulates salt tolerance of soybean plants by modulating photosynthetic performance, osmoregulation, and the enzymatic antioxidant defence system. Acta Biologica Slovenica 68 (3)

**Received:** 15.01.2025 / **Accepted:** 10.07.2025 / **Published:** 11.07.2025

**https://doi.org/10.14720/abs.68.3.21635**

## Melatonin stimulira toleranco soje na sol s pomočjo moduliranja fotosinteze, osmoregulacije in encimskega antioksidativnega obrambnega sistema

**Izvleček**

Abiotski stres in podnebne spremembe predstavljajo resno grožnjo za kmetijski sektor. Zato je bil v rastlinjaku Nacionalnega raziskovalnega centra v Dokkiju v Kairu v Egiptu izveden poskus v lončkih, da bi preučili vpliv listnega škropiva melatonina (1,25; 2,50; 5,0 mM) na sojine rastline, izpostavljene namakanju s slano raztopino (6,25 dS/m). Rezultati kažejo, da je slanost (S1: 6,25 dS/m) znatno zmanjšala parametre vegetativne rasti, fotosintezne pigmente, indolocetno kislino (IAA), indeks stabilnosti membrane (MSI), količino in kakovost pridelka semen, hkrati pa se je znatno povečala vsebnost karotenoidov, fenolov, ozmolitov in aktivnost antioksidativnih encimov. Tretiranje z melatoninom je kazala nasprotne učinke na merjene parametre kot slanost. Tretiranje z melatoninom so znatno izboljšale rast in produktivnost sojinih rastlin, namakanih s vodovodno vodo ali slano raztopino (6,25 dS/m), v primerjavi z ustreznimi kontrolnimi skupinami. Optimalna koncentracija je bila 2,50 mM melatonina. S tem se je suha masa rastlin povečala za 2,04-krat pri rastlinah, namakanih z vodovodno vodo, in za 2,79-krat pri rastlinah namakanih s slano raztopino (6,25 dS/m), prav tako pa se je pridelek semen na rastlino povečal za 116,13 % pri rastlinah, namakanih z vodovodno vodo (0,23 dS/m) in za 74,64 % pri rastlinah, namakanih s slano raztopino (6,25 dS/m), v primerjavi z ustreznimi kontrolnimi skupinami. Poleg tega je melatonin povečal toleranco soje na slanost s povečanjem skupnih fotosinteznih pigmentov, IAA, fenolnih vsebin, ozmolitov (proste aminokisline, prolin, skupni raztopnljeni sladkorji), indeksa stabilnosti membrane, aktivnosti antioksidativnih encimov (superoksid dismutaza, katalaza, peroksid), kakovosti semen (vsebnost olja, beljakovin in ogljikovih hidratov) ter zmanjšanjem vodikovega peroksida, malondialdehid, puščanje membrane (ML) tako v sojinih rastlinah, namakanih z vodovodno vodo (0,23 dS/m), kot v rastlinah, namakanih s slano raztopino (6,25 dS/m). Priporočljivo je, da se melatonin uporablja za zmanjšanje škodljivega učinka slanosti na sojo.

**Ključne besede**

*Glycine max L.*, abiotski stres, toleranca na slanost, N-acetil-5-metoksitriptamin

# Introduction

Salinity stress has a deleterious impact on the productivity of various crops, especially in dry and semi-dry areas (Eisa et al., 2023). The farming land area affected by salinity stress will be expanded and continue to expand due to continuously unsustainable agricultural methods and changes in the climate (Qadir et al., 2014), posing a severe and obviously increasing threat to agricultural areas (Bui, 2013). Consequently, 30% of farmland will be impacted over the next 25 years, and up to 50% of farmland will be unsuitable by 2050 (Chandrasekaran et al., 2014). The principal physiological activities of many plants are altered by salinity-induced osmotic and ionic stress (Zhao et al., 2021), which includes sodium ion toxicity, metabolic disruptions, nutritional disorder, physiological water shortages, and photo-inhibition (Shabala and Cuin, 2007; Pandolfi et al., 2012). Further, salinity generated oxidative stress, characterised by the formation of reactive oxygen species (ROS) over a threshold level, resulting in multifaceted destruction to plant cells, including a reduction of photosynthesis (Gururani et al., 2015), increasing lipid peroxidation, electrolyte leakage, damage to nucleic acids(Tanveer and Shabala, 2018; Varghese et al., 2019) and, ultimately, programmed cell death (Colombage et al., 2023). So, plants evolved different mechanisms to overcome ROS-induced damage, including an enzymatic and non-enzymatic scavenging defence system for ROS detoxification as well as increased accumulation of osmoprotectants. The antioxidant enzyme system includes superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, and glutathione peroxidase. Whereas, the non-enzymatic defensive system includes ascorbic acid, vitamin E, glutathione, and flavonoids, as mentioned by Gu et al. (2022). Jagesh et al. (2010) demonstrated that compatible solutes significantly contribute to cellular adaptation under unfavourable envi-

ronmental conditions by elevating osmotic pressure in the cytoplasm, maintaining proteins and membranes, and preserving the relatively high water content essential for plant growth and cellular functions. Accumulation of compatable solutes as proline, free amino acids, soluble proteins, and soluble sugars, etc plays vital roles in keeping osmotic pressure and increase plant resistance to stress (Chen et al., 2020; Wang et al., 2022) by reducing cellular damage and facilitating water uptake (Abd El-Mageed et al., 2022).

Melatonin (N-acetyl-5-methoxytryptamine) is a derivative of the L-tryptophan amino acid (Imran et al., 2021; Awan et al., 2023) and has been identified as a growth regulator with auxin-like properties (Pelagio-Flores et al., 2012), and its growth-promoting efficacy is approximately 10 - 50% that of IAA (Hernández-Ruiz et al., 2004). Melatonin is a lipophilic and hydrophilic molecule that can be distributed in the cytoplasm and lipid membranes.Itis locatedon the hydrophilic side of the lipid bilayer. Melatonin molecules at low concentrations align themselves parallel to the lipid tail, and at high concentrations, parallel to the bilayer. This indicates that the arrangement of melatonin in lipid membranes depends on its concentration (Huang et al., 2019). Melatonin treatments stimulated plant tolerance to abiotic stresses by increasing levels of non-enzymatic antioxidants, boosting activity of antioxidant enzymes, and increasing theproduction of compatible solutes (Chen et al., 2020; Muhammad et al., 2023). Tan et al. (2007) noted that melatonin presumably acts as an indirect antioxidant and a direct scavenger of free radicals to protect plants from pollutants in the soil and water. According to their findings, melatonin molecules have the ability to scavenge up to 10 free radicals, in contrast to traditional antioxidants that can only neutralise one radical per molecule. Likewise, recently, Sachdev et al. (2021) mentioned that melatonin is recognised as one of the most powerful antioxidants compared to other non-enzymatic antioxidants, as tocopherols and ascorbates, that may be linked to the potential of melatonin to be effectively transferred into various cell compartments. So, melatonin treated plants were more tolerant to salinity stress because melatonin have the power to lower levels of malondialdehyde (MDA) content, hydrogen peroxide ($H_2O_2$) (Wei et al., 2021), reactive nitrogen species (RNS) and reactive oxygen species (ROS)(Wang et al., 2012), electrolyte leakage, and cell membrane damage (Arnao and Hernández-Ruiz, 2015; Shi et al., 2015). Additionally, melatonin effectively regulates seed germination, plant growth and productivity, and the antioxidant defence system in plants even under stressful conditions (Sadak, 2016 Xiao et al., 2019). Moreover, melatonin enhanced an array of stress-resilient adaptation reactions, as increasing the photosynthetic rate, stomatal conductance, transpiration rate, exudation of organic acid anions, phenolic compounds, mineral uptake, and regulation of the hormonal system, sugar metabolism and antioxidant enzyme activity (Imran et al., 2021; Colombage et al., 2023; Awan et al., 2023). In addition, under environmental stress conditions, application of melatonin boosted the crop yield by 18-20% (Yang et al., 2022) and improved fatty acid contents either under drought or salinity stress conditions (Wei et al., 2015; Zou et al., 2019). Similarly, seed priming with melatonin increased maize yield and its salt tolerance (Hussain et al., 2022).

Soybean (*Glycine max L.*) is one of the most significant and economical oilseed crops grown for protein (40%) and oil (20%) globally (Zhao et al., 2021). It provides around 60% of the edible oil and about 70% of the plant protein used by humans and animals worldwide (Liu et al., 2020). In addition, soybeans are a key source of food and feed due to their high economic value and nutritional value, such as minerals, nutrients, and vitamins (Yang et al., 2018). Global soybean production was anticipated to be 311.1 million tonnes in 2020 and is predicted to increase to 371.3 million tonnes in 2030, representing a 1.8% increase in growth rate from 2010 to 2020 (Siamabele, 2021). Soybean is highly sensitive to salinity stress, which may cause a 40% yield loss depending on the salinity level. Since salinity stress significantly decreased soybean plant growth, physiological, biochemical attributes and consequently the quality and quantity of the yielded seeds (Zhang et al., 2020; Imran et al., 2021).

So, this experiment aimed to investigate the possible physiological role of melatonin in reducing the deleterious influence of salinity stress on soybean production and quality.

# Materials and Methods

## Experimental design

During the summer season of 2023 and 2024, at the National Research Centre greenhouse in Dokki, Cairo, Egypt, a pot experiment was carried out to investigate the effects of melatonin foliar spraying on soybean plants that were irrigated with saline solution. Similar-sized and colored soybean seeds (Giza 22 cultivar) were chosen, sterilised for two minutes with 1% sodium hypochlorite, and

then washed under running water. Ten identical air-dried seeds were sown in plastic pots filled with seven kilograms of clay soil that had been homogenised in a 3:1 (v/v) ratio with sand. Soil's chemical and physical characteristics were identified. The soil's texture was clay-loam, with 1.4% coarse sand, 31.7% fine sand, 39.6% silt, and 27.3% clay. Its pH was 7.5, its organic matter content was 1.93%, its $CaCO_3$ content was 7.88%, and its accessible N, P, and K contents were 45.6, 7.8, and 415.0 mg $kg^{-1}$, respectively. Three days before planting, the soil was fertilized with the following amounts: (1) 800 kg $ha^{-1}$ of ammonium sulfate (20.5% N); (2) 240 kg $ha^{-1}$ of super phosphate (15% $P_2O_5$); and (3) 120 kg $ha^{-1}$ of potassium sulfate (48% $K_2O$), which was well mixed into each pot. In addition, the salt mixture used for chloride salinisation, as Stroganov's (1962), contained 10% $MgSO_4$, 1% $CaSO_4$,78% NaCl, 2% $MgCl_2$, and 9% $CaCO_3$. The component of specific anions and cations in the chloride mixture is 38% $Na^+$, 6% $Mg^{2+}$, 6% $Ca^{2+}$, 5% $SO^{2-}$, 40% $Cl^-$, and 5% $CO^{2-}$.

Regarding the cultivation of soybeans, the instructions provided by the Egyptian Ministry of Agriculture and Land Reclamation were adhered to. Soybean seedlings were thinned at 15 days from sowing, leaving four plants per pot. The experiment was done as a factorial with two factors and arranged in a completely randomised design with six replicates. The experiment comprised two categories. The first category includes irrigation with tap water or saline solution (6.25 dS/m). The second category includes foliar application of plants with different doses of melatonin (0, 1.25 mM, 2.5 mM and 5.0 mM) at 30 and 45 days from sowing. The irrigation with saline solution started 30 days after sowing.

## Data recorded

After 60 days from sowing, plant samples were collected to assess some morphological parameters, including plant height, number of branches and leaves/plant, plant dry weight, as well as some biochemical composition was determined in leaf tissues. At harvest, the number and weight of pods /plants, seed yield/plant, and 100-seed weight were measured on random samples of ten plants in each treatment. Some biochemical analyses of the yielded seeds were done (oil, carbohydrate, and protein content).

## Biochemical analysis

Photosynthetic pigments, such aschlorophyll a, chlorophyll b and carotenoid contents, were estimated in fresh leaf tissues according to Li and Chen (2015). Indole acetic acid content was measured in fresh leaf tissues usingthe method described by Gusmiaty et al.(2019).Total phenolic content was determined usingthe method mentioned by Gonzalez et al. (2003). Sorrequieta et al. (2009) method was used to determine free amino acids. Proline content was measured usingthe Kalsoom et al. (2016) method. The technique outlined by Chow and Landhausser (2004) was utilised to extract and determine the total soluble sugars (TSS).Membrane leakage was determined according to Vahala et al. (2003). The membrane leakage was determined according to the method recorded by Karimi et al. (2012). The Hydrogen peroxide level was estimated calorimetrically using the method reported by Yu et al. (2003). The level of lipid peroxidation was assessed by estimating the malondialdehyde (MDA) concentration following the method of Hodges et al. (1999). Enzyme extractions were carried out using Chen and Wang's (2006) methodology. Peroxidase (POX) (EC 1.11.1.7) activity was evaluated usingthe method mentioned by Kumar and Khan (1982). Superoxide dismutase (SOD) (EC 1.12.1.1) activity was assayed as by method mentioned by Chen and Wang (2006). Using spectrophotometry, the activity of catalase (CAT) (EC 1.11.1.6) was ascertained by tracking the absorbance drop at 240 nm (Chen and Wang, 2006). The oil of the soybean seeds was determined according to Das et al. (2002). Total carbohydrates were evaluated according to the method reported by Albalasmeh et al. (2013). Total nitrogen was determined using the micro-Kjeldahl method, as described by AOAC (Association of OfficialAgricultural Chemists) (2000). Total protein content was calculated by multiplying nitrogen per cent by 6.25.

## Genetically studies

Fresh and young leaves of soybean were used to isolate the genomic DNA using a plant genomic DNA extraction kit according to Sadak et al. (2023). A total of 10 ISSR primers were screened for the production of polymorphic products from all treated plants under study, but 5 ISSR primers ((GA) 6 CC, (CAC)3 GC, (GT)6CC, (CA)6 AC and (CT)8 GC) showed positive results. The primers were selected based on the PCR reactions that produced clear and repeatable bands.

Polymorphism % was calculated according to this equation:
Polymorphism percentage (PB%) = (UB + PB)/Total bands
Where UB=Number of unique bands, PB=Number of polymorphic bands

### Statistical analysis

All results in this study had analyses of variance (two-way ANOVA with interactions) computed using the analysis program SPSS v20.0 (SPSS Inc., Chicago, USA). Duncan's Multiple Range Test was applied to determine significant differences between means when ANOVA was significant at $P \leq 0.05$ and represented by the standard error (SE) of the means. In order to identify potential links between different parameters, a Pearson's correlation analysis was also conducted.

## Results

It was obvious from Figure 1 that salinity stress (S1: 6.25 dS/m) significantly declined plant height (cm), number per plant, leaves per plant, and plant dry weight (g) compared to those irrigated with tap water (S0:0.23 dS/m). On the other hand, foliar spraying of melatonin at all levels significantly increased all growth parameters under investigation relative to the control, and the most pronounced treatment was 2.50 mM melatonin.

Regarding interaction between salinity and melatonin, salinity stress significantly reduced plant height from 37.63 cm to 23.90 cm, branches number per plant from 3.67 to 2.00, leaves number per plant from 14.00 to 8.67 and dry weight from 3.94 to 1.87, showing a decline by 13.73%, 45.46%, 38.10%, and 52.64% respectively comparing with those irrigated with tap water (Table 1). On the other hand, foliar spraying of melatonin (1.25, 2.50 and 5.00 mM) significantly improved all the studied morphological features in soybean plants, either irrigated with tap water or saline solution,relative to their corresponding controls. Furthermore, 2.50 mM melatonin is the optimum treatment since it increased plant dry weight by 2.04 times in plants irrigated with tap water and by 2.79 times in plants irrigated with saline solution relative to the corresponding controls.

Regarding photosynthetic pigments, it was clear from Figure 2 that salinity stress (S1: 6.25 dS/m) significantly decreased chlorophyll a, chlorophyll b, and total photosynthetic pigments compared to those irrigated with tap water (S0). Salinity stress significantly increased carotenoid content compared to the control. On the other hand, melatonin treatments (M1, M2, and M3) significantly increased all components of photosynthetic pigments relative to control (M0). The highest significant increases in total photosynthetic pigments were achieved by 2.50 mM melatonin.

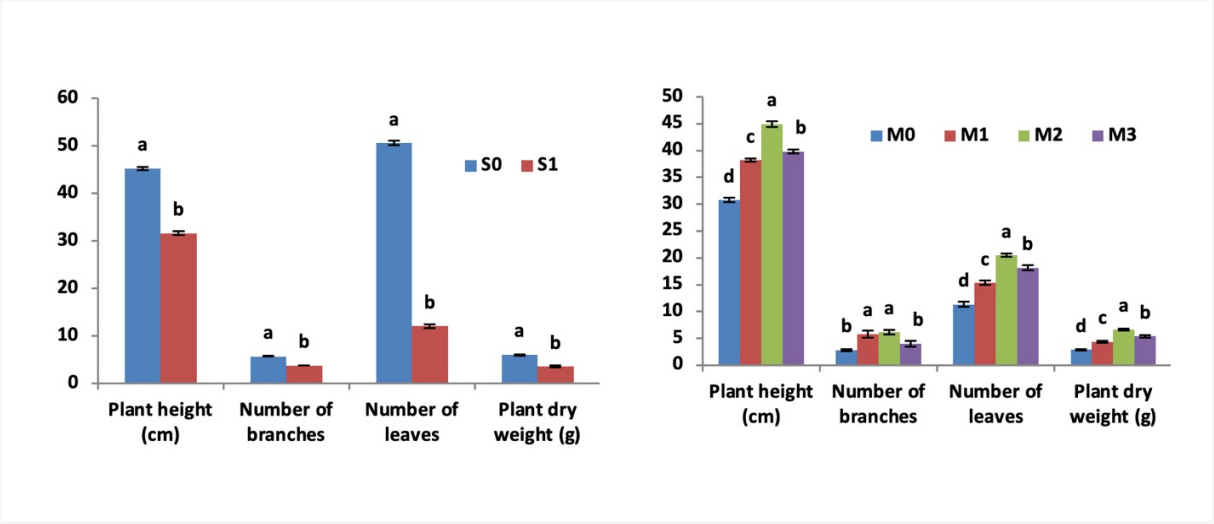


**Figure 1.** Impact of either salinity or melatonin on some growth parameters of soybean plants (S0: 0.23dS/m; S2: 6.25 dS/m; M0: 0mM; M1:1.25 mM; M2: 2.50 mM; and M3: 5.00 mM). Means followed by the same letters for each parameter are not significantly different by the Duncan test ($P \leq 0.05$) and represented by ±SE.

**Slika 1.** Vpliv slanosti ali melatonina na nekatere parametre rasti sojinih rastlin (S0: 0,23 dS/m; S2: 6,25 dS/m; M0: 0 mM; M1: 1,25 mM; M2: 2,50 mM; in M3: 5,00 mM). Povprečja, ki jim za vsak parameter sledijo enake črke, se po Duncanovem testu ($P \leq 0,05$) med seboj ne razlikujejo pomembno in so predstavljena z ±SE.

**Table 1.** Impact of interaction between salinity stress and melatonin on some growth parameters of soybean plants.

**Tabela 1.** Vpliv interakcije med stresom zaradi slanosti in melatoninom na nekatere parametre rasti sojinih rastlin.

| Treatments | | Plant height (cm) | Number of branches | Number of leaves | Plant dry weight (g) |
|---|---|---|---|---|---|
| Salinity (dS/m) | Melatonine (mM) | | | | |
| S0 | M0 | 37.63±1.17 c | 3.66±1.15cd | 14.00±1.00de | 3.94±0.72d |
| | M1 | 44.77±1.10 b | 6.66±1.52ab | 19.00±1.00c | 5.64±0.57c |
| | M2 | 52.60±1.18 a | 7.33±1.15a | 25.66±0.57a | 8.04±0.62a |
| | M3 | 46.06±0.46 b | 5.00±1.00bc | 23.66±1.15b | 6.66±0.39b |
| S1 | M0 | 23.90±0.36 bf | 2.00±1.00d | 8.66±0.57g | 1.86±0.19f |
| | M1 | 31.66±0.90 e | 5.00±1.00bc | 11.66±0.58f | 3.13±0.45e |
| | M2 | 37.26±1.52 c | 5.00±1.00bc | 15.33±1.15d | 5.22±0.48c |
| | M3 | 33.56±1.05 d | 3.00±1.00d | 12.66±0.57ef | 4.13±0.50d |

S0: 0.23 dS/m ; S1: 6.25 dS/m M0:0 mM; M1:1.25 mM; M2: 2.50mM; and M3: 5.00 mM;. Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE

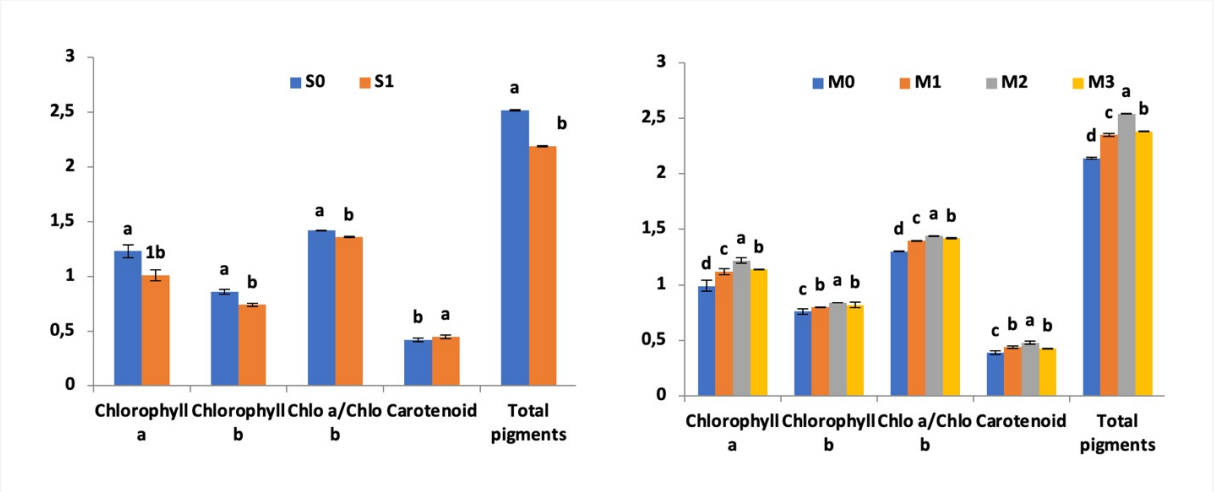


**Figure 2.** Impact of either salinity or melatonin on photosynthetic pigments(mg/g fresh leaf tissues)of soybean plants. (S0: 0.23 dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50 mM; and M3: 5.00 mM). Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE.

**Slika 2.** Vpliv slanosti ali melatonina na fotosintetne pigmente (mg/g svežega listnega tkiva) sojinih rastlin. (S0: 0,23 dS/m in S2: 6,25 dS/m; M0: 0 mM; M1: 1,25 mM; M2: 2,50 mM; in M3: 5,00 mM). Povprečja, ki jim sledijo iste črke za vsak parameter, se po Duncanovem testu (P ≤ 0,05) med seboj ne razlikujejo pomembno in so predstavljena z ±SE.

Regarding interaction between salinity and melatonin, it was noted that salinity stress (S1: 6.25 dS/m) caused a substantial decreasein the effect on photosynthetic pigment content (chlorophyll a, chlorophyll b, Chl a / Chl b, and total photosynthetic pigments) comparedto control plants (Table 2). The percentages of reductions were 22.32%, 15.85%, 7.41%, and 14.28% respectively, compared with unstressed controls. Meanwhile, it significantly increased carotenoid content by 10.81% relative to the control. On the other hand, foliar application with different levels of melatonin (1.25, 2.50, and 5.00 mM) significantly improved different photosynthetic pigment constituents either in plants irrigated with tap water or saline solution relative to corresponding controls (Table 2). Melatonin application at 2.5 mM was noticed as the most effective treatment over the control and the other melatonin treatments. This increased total

photosynthetic pigments by 17.31% in plants irrigated with tap water and by 19.69% in plants irrigated with saline solution relative to the corresponding controls.

Further, salinity stress (S1: 6.25dS/m) significantly decreased IAA, accompanied by significant increases in phenolic content and osmolytes (free amino acids, proline, and total soluble sugars) relative to those irrigated with tap water (Figure 3). On the other hand, all applied melatonin treatments significantly boosted IAA, phenolic content, free amino acids, proline, and total soluble sugars relative to the control. The optimum treatment was melatonin at 2.50 mM.

**Table 2.** Impact of interaction between salinity stress and melatonin on photosynthetic pigments of soybean plants.

**Tabela 2.** Vpliv interakcije med stresom zaradi slanosti in melatoninom na fotosintetske pigmente sojinih rastlin.

| Treatments | | Chlorophyll a | Chlorophyll b | Chlorophyll a/ Chlorophyll b | Carotenoid | Total photosynthetic pigments |
|---|---|---|---|---|---|---|
| Salinity (dS/m) | Melatonine (mM) | (mg/g fresh leaf tissues) | | | | |
| S0 | M0 | 1.12±0.007c | 0.82±0.010c | 1.35±0.006e | 0.37±0.006g | 2.31±0.011d |
| | M1 | 1.23±0.004b | 0.86±3.000b | 1.43±0.004b | 0.42±0.000e | 2.52±0.004b |
| | M2 | 1.33±0.005a | 0.90±0.000a | 1.47±0.006a | 0.47±0.003b | 2.71±0.010a |
| | M3 | 1.24±0.002b | 0.86±0.006b | 1.43±0.009bc | 0.42±0.000e | 2.53±0.008b |
| S1 | M0 | 0.87±0.004f | 0.69±0.002f | 1.25±0.003f | 0.41±0.012f | 1.98±0.006g |
| | M1 | 1.00±0.021e | 0.73±0.011e | 1.37±0.008d | 0.45±0.002c | 2.19±0.034f |
| | M2 | 1.11±0.000c | 0.78±0.000d | 1.41±0.002c | 0.48±0.005a | 2.37±0.004c |
| | M3 | 1.04±0.006d | 0.73±0.002e | 1.42±0.013bc | 0.44±0.000d | 2.22±0.004e |

M0:0 mM; M1:1.25 mM; M2: 2.50; and M3: 5.00 mM; S0: 0.23 dS/m and S2: 6.25 dS/m. Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE

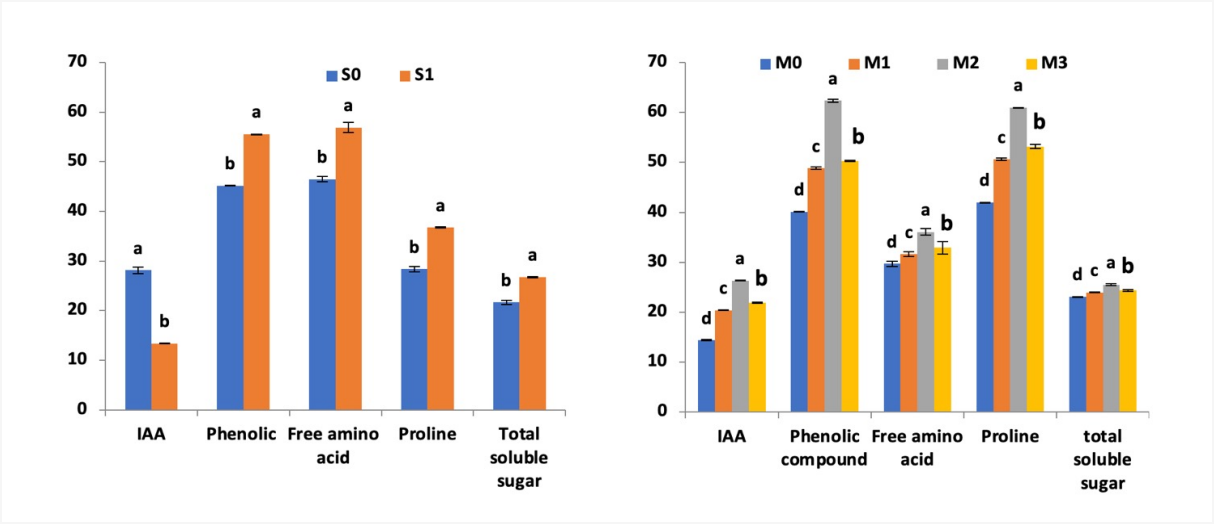


**Figure 3.** Impact of either salinity or melatonin on IAA (µg/g fresh leaf tissue), phenolic content (mg/100 g dry leaf tissue), and some osmolytes (mg/g dry leaf tissue) of leaf tissues of the soybean plant. (S0: 0.23dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50mM; and M3: 5.00 mM). Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE.

**Slika 3.** Vpliv slanosti ali melatonina na IAA (µg/g svežega listnega tkiva), vsebnost fenolov (mg/100 g suhega listnega tkiva) in nekaterih osmolytov (mg/g suhega listnega tkiva) v listnem tkivu soje. (S0: 0,23 dS/m in S2: 6,25 dS/m; M0: 0 mM; M1: 1,25 mM; M2: 2,50 mM; in M3: 5,00 mM). Povprečja, ki jim sledijo iste črke za vsak parameter, se po Duncanovem testu (P ≤ 0,05) med seboj ne razlikujejo bistveno in so predstavljena z ±SE.

Regarding interaction between salinity and melatonin treatments, it was noted that salinity significantly reduced IAA, and significantly increased phenolic content, free amino acid, proline, and total soluble sugar. The percentagedecrease of IAA reached 57.26%. Whereas, increases in phenolic content, free amino acids, proline and total soluble sugar reached 38.92%, 34.03%, 24.43% and 14.382% respectively in soybean leaves as compared to unstressed plants (S0). In addition, foliar application with the different levels of melatonin (1.25, 2.50, and 5.00 mM) caused significant increments in IAA, phenolic content, free amino acid, proline, and total soluble sugar, either in plants irrigated with tap water or saline solution (Table 3). Clearly, 2.50 mM melatonin was the superior treatment in improving IAA, phenolic content and different osmolyte contents, either in soybean plants irrigated with tap water or saline solution (Table 3).

Figure 4 shows that salinity stress (S1: 6.25 dS/m) significantly increased ML, $H_2O_2$, MDA, SOD, CAT, POX and significantly decreased MSI in leaf tissues of soybean plants relative to those irrigated with tap water. Whereas, melatonin treatments (1.25, 2.50 and 5.00 mM) significantly reduced MSI, $H_2O_2$, and MDA accompanied by significant increases in SOD, CAT, POX relative to control.

Regarding interaction between salinity stress and melatonin application, it was noted that salinity stress caused marked increases in ML, $H_2O_2$, MDA, SOD, CAT, POX relative to those irrigated with tap water.These increases were 13.59%, 57.37%, 32.55%, 51.61%, 35.65%, 67.78% respectively. Salinity stress significantly decreased MSI by 18.09% relative to the control. On the other hand, melatonin treatments caused non significant decreases in ML, and significant decreases in $H_2O_2$ and MDA accompanied by significant increases in MSI, SOD, CAT, POX in soybean plants either irrigated with tap water or saline solution relative to corresponding controls. Obviously, 2.50 mM melatonin is the optimum treatment in decreasing ROS, $H_2O_2$, MDA, ML and increasing MSI and antioxidant enzymes activity (SOD, CAT, POX).

It was obvious from Figure 5 that salinity stress significantly decreased seed yield quantity (number of pods/plant, weight of pods and seeds /plant and 100 seeds weight) and quality (oil, protein and carbohydrate content) relative to those irrigated with tap water. On the opposite trend, melatonin treatments at all levels significantly increased seed yield quality and quantity.

Table 5 shows the interaction impact between salinity and melatonin treatments on the quality and quantity of the soybean seeds.The irrigation of soybean plants with 6.25 dS/m saline solution caused significant decreases in the pod number/plant and weight of pods and seeds/plant and 100 seeds weight (g), in addition to oil%, protein%

**Table 3.** Impact of interaction between salinity stress and melatonin on IAA, phenolic content, and some osmolytes of leaf tissues of soybean plants.
**Tabela 3.** Vpliv interakcije med stresom zaradi slanosti in melatoninom na IAA, vsebnost fenolov in nekatere osmolite v listnih tkivih sojinih rastlin.

| Treatments | | IAA | Phenolic content | Osmolytes | | |
|---|---|---|---|---|---|---|
| | | | | Free amino acid | Proline | Total soluble sugar |
| Salinity (dS/m) | Melatonine (mM) | µg/g fresh leaf tissue | mg/100 g dry leaf tissue | mg/100 g dry leaf tissue | | mg/g dry leaf tissue |
| S0 | M0 | 20.17±0.48 | 33.55±0.07g | 35.90±0.24f | 26.48±1.25h | 21.57±0.06 |
| | M1 | 27.46±0.47 | 42.76±0.40f | 46.29±0.66e | 27.41±0.50g | 21.39±0.23 |
| | M2 | 34.72±0.06 | 58.86±0.50b | 55.14±0.48c | 31.11±0.54e | 21.73±0.11 |
| | M3 | 30.14±0.48 | 45.55±0.19e | 48.59±0.05d | 28.55±0.86f | 22.09±0.46 |
| S1 | M0 | 08.62±0.00 | 46.61±0.24d | 48.12±0.02d | 32.95±1.10d | 24.65±0.02 |
| | M1 | 13.29±0.33 | 54.92±0.22c | 54.95±0.03c | 35.75±1.13c | 26.51±0.00 |
| | M2 | 17.97±0.01 | 65.80±0.58a | 66.81±0.45a | 41.05±1.86a | 29.27±0.57 |
| | M3 | 13.62±0.005 | 54.89±0.25c | 57.72±1.23b | 37.21±1.48b | 26.68±0.16 |
| LSD at 0.05 | | 0.55 | 0.60 | 0.99 | 0.69 | 0.49 |

S0: 0.23dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50mM; and M3: 5.00 mM;.Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE

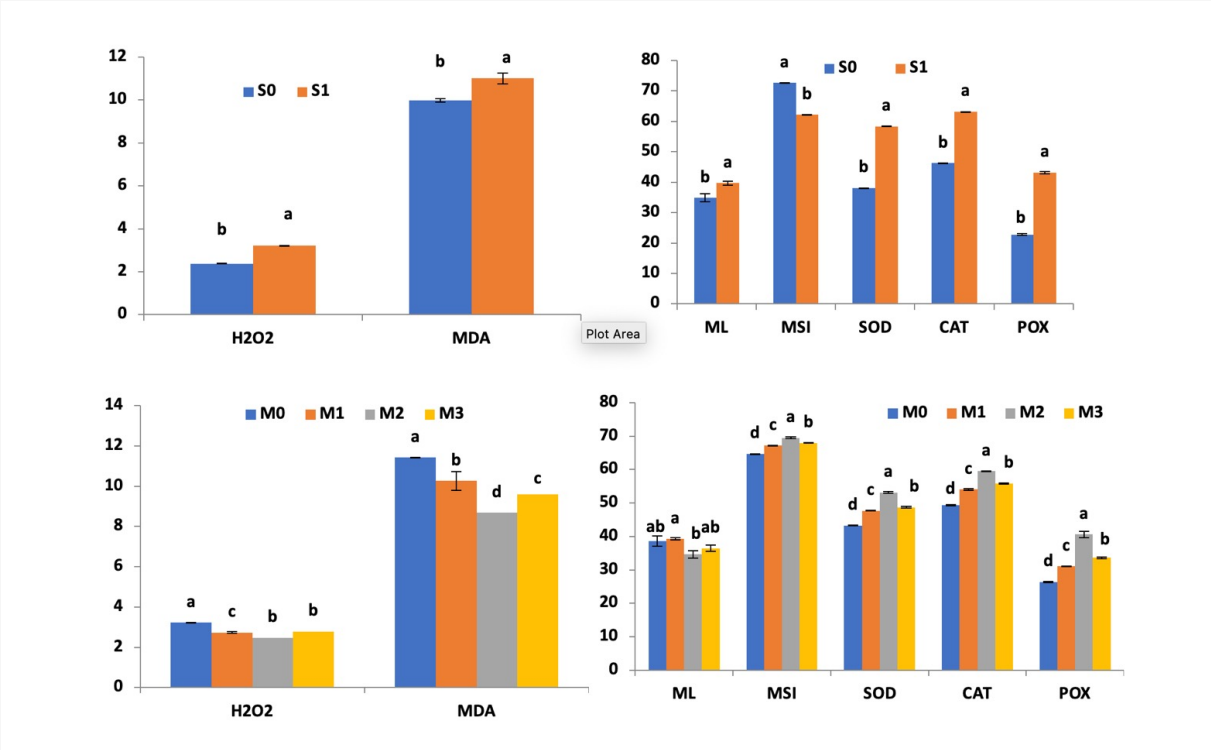


**Figure 4.** Impact of either salinity or melatonin on membrane leakage (ML%), membrane stability (MSI%), $H_2O_2$, MDA (nmole/g fresh leaf tissue), some antioxidant activity (SOD, CAT, POX) of leaf tissues of soybean plants. S0: 0.23 dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50 mM; and M3: 5.00 mM; Means followed by the same letters for each parameter are not significantly different by Duncan test (P≤ 0.05) and represented by ±SE.

**Slika 4.** Vpliv slanosti ali melatonina na prepustnost membrane (ML%), stabilnost membrane (MSI%), $H_2O_2$, MDA (nmol/g svežega listnega tkiva) in nekatere antioksidativne aktivnosti (SOD, CAT, POX) listnega tkiva sojinih rastlin. S0: 0,23 dS/m in S2: 6,25 dS/m; M0: 0 mM; M1: 1,25 mM; M2: 2,50 mM; in M3: 5,00 mM; povprečja, ki jim sledijo iste črke za vsak parameter, se po Duncanovem testu (P ≤ 0,05) med seboj ne razlikujejo pomembno in so predstavljena z ±SE.

**Table 4.** Impact of interaction between salinity stress and melatonin on membrane leakage (ML), membrane stability (MSI), $H_2O_2$, MDA, some antioxidant activity (SOD, CAT, POX) of leaf tissues of soybean plants.

**Tabela 4.** Vpliv interakcije med stresom zaradi slanosti in melatoninom na membransko prepustnost (ML), stabilnost membrane (MSI), $H_2O_2$, MDA, nekatere antioksidativne aktivnosti (SOD, CAT, POX) listnih tkiv sojinih rastlin.

| Treatments | | ML | MSI | $H_2O_2$ | MDA | SOD | CAT | POX |
|---|---|---|---|---|---|---|---|---|
| Salinity (dS/m) | Melatonine (mM) | % | | (nmole/g fresh leaf tissue) | | U/min/g fresh leaf tissue | | |
| S0 | M0 | 36.20±0.10 | 71.14±0.51c | 2.51±0.004e | 9.83±0.19d | 34.39±0.23h | 41.84±0.21h | 19.74±0.10e |
| | M1 | 35.17±0.25 | 72.38±0.23b | 2.39±0.019f | 9.19±0.02e | 38.82±0.19f | 45.29±0.65g | 21.13±0.40e |
| | M2 | 33.49±0.21 | 74.12±0.50a | 2.14±0.004g | 8.29±0.07g | 41.89±0.26e | 50.13±0.49e | 26.09±0.45d |
| | M3 | 34.58±0.11 | 72.62±0.00b | 2.48±0.039e | 8.61±0.01f | 37.39±0.23g | 47.63±1.03f | 24.12±0.49d |
| S1 | M0 | 41.12±0.41 | 58.27±0.35g | 3.95±0.004a | 13.03±0.18a | 52.14±0.50d | 56.76±0.39d | 33.12±0.50c |
| | M1 | 43.39±0.60 | 61.93±0.57f | 3.06±0.003c | 11.34±0.28b | 56.60±0.24c | 62.89±0.25c | 41.13±0.50b |
| | M2 | 35.88±0.09 | 64.88±0.26d | 2.80±0.041d | 9.10±0.22e | 64.44±0.81a | 68.89±0.75a | 55.13±0.49a |
| | M3 | 38.40±0.11 | 63.32±0.30e | 3.11±0.015b | 10.57±0.5c | 60.00±0.64b | 64.07±0.44b | 43.12±0.46b |

S0: 0.23dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50mM; and M3: 5.00 mM;.Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE

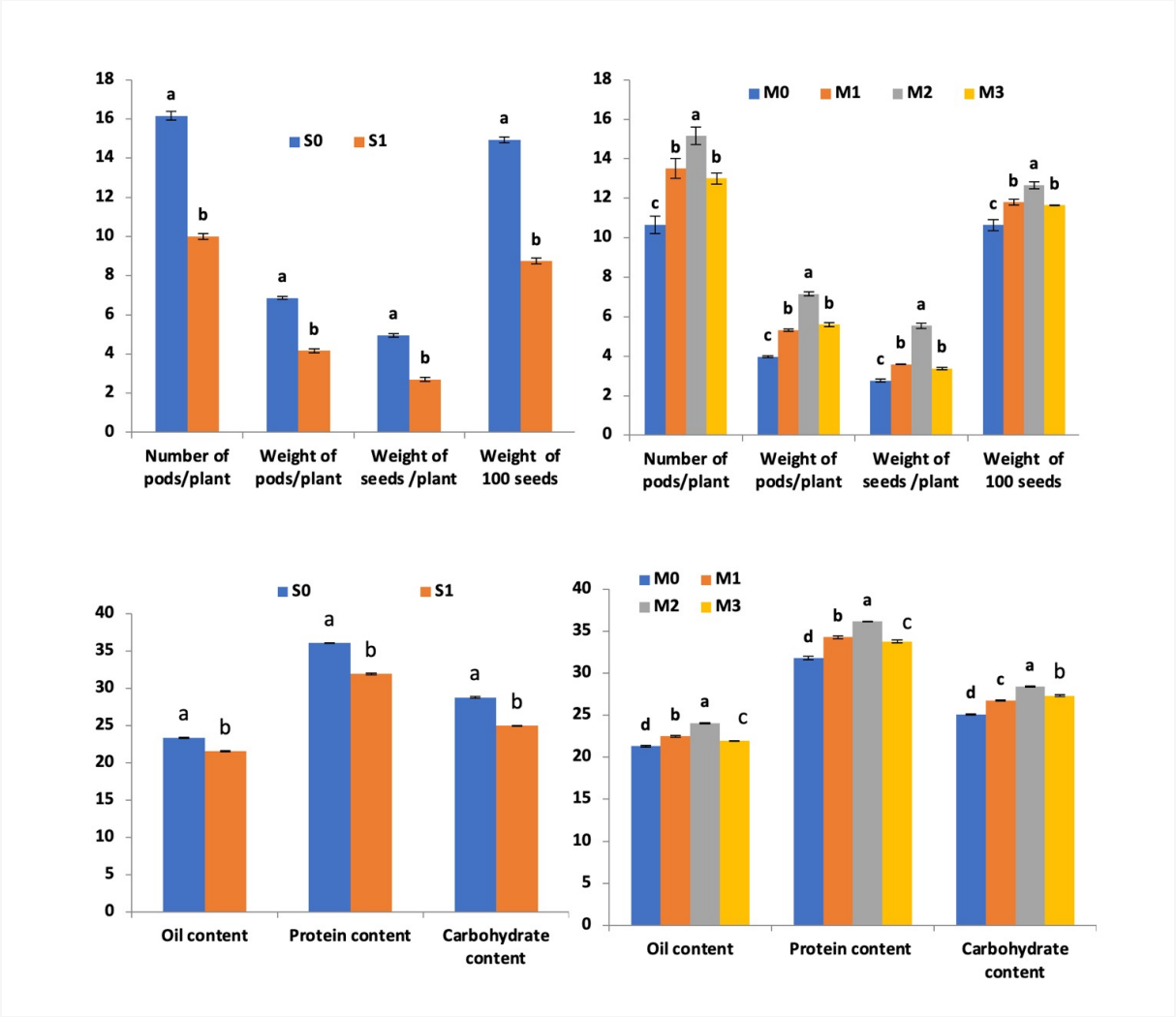


**Figure 5.** Impact of either salinity or melatonin on seed yield quantity and quality of soybean. S0: 0.23dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50mM; and M3: 5.00 mM;. Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE.

**Slika 5.** Vpliv slanosti ali melatonina na količino in kakovost pridelka semen soje. S0: 0,23 dS/m in S2: 6,25 dS/m; M0: 0 mM; M1: 1,25 mM; M2: 2,50 mM; in M3: 5,00 mM. Povprečja, ki jim sledijo iste črke za vsak parameter, se po Duncanovem testu (P ≤ 0,05) med seboj ne razlikujejo pomembno in so predstavljena z ±SE.

and total carbohydrates%, of the yielded seeds compared with unstressed control plants. The negative impact of salinity stress on productivity of soybean plant were stated as significant decreases in pods number per plant, pods weight per plant and seed weight/plant (g) from 13.00, 4.80 and 3.41 g to 8.33, 3.14 and 2.13 g respectively, showing a decline by a ratio of 35.89%, 34.71% and 37.53% comparing with control plant. Meanwhile, different levels of melatonin significantly increased all yield parameters under investigation, either in plants irrigated with tap water or saline solution, compared with corresponding controls (Table 5). Results clearly show that 2.50 mM of melatonin was a superior treatment in increasing seed yield quality and quantity. This significantly increased seed yield /plant by 116.13% in plants irrigated with tap water and by 74.64% in plants irrigated with saline solution. Likewise, 2.50 mM of melatonin significantly increased oil, protein and carbohydrate content by 14.83%, 16.47%, 14.86% in plants irrigated with tap water and by 10.55%, 10.71%, 11.25% in plants irrigated with saline solution relative to corresponding controls.

Table 6 shows the effects of melatonin on DNA fragments of soybean plants irrigated with tap water or saline solution using 5 ISSR primers. It was noticed that every primer detected a different number of bands with different ranges of molecular weights and a high ratio of polymorphism. Eighty-five bands (Totalband TB) with molecular weights ranging from 244 to 2,475 bp were detected. Moreover, these total bands were distributed between 50 polymorphic bands (PB) with an average of 10.0 and 20.0, unique bands (UB) with an average of 4.0 and 15.0, and monomorphic bands (MB) with an average of 3.0. Moreover, the highest levels of polymorphism (PB% was 85.00%) were detected using IS-02 (B) and the lowest levels of polymorphism (PB% was 76.47%) were detected using IS-02 (E).

Correlation coefficients and significance between different traits of soybean plants grown under interaction between salinity stress and melatonin treatments were determined by using Pearson correlation coefficients (PCCs) and illustrated in Table 7. The results show that there was a clear, significant correlation, either positive or negative, between all studied traits. The correlation coefficients between many characters reached to be highly positive correlation as shown in Table (7) between seed yield and total photosynthetic pigments (r=0.92**), IAA (r=0.93**), membrane stability index (r=0.81*),

**Table 5.** Impact of interaction between salinity stress and melatonin on seed yield quantity and quality of soybean.

**Tabela 5.** Vpliv interakcije med stresom zaradi slanosti in melatoninom na količino in kakovost pridelka semen soje.

| Treatments | | Number of pods/plant | Weight of pods/plant | Weight of seeds /plant | Weight of 100 seeds | Oil content | Protein content | Carbohydrate content |
|---|---|---|---|---|---|---|---|---|
| Salinity (dS/m) | Melatonine (mM) | | (g) | | | (%) | | |
| S0 | M0 | 13.00±1.00 | 4.08±0.16d | 3.41±0.24c | 13.18±0.50c | 22.25±0.10d | 33.38±0.24d | 26.44±0.08c |
| | M1 | 16.66±1.15 | 6.71±0.24b | 4.43±0.17b | 14.75±0.21b | 23.38±0.23b | 35.67±0.34c | 28.99±0.37b |
| | M2 | 19.33±1.16 | 8.94±0.35a | 7.37±0.25a | 15.83±0.50a | 25.55±0.06a | 38.88±0.26a | 30.37±0.25a |
| | M3 | 15.66±0.57 | 7.00±0.27b | 4.60±0.26b | 14.73±0.26b | 22.26±0.10d | 36.28±0.23b | 29.25±0.10b |
| S1 | M0 | 8.33±0.57 | 3.13±0.10f | 2.12±0.05e | 8.10±0.51f | 20.38±0.23f | 30.15±0.47f | 23.73±0.11f |
| | M1 | 10.33±1.07 | 3.95±0.07e | 2.75±0.10d | 8.84±0.28e | 21.59±0.05e | 32.88±0.26d | 24.49±0.13e |
| | M2 | 11.00±0.56 | 5.37±0.59c | 3.71±0.48c | 9.48±0.20d | 22.53±0.08c | 33.38±0.23d | 26.40±0.04c |
| | M3 | 10.33±0.40 | 4.20±0.14e | 2.17±0.15e | 8.57±0.27ef | 21.65±0.03e | 31.23±0.39e | 25.39±0.24d |
| LSD at 0.05 | | 1.73 | 0.50 | 0.50 | 0.63 | 0.23 | 0.54 | 0.34 |

S0: 0.23dS/m and S2: 6.25 dS/m; M0:0 mM; M1:1.25 mM; M2: 2.50mM; and M3: 5.00 mM.Means followed by the same letters for each parameter are not significantly different by the Duncan test (P≤ 0.05) and represented by ±SE

**Table 6.** Effect of melatonin on reproducible DNA fragments of soybean grown under normal and salinity conditions using ISSR molecular markers.

**Tabela 6.** Vpliv melatonina na reproducibilne fragmente DNA soje, gojene v normalnih in slanih pogojih, z uporabo molekularnih markerjev ISSR.

| Primers | Marker weights (bp) | Amplified bands | | | | |
|---|---|---|---|---|---|---|
| | | TB | MB | UB | PB | PB % |
| $(GA)_6$ CC | 338-2,056 | 17 | 3 | 4 | 10 | 82.35 % |
| $(CAC)_6$ GC | 244-2,475 | 20 | 3 | 7 | 10 | 85.00 % |
| $(GT)_6$ CC | 249-2,295 | 18 | 3 | 3 | 12 | 83.33 % |
| $(CA)_6$ AC | 394-1,216 | 13 | 2 | 4 | 7 | 84.62 % |
| $(CT)_8$ GC | 294-1,691 | 17 | 4 | 2 | 11 | 76.47 % |
| Total | | 85 | 15 | 20 | 50 | - |
| **Average** | | 17.0 | 3.0 | 4.0 | 10.0 | 82.35 % |

carbohydrate (r=0.91**), oil (r=0.95**), protein (r=0.96**) content. Meanwhile, there is a highly negative correlation between seed yield and membrane leakage (r=-0.76*), $H_2O_2$ (r=-0.80*), and MDA (r=-0.79*). In addition, a moderate negative and non significant correlation appeared between seed yield and proline (r=-0.35), total soluble sugar (r=-0.53), SOD (r=-0.49), CAT (r=-0.43), POX (r=-0.41).

Regarding the correlation between membrane stability index (MSI), ML, $H_2O_2$, MDA, some antioxidant activity (SOD, CAT, POX), the nutritive value of the yielded seeds as shown in Table 7. Results showed that there were a high negative correlation between MSI and $H_2O_2$, MDA and all antioxidant activity enzymes (SOD, CAT, POX) represented by (r=-0.86**, - 0.94**, -0.88**, - 0.77*,- 0.73*, - 0.66).

Also, Table 7 shows that the membrane stability index showed a high positive and significant correlation to carbohydrate oil and protein, represented by (r=0.94**, 0.89*, 0.80*), respectively. Moreover, a high positive and significant correlation appeared between the three parameters of nutritive value of the yielded seeds (carbohydrate, oil and protein content).

# Discussion

The reduction in the growth of soybean plant under salinity (Table1) could be attributed to the negative impact of both ion toxicity and osmotic stress (Hasanuzzaman et al., 2013) which changed the structure of cell wall (Sweet et al.,1990), declined the cell elongation and division (Radi et al.,2013); reduced absorption of $CO_2$, and decreased the accumulation of photoassimilates (Golldack et al., 2014), inhibited vital metabolic and physiological processes and thus limiting plant biomass (Kumari et al., 2018). In addition, salinity stress may change antioxidant balances, hormonal contents, and photosynthesis, thereby causing a decrease in plant growth (Acosta-Motos et al., 2017).

The depressive effect of salinity stress on photosynthetic pigments of fresh leaf tissues (Table 2) may be attributed to the enhancement of proteolytic enzymes such as chlorophyllase, which degrade chlorophyll and/or damage the photosynthesis apparatus (Radi et al.,2013). Additionally, Santos (2004) mentioned that salinity decreased chlorophyll content due to a decrease in the synthesis of 5-amin-

**Table 7.** Correlation coefficient and significance between different characters of soybean plants treated with melatonin and grown under salinity stress.

**Tabela 7.** Korelacijski koeficient in pomembnost med različnimi značilnostmi sojinih rastlin, obdelanih z melatoninom in gojenih v slanih razmerah.

| | Seeds Wt. | Total pig. | IAA | FAA | Pr. | TSS | ML | MSI | $H_2O_2$ | MDA | SOD | CAT | POX | TCHO % | Oil% | Prot.% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Seeds wt | 1.00 | | | | | | | | | | | | | | | |
| Total pig. | 0.92** | 1.00 | | | | | | | | | | | | | | |
| IAA | 0.93** | 0.98** | 1.00 | | | | | | | | | | | | | |
| FAA | 0.04 | 0.05 | -0.13 | 1.00 | | | | | | | | | | | | |
| proline | -0.35 | -0.36 | -0.53 | 0.90** | 1.00 | | | | | | | | | | | |
| TSS | -0.53 | -0.50 | -0.66 | 0.79* | 0.97** | 1.00 | | | | | | | | | | |
| ML | -0.76* | -0.85** | -0.85** | 0.07 | 0.39 | 0.49 | 1.00 | | | | | | | | | |
| MSI | 0.81* | 0.92** | 0.95** | -0.33 | -0.66 | -0.73* | -0.86** | 1.00 | | | | | | | | |
| $H_2O_2$ | -0.80* | -0.93** | -0.90** | 0.11 | 0.43 | 0.51 | 0.79* | -0.94** | 1.00 | | | | | | | |
| MDA | -0.79* | -0.95** | -0.89** | -0.11 | 0.24 | 0.34 | 0.89** | -0.88** | 0.94** | 1.00 | | | | | | |
| SOD | -0.49 | -0.50 | -0.66 | 0.83* | 0.98** | 0.97** | 0.52 | -0.77* | 0.56 | 0.40 | 1.00 | | | | | |
| CAT | -0.43 | -0.44 | -0.59 | 0.87** | 0.99** | 0.97** | 0.50 | -0.73* | 0.51 | 0.34 | 0.99** | 1.00 | | | | |
| POX | -0.41 | -0.38 | -0.56 | 0.87** | 0.99** | 0.99** | 0.40 | -0.66 | 0.43 | 0.24 | 0.97** | 0.98** | 1.00 | | | |
| TCHO% | 0.91** | 0.98** | 0.99** | -0.08 | -0.49 | -0.62 | -0.89** | 0.94** | -0.89** | -0.90** | -0.62 | -0.56 | -0.52 | 1.00 | | |
| Oil% | 0.95** | 0.91** | 0.87** | 0.11 | -0.25 | -0.43 | -0.74* | 0.80* | -0.85** | -0.81* | -0.38 | -0.35 | -0.31 | 0.86** | 1.00 | |
| Protein% | 0.96** | 0.97** | 0.97** | -0.03 | -0.43 | -0.58 | -0.75* | 0.89* | -0.89** | -0.85** | -0.57 | -0.50 | -0.47 | 0.95** | 0.91** | 1.00 |

** Correlation is significant at the 0.01 level.

* Correlation is significant at the 0.05 level.

olinolic acid (precursor of chlorophyll). Further, salinity stress reduced biosynthesis of the chlorophyll pigment by harming enzyme activity and triggered functional and structural changes in the protein-pigment-lipid complex (Alharbi et al., 2021). Moreover, the increase in carotenoid content under salinity stress (as shown in Table 2) protects the plant from photo-inhibition (Sharma and Hall, 1991) because carotenoids serve as antioxidants (Mohammadi et al., 2019) and enhance free radical scavenging (Awad et al., 2017).

The decreasing in vegetative growth parameters (Table 1) and photosynthetic pigments (Table 2) occurred simultaneously with the lowering of indole acetic acid (IAA) (Table 3) due to the influence of salinity stress, which could be related to the reduction in the synthesis of indole acetic acid and/or increments in their breakdown or transformation into inactive form. However, the decrease in auxin concentrations under salinity stress may be complex, diminished by stress-responsive transcription variables and microRNAs that modulate auxin- and environment-mediated roots (Kazan, 2013).

In response to different abiotic stresseslike salinity, plants have evolved diverse physiological and biochemical mechanisms to withstand stressors. The increases in phenolic compounds under salinity, as shown in Table 3, may be explained by Dawood and El Awadi (2014), who mentioned that salinity induced alterations in the metabolic process and boosted the synthesis of phenolic compounds. Actually, variations in net carbon assimilation under salinity stress are usually linked to the accumulation of reactive oxygen species (ROS), which can significantly affect the synthesis of secondary carbon-based compounds, particularly leaf polyphenols (Radi et al., 2013). Phenolic substances act as antioxidants, so they are crucial in scavenging free radicals produced by salt stress (Huang et al., 2005 and Sadak et al., 2012).

In addition, Table 3 shows that salinity stress induced the accumulation of some osmoprotectants, i.e. free amino acids, proline, and soluble sugar. The accumulation of osmoprotectants may helpneutralise or mitigate cellular damage from harmful substances by facilitating water uptake (Abd El-Mageed et al., 2022). The accumulation of free amino acid due to abiotic stress may be an essential component of an adaptive mechanism which promotes osmotic adjustment (Dubey, 1994) and reducing the loss of water and remitting the impact of stress (Wang et al., 2022). Proline accumulation is implicated in the production of essential proteins required for stress responses (Iyer and Caplan, 1998). In addition, proline serves as an osmotic protectant, and assists in maintaining the turgor of cells (Jagesh et al., 2010), provides a potent hydration ability of cells, and preserves proteins from deterioration and dehydration during osmotic stress (Hoekstra et al., 2001). Furthermore, proline maintains the stability of the enzyme system by acting as a protectant for several enzymes (Man et al., 2011). The increases in proline level under saline conditions may have resulted from a decline in proline oxidase activity (Roodbari et al., 2013) or from activation of enzyme production, particularly delta one proline five carboxylate synthetase (P5CS) and proline five carboxylate reductase (P5CR) (Hesami et al., 2020). Soluble sugar is a powerful osmoregulator and acts as a carbon frame and a source of energy for the production of other organic solutes in plants (Keiluweit et al., 2015). According to Jouve et al. (2004), increasing levels of sugar in salt-stressed plants might maintain and stabilise cellular membrane turgor. Likewise, carbohydrates have the potential to promote membrane stability and act as ROS scavengers (Bohnert and Jensen 1996). Dawood et al. (2021) reported that chickpea plants tolerate salinity stress (3000 mg/L NaCl) by causing significant increases in osmoprotectant (proline and soluble carbohydrates), phenolic content and antioxidant activity.

Salinity stress induced the production of ROS in the form of $H_2O_2$ that accelerates MDA production, membrane leakage, and increased activity of antioxidant enzymes (POX CAT, SOD) as shown in Table 4. These results may be explained by Muhammad et al. (2023), who mentioned that salinity stress induced osmotic stress and ion toxicity, which led to oxidative damage and production of ROS. Plant metabolism is severely harmed by an excess of ROS, causing permanent damage to vital macromolecules (Devireddy et al., 2021; Sachdev et al., 2021). Hydrogen peroxide ($H_2O_2$), the most crucial ROS, is included in a variety of processes essential to plant growth at low levels, but an excess of $H_2O_2$ is harmful. Also, under stress, lipid peroxidation (MDA) serves as an indicator of damage tothe cell membrane. The changes in MDA level are typically regarded as a reliable biomarker of oxidative damage, indicating cell membrane stability (Chen et al., 2018), and membrane leakage measures the membrane integrity (Demidchik et al., 2014). The excess accumulation of ROS reacts with phospholipids and fatty acids to hasten the lysis of the cell membrane, creating malondialdehyde, hence enhancing membrane permeability and damaging the stability of membrane structure (Jiang et al., 2021; Ozturk et al., 2021). Alharby et al. (2021)

stated that salinity stress at 6 $dSm^{-1}$ significantly increased electrolyte leakage (EL) by 69%, hydrogen peroxide ($H_2O_2$) by 75% and malondialdehyde (MDA) concentration by 56% in soybean plants. On the other hand, plants have evolved a range of defence mechanisms to combat the detrimental effects of stress while also shielding themselves from the harmful effects of elevated ROS levels by boosting their antioxidant defence system (Muhammad et al., 2023). The antioxidant defence system has both enzymatic and non-enzymatic components that effectively scavenge reactive oxygen species (ROS). The active antioxidant enzymes, including SOD, CAT, and POD, scavenging excess cellular reactive oxygen species (ROS) and minimize membrane lipid peroxidation (Khan et al., 2020; Muhammad et al., 2023, Sadak et al., 2024). According to Li et al. (2017) and Azizi et al. (2022), SOD is thought to be the first line of protection against oxidative stress. It can transform $O^{2-}$ to $H_2O_2$ into water and oxygen by CAT. The SOD and POD are powerful antioxidants that scavenge ROS from cells and preserve the integrity of cell membranes from lipid peroxidation (Xiao et al., 2019). In *Brassica juncea* plants, higher SOD activity increased the plants ability to withstand salinity and drought stress (Alamri et al., 2020). Recently, Zaki et al. (2023) mentioned that salinity stress significantly increased sunflower defence system in the form of catalase, peroxidase, superoxide dismutase, and glutathione reductase.

Salinity stress showed a deleterious effect on the quality and quantity of the soybean seeds, as shown in Table 5. Salinity stress caused morphological, biochemical and physiological changes in the plant that ultimately posed negative effects on different crops' yield (Hasanuzzaman et al., 2021). Furthermore, the inhibitory effects of salinity stress on various physiological processes, such as reduced photosynthesis, stomatal obstruction to water flow, excess production of ROS, and nutritional and hormonal imbalances, may cause plants to develop more slowly (Arif et al., 2020; Sofy et al., 2020) and consequently reduced crop yield. Further, Sadak and Dawood (2022) reported that salinity stress (4dS/m) caused asignificant decrease in wheat grain yield/plant by 8.25% but salinity stress at 8dS/m caused a significant decrease in wheat grain yield/plant by 15.73% as compared to unstressed plants. It is commonly known that decreased synthesis of photosynthetic components, slower transition of photosynthetic components, and changes in cytosolic metabolism cause yield reduction under salinity stress (Akram et al., 2017). Furthermore, the osmotic stress due salinity influences caused retardation of growth promoter, ion uptake, absorption of water, and reduction in photosynthetic activitieswhich caused changes the growth characteristics, yield, yield attributes, oil and protein content of the yielded soybean seeds (Taha et al., 2020). Zhang et al. (2021) reported that salinity stress stimulated ion toxicity, osmotic stress, and oxidative damage, causing inhibition of cell expansion and division, and consequently decreased plant growth and productivity of sugar beets. The excess production of ROS encouraged the mutation of DNA (Ke et al., 2018), degradation of chlorophyll and protein, which reduced the yield of plants (Liang et al., 2015; Ahmad et al., 2020). Excess accumulation of ROS causes damage to membranes, breaks the DNA chain, and inhibitsthe activity of different vital enzymes (Li et al., 2017). Salinity stress caused changes in carbohydrate and protein metabolism (Elkelish et al., 2019), as well as decreasing the size of the grain along with decreasing oil and protein content (Ghassemi-Golezani et al., 2010; Parveen et al., 2016). Likewise, a common occurrence under salt stress is the disruption of N metabolism and nitrate absorption within the plant, which can also result in decreased protein content (Farhangi-Abriz et al., 2016). In addition, the majority of plants are susceptible to salt stress, and excessive salinity levels reduce the amount of carbohydrates in plants (Hassanein et al., 2009).

Regarding the effect of melatonin, it was noted from Table 1 that melatonin application improved some vegetative growth parameters of soybean plants, either irrigated with tap water or saline solution. These improvements may be explained by the fact that melatonin stimulates the formation of globulins and microtubule filaments in seeds or boosts the enzyme activity of isocitrate lyase and malate synthase in the glyoxylate cycle, which consequently encourages cell elongation and division (Cui et al., 2018). Wei et al. (2015) revealed that melatonin regulates cell division, photosynthesis, carbohydrate metabolism, ascorbate metabolism, and biosynthesis of fatty acids, which enhance soybean growth, yield of seeds, and plant tolerance to stress (salt and drought).

It was noted from Table 2 that melatonin application improved all components of photosynthetic pigments of soybean plants,whether irrigated with tap water or saline solution. These improvements of photosynthetic pigments may be explained by Xu et al. (2010) who reported that melatonin application exerted an essential role in the regulation of photosynthesis and preservation of chlorophyll, boosting the antioxidant defence system and thus reducing

the formation of reactive oxygen species. The melatonin molecule significantly prevents chlorophyll degradation by enzymes such as chlorophyllase and peroxidase (Sharifet al., 2018), reduces the up-regulation of senescence-associated genes, maintains the photosynthetic apparatus and enhances the level of transcription of genes involved in photosynthesis (Shi et al., 2015). Further, exogenous application of melatonin enhanced photosynthesis and salt tolerance of plants by reducing the deleterious effects of salinity stress on gas exchange, photosystem II photochemistry, and chlorophyll biosynthesis (Su et al., 2023). Similarly, melatonin treatment improved different photosynthetic pigment components of white lupine under water stress (Sadak and Ramadan 2021).

It was noted from Table 5 that melatonin application improved IAA, phenolic content, and accumulation of some osmoprotectant (free amino acid, proline, soluble sugar) of soybean plants, either irrigated with tap water or saline solution. Melatonin has a similar chemical structure to auxin-IAA (Sarropoulou et al., 2012) and may possess some auxin-like effects (Kolár and Machackova, 2005). Melatonin stimulates the production of auxin, which has an impact on root growth, water absorption, and the permanent process of cell wall elongation (Ahmad et al., 2019). In addition, it has been demonstrated that treating *Brassica juncea* (Chen et al., 2009), tomato plants (Wen et al., 2016), and flax (Sadak and Bakry, 2020) with melatonin increased auxin concentration by 1.4-2.0 times.

Tan et al. (2012) indicate that melatonin has a signalling role; it may also have beneficial impacts by inducing distinct metabolic pathways and stimulating the synthesis of different compounds, especially under abiotic stress. Szafranska et al. (2012) mentioned that pre-sowing *Vigna radiata* L. seeds with melatonin enhanced the synthesis of phenolic compounds and shielded the roots of cold seedlings after re-warming. Likewise, Dawood and El Awadi (2014) mentioned that melatonin treatments increased IAA and phenolic contents in leaf tissues of faba bean plants either irrigated with tap water or saline solution. Accordingly, melatonin acts as an osmotic regulator in plants, facilitating the preservation of ion homeostasis and growth regulation under abiotic stressors (Timothy et al., 2015). The production of osmotic substances, such as free amino acids, proline, soluble proteins, soluble sugars, etc,is a vital source of energy for plant growth and development and maintaining essential nutrients for cell survival (Gul et al., 2022). They play an important role in keeping cell stability and protecting cells from damage caused by salinity stress (Chen et al., 2020). Melatonin application increased proline content in leaves through boosting the formation of pyrroline-5-carboxylate synthetase 1 (P5CS1), an enzyme involved in proline biosynthesis (Alyammahi and Gururani, 2020) and decreased catabolism of proline (Aghdam et al., 2019). It is well-established that proline protects plants against salinity by acting as a compatible osmolyte, cell redox equilibrium, enzyme protectant, free radical scavenger, cytosolic pH buffer, and subcellular structure stabiliser (Alam et al., 2019). The increases in osmotic regulation and plant salt tolerance lead to an increase in proline content (Chen et al., 2020). Melatonin may act as a scavenger of reactive oxygen species to reduce excess intracellular ROS, decrease protein breakdown, or encourage the formation of new proteins under salinity stress, which may be responsible for the increase in soluble protein content (Gao et al., 2019).

The collected results showed that melatonin treatments significantly decreased $H_2O_2$, MDA levels, membrane leakage and increased membrane stability in soybean plants grown under salinity stress (Table 6). It is worth mentioning that melatonin plays an important role inenhancing the redox state of cells, lowering reactive oxygen species (ROS) and reactive nitrogen species levels (RNS) and maintaining biological membranes of plant cells, as mentioned by Liu et al.(2021). Melatonin is situated between the polar heads of polyunsaturated fatty acids in cell membranes and hence lowers the level of lipid peroxidation and maintains natural membrane fluidity (Banerjee et al., 2021). Moreover, melatonin could play a role in decreasing lipid peroxidation through its capability to interact with lipid peroxyl (LOO_) and lipid alcoxyl (LO_) radicals and thereby the peroxidation cycle is interrupted and stopped (Malmir et al., 2021). Melatonin has the capability to maintain membrane integrity via suppressing electrolyte leakage and lipid peroxidation products in cucumber seedlings grown under water deficit stress (Zhang et al., 2020). Additionally, melatonin maintains the osmotic balance of cells by decreasing lipid peroxidation of the cell membrane and electrolyte leakage; these mechanisms alleviate the deleterious impacts of salinity stress and enhance the overall water status of the plant (Alharbi et al., 2021; Xiang et al., 2021). Increased plant tolerance to stress by using melatonin treatment may be attributed to its antioxidant properties which reduced the deleterious effects of reactive oxygen species by enhancing activity of antioxidant enzymes, and increasing plant antioxidant capacity to protect plant tissues from oxidative damage

(Tan et al., 2007; Silalert and Pattanagul, 2021; Altaf et al.,2021). Furthermore, melatonin alleviated the deleterious effects of salinity stress on maize plants by stimulating the gene transcription level of antioxidant enzymes (Chen et al., 2018). Application of melatonin on rice seedlings grown under drought stress boosted antioxidant enzymes activity as CAT, SOD, POD, and APX via enhancing the expression levels of ALM1, OsPOX, OsCATC, and OsAPX2 genes (Luo et al., 2022) as well as increase salinity tolerance in sorghum seedlings via enhancing the antioxidant defense systems and regulation of resistance-related gene expression (Jahan et al., 2020). Unlike other widely used antioxidants, a single melatonin molecule can scavenge up to 10 reactive oxygen species (ROS) or reactive nitrogen species (RNS) because melatonin interacts with ROS and scavenges them directly (Campos et al., 2012). According to PCR analysis, Jafari et al. (2022) concluded that melatonin treatment enhanced the expression of genes involved in ROS scavenging, cell redox regulation,and proline biosynthesis. Furthermore, melatonin is an incredibly effective antioxidant that may efficiently inhibit $H_2O_2$ formation by raising the activities of CAT, POD, and APX under stress, and boosting antioxidant defence systems via melatonin-induced production of nitric oxide (Tan et al., 2007; Kaya et al., 2019). Generally, melatonin is essential for mitigating stress, reducing oxidative stress, maintaining the stability of membranes, decreasing the accumulation of ROS, $H_2O_2$ and MDA, electrolyte leakage andincreasing antioxidant enzymesactivity (Wei et al., 2021; Farouk and AL-Huqail, 2022). Collectively from previous results, melatonin boosted soybean plant growth, photosynthetic pigments, IAA, some osmoprotectants, antioxidant defence system, membrane stability and reduced $H_2O_2$, MDA, and membrane leakage; all of these results induced an increase in seed yield.

Melatonin treatments increased seed yield (Table 5) because melatonin may play an efficient role in improving the transfer of water and minerals from the roots to the shoots and accelerating regular plant metabolism (Jahan et al., 2020), thereby enhancing photosynthetic production and biomass accumulation. Melatonin treatment enhanced the sucrose content through C assimilation, increased light energy absorption and utilisation induced by drought stress, and contributed to increased growth and productivity (Zou et al., 2021). Furthermore, it minimisedoxidative damage to maize, reduced the decomposition of chlorophyll, and enhanced photosynthetic rate, PSII reaction centres, and quantum yields (Guo et al., 2020; Li et al., 2021). According to Cao et al. (2021), the exogenous administration of melatonin monitored the expression of genes related to sugar metabolism, increased the activity of enzymes related to sugar metabolism, encouraged sugar production and metabolism, repaired membrane damage caused by drought stress, decreased MDA production, and rectified electrolyte leakage. As a result, plant dry matter buildup recovered, allowing soybean plants to continue producing a consistent yield. Further, the application of melatonin-activated genes is included in photosynthesis, carbohydrate metabolism, and fatty acid biosynthesis in soybeans (Wei et al., 2018).

Regarding genetic analysis, ISSR is an effective marker type for locating polymorphisms in DNA sequences, as mentioned by Mudibu et al. (2011). Gaafar et al. (2017)used five ISSR primers to evaluate the effect of different gamma ray doses on ISSR polymorphism of two Egyptian soybean varieties, and reported that there were variable amplified DNA bands detected by γ-radiation (as stress). Moreover, ISSR molecular markers were used by Dawood et al. (2021) to study the molecular changes in chickpea brought about by the effect of proline or glycinebetaine with salinity stress and found that a variety of unique polymorphic band types were detected in response to the salinity. Likewise, El Sayed et al.(2023) studied the effect of interacting hydrogel and drought stress on reproducible DNA fragments insoybean, and they obtained variable numbers of reproducible fragments with various molecular weights.

Regarding the correlation coefficient between different parameters of soybean results in Table 6 and illustrated in Supp. Fig. S1, they are in good agreement with those reported by Tint et al. (2011), who observed that membrane leakage showed significant correlation with seed yield. In addition, the correlation coefficient between seed yield and oil content was positive and significant, as mentioned by Oluk et al. (2020). On the other hand, our results are in the opposite direction to those reported by Masoumi et al. (2011), who stated that there are significant positive correlations between seed yield and different components of the plant's enzymatic antioxidant defence system.

## Conclusion

The collected results indicate that melatonin treatments reducedthe deleterious effect of salinity on the growth, photosynthetic pigments, quality and quantity of the yielded seeds of soybean through decreasing production

of $H_2O_2$, MDA, membrane leakage, and maintain membrane stability, activity of antioxidant enzymes, accumulation of osmoprotectants (free amino acid, proline, soluble sugar), increasing IAA, phenolic content and thereby increased its salinity tolerance.

## Supplementary Materials

Supplementary Figure S1. Impact of foliar spraying with melatonin on reproducible DNA fragments of soybean plants grown under salinity stress using ISSR molecular markers.

## Author Contributions

Conceptualization, M.E.E., M.Sh.S., M.A.K., and M.G.D.; methodology, M.E.E., M.Sh.S., and M.G.D; software, M.E.E., M.Sh.S., M.A.K., and M.G.D.; validation, M.E.E., M.Sh.S., M.A.K., and M.G.D.; formal analysis, M.A.K., and M.G.D; investigation, M.E.E., M.Sh.S., M.A.K., and M.G.D.; resources, M.E.E., M. Sh.S., M.A.K., and M.G.D; data curation, M.Sh.S., and M.G.D.; writing—original draft preparation, M. G.D. writing—review and editing, M.Sh.S., and M.G.D. visualization, M.E.E., M.Sh.S., M.A.K., and M. G.D. supervision, M.E.E., and M.Sh.S.; project administration, M.E.E., and M.Sh.S.; funding acquisition, M.E.E., and M.Sh.S. All authors have read and agreed to the published version of the manuscript.

## Funding

The Authors express their appreciations to the National Research Centre who financed the project ofSome effective approaches to enhance oilseed plants tolerance against environmental stress (Project No. 13050107).

## Data Availability

All data are included in the MS.

## Declaration of competing interest

The authors declare that they have no competing interest.

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ABS

Review

# Phytochemical and pharmacological potential of *Solanum nigrum*: A concise review

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## Abstract

Medicinal plants have been a great source of folk medicines for humans for centuries worldwide. Solanaceae family is the most prominent family of plants, having a large group of more than 1400 species present in tropical and temperate regions worldwide, including *Solanum nigrum*. This edible medicinal herb exhibits a wide range of therapeutic potential, predominantly encompassing anti-tumour, antioxidative, hepato-protective, antimicrobial, immune-modulatory, neuroprotective and anti-inflammatory properties. Phytoconstituents and therapeutic properties of *S. nigrum* were elaborated in the present review. The information documented in this review was accessed using Google, Science Direct, Google Scholar and NCBI. Pharmacologically and medicinally important compounds are present within the *S. nigrum*, having great importance, predominantly steroidal compounds and many alkaloids. So far, around 188 phytoconstituents have been identified from *S. nigrum*. Among them, the major bioactive constituents are steroidal saponins, alkaloids, phenols, and polysaccharides. This review revealed different properties of *S. nigrum* of pharmaceutical value (cardioprotective, anti-ulcer, anti-microbial, antiviral, anticancer, anti-mycotoxin and nutritive importance) and their biological roles were elucidated. The current reviewed information will provide the therapeutic potential of *S. nigrum*. It will undoubtedly be helpful in developing new therapeutic and treatment options by discovering new drugs.

## Keywords

Phytoconstituents, Cardio-protective, Antiviral, Antimicrobial, *S. nigrum*, Mycotoxins.

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**Citation:** Jamal, A., Nawaz, A., Arif, A., Shahid, M. N., (2025). Phytochemical and pharmacological potential of *Solanum nigrum*: A concise review. Acta Biologica Slovenica 68 (3)

**Received:** 29.10.2024 / **Accepted:** 25.04.2025 / **Published:** 30.04.2025

**https://doi.org/10.14720/abs.68.3.20074**

## Fitokemični in farmakološki potencial *Solanum nigrum*: Kratek pregled

**Izvleček**

Zdravilne rastline so že stoletja velik vir ljudskih zdravil za ljudi po vsem svetu. Družina Solanaceae je družina rastlin z več kot 1400 vrstami, ki so prisotne v tropskih in zmerno toplih območjih po vsem svetu. Družina vključuje vrsto *Solanum nigrum*. Le-ta je užitna zdravilna rastlina z velikim terapevtskim potencialom, ki zajema predvsem protitumorske, antioksidativne, hepatološko zaščitne, protimikrobne, imunomodulatorne, nevrozaščitne in protivnetne lastnosti. V tem pregledu so opisane bioaktivne snovi in terapevtske lastnosti *S. nigrum*. Informacije, dokumentirane v tem pregledu, so bile pridobljene z uporabo spletnih strani google, science direct, google scholar in NCBI. *S. nigrum* vsebuje farmakološko in medicinsko pomembne spojine - predvsem steroidne spojine in številne alkaloide. Doslej je bilo v *S. nigrum* identificiranih približno 188 bioaktivnih snovi. Med njimi so glavne bioaktivne sestavine steroidni saponini, alkaloidi, fenoli in polisaharidi, ki imajo različne farmacevtske lastnosti (kardio-zaščitne, protivnetne, protimikrobne, protivirusne, protirakave, protimikotoksinske in hranilne lastnosti). S pregledom smo želeli izpostaviti terapevtski potencial *S. nigrum*, kar bo nedvomno v pomoč pri razvoju novih terapevtskih pristopov in načinov zdravljenja z odkrivanjem novih zdravil.

**Ključne besede**

bioaktivne snovi, kardio-zaščitni, protivirusni, protimikrobni, *S. nigrum*, mikotoksini

# Introduction

Plants have endured a fundamental effect on human civilization since antiquity. Approximately 80% of the global population relies on plant resources (Hussain et al., 2018). Medicinal plants utilised for various diseases provide a cost-effective alternative for healthcare systems in underdeveloped nations, owing to the lack of sophisticated medical facilities, their efficiency, cultural significance, and availability (Ahmed et al., 2018). The conventional use of herbal medicines is more prevalent in developed nations in the contemporary period. In Asia, 40-50% of the population relies on the intake of traditional medicine. Simultaneously, 60% of the population depends on herbal remedies, particularly among youngsters, to address various ailments such as malaria. The selection of herbal medications is contingent upon consumer cultural preferences and the accessible pharmacopoeias. The affordability of traditional remedies relative to contemporary pharmaceuticals renders them a viable option for many nations. The existing literature on plants and their pharmacopoeias will aid in bridging the gap for discovering novel medications in contemporary research (Gupta et al., 2018).

Pakistan is a country that boasts diverse climates and rich biodiversity, with over 6000 plant species. Approximately 500 to 600 plant species are documented as possessing therapeutic properties and can be utilised to address various illnesses. A multitude of studies have explored the therapeutic potential of these medicinal plants and documented the utilisation of plant resources (Amjad et al., 2020). General information on traditional herbal therapies typically transfers from one generation to another generation verbally. During information transfer, the likelihood of knowledge exclusion may occur, posing a significant risk that must be mitigated to enhance the healthcare system (Sehrawat et al., 2020). In recent decades, a notable tendency has emerged nationwide regarding the systematic and commercial utilisation of herbal plant items to bolster the state's economy. An intriguing development in scientific and commercial sectors has emerged owing to the cultural acceptance and economic viability of plant-based herbal products nationwide (Suntar, 2020). The country encompasses diverse civilisations across numerous rural and remote regions. Individuals in underdeveloped regions lack access to basic healthcare services, which is a key factor contributing to the usage of traditional herbal remedies (Bhat et al., 2020). Various crude and synthetic pharmaceuticals are sourced from medicinal plants, which serve as a vital source of metabolites deployed in drug discovery, the pharmaceutical industry, nutraceuticals, sup-

plement formulation, and drug intermediates. The emergence of novel ailments has prompted the development of new bioactive compounds from various plants, as existing bioactive natural products are inadequate. It is imperative to synthesise pharmaceuticals from natural sources by employing efficient techniques. A multitude of extraction techniques are now utilised to identify bioactive chemicals in plants, followed by isolation and characterisation to get specific compounds from natural products for disease treatment (Odukoya et al., 2018).

The initial documented incidence of *S. nigrum* therapeutic use was identified in traditional Chinese medicine. *S. nigrum* possesses several therapeutic qualities, including treating carbuncular inflammation, skin dermatitis, dysuria, chronic bronchitis, exorbitant leukorrhea, prostatitis, and dysentery. *S. nigrum* is known to possess many therapeutic potential and biological activities, including antimicrobial, antiviral, anticancerous, antimycotic, anti-inflammatory, immunomodulatory and others (Figure 1). Numerous plants or traditional treatments, including *S. nigrum*, have been utilised in concoctions, powders, granules, and tablets. As a traditional medicine, practitioners have amassed much clinical knowledge on the use of *S. nigrum*. The whole plant of *S. nigrum* successfully fosters blood stability and lessens swelling while also alleviating heat and facilitating detoxification (Wang et al., 2007). Novel phenolic chemicals, benzoiisovanillin and syringic acid, might contribute to its pharmacological effects (Khan et al., 2016). The fruits of *S. nigrum* resulted in the extraction of four novel steroidal alkaloids, designated as solanine A, 7α-OH khasianine, 7α-OH solamargine, and 7α-OH solasonine, which have cytotoxic properties against human cancer cell lines (Gu et al., 2018).

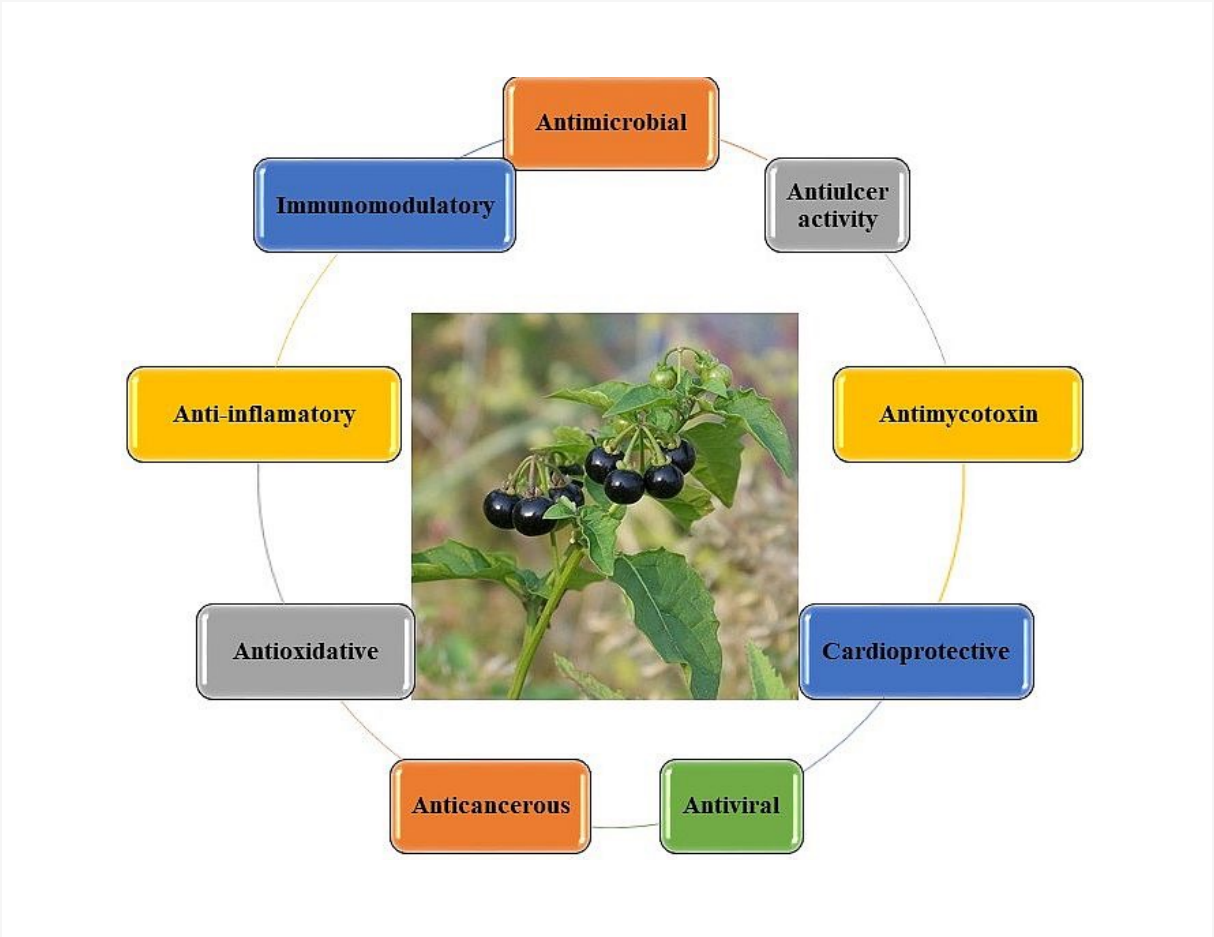


**Figure 1.** *S. nigrum* potential therapeutic applications.

**Slika 1.** Potencialna terapevtska uporaba *S. nigrum*.

## Phytoconstituents

The remarkable *S. nigrum* possesses a substantial array of chemicals, as shown by phytochemical analysis of the entire plant, which includes glycosides, proteins, carbohydrates, coumarins, alkaloids, flavonoids, tannins, saponins, and phytosterols (Figure 2). *S. nigrum* has recently been uncovered to include saponins, alkaloids, and glycoproteins, which have significant antiproliferative action (Joshi, 2019). *S. nigrum* comprises several phytoconstituents that vary in nature and exhibit a range of biological activity. It contains several polyphenolic components, including protocatechuic acid, gallic acid, catechin, caffeic acid, rutin, naringenin and epicatechin, contributing to its global antioxidant and antiproliferative properties. Steroidal alkaloids, steroidal aminoglycosides and steroidal glycosides, including solasonine, solamargine, solavilline, solanine and solasdamine, are exclusively found in *S. nigrum* (Saibu et al., 2021). A variety of flavonoids, glycoproteins, and steroidal saponins were identified and are responsible for multiple medicinal properties. A variety of proteins, carbohydrates, phytosterols, crude polyphenols, gentisic acid, polysaccharides, luteolin, apigenin, m-coumarins, kaempferol, and anthocyanidin have also been have also been uncovered for their role. Glycoproteins and steroidal alkaloids have a heightened propensity to combat certain malignancies (Xiang et al., 2019).

Around 188 chemical constituents have been separated and identified from *S. nigrum*, containing steroids, alkaloids, organic acids, flavonoids, phenylpropanoids and their glycosides, and other compounds. The leaves have a higher polyphenol concentration than the stem and fruit. The leaves of *S. nigrum* exhibited the most significant concentrations of gentisic acid, luteolin, apigenin, kaempferol, and m-coumaric acid. Nonetheless, anthocyanidin has been reported solely in purple fruits (Huang et al., 2010). Steroidal chemicals, including steroidal saponins and steroidal alkaloids, are regarded as the principal bioactive constituents of *S. nigrum*, illuminating diverse pharmacological effects, including anticancer, anti-inflammatory, and antiviral activity. The compounds gleaned from *S. nigrum* have been documented and enumerated in the (Table 1).

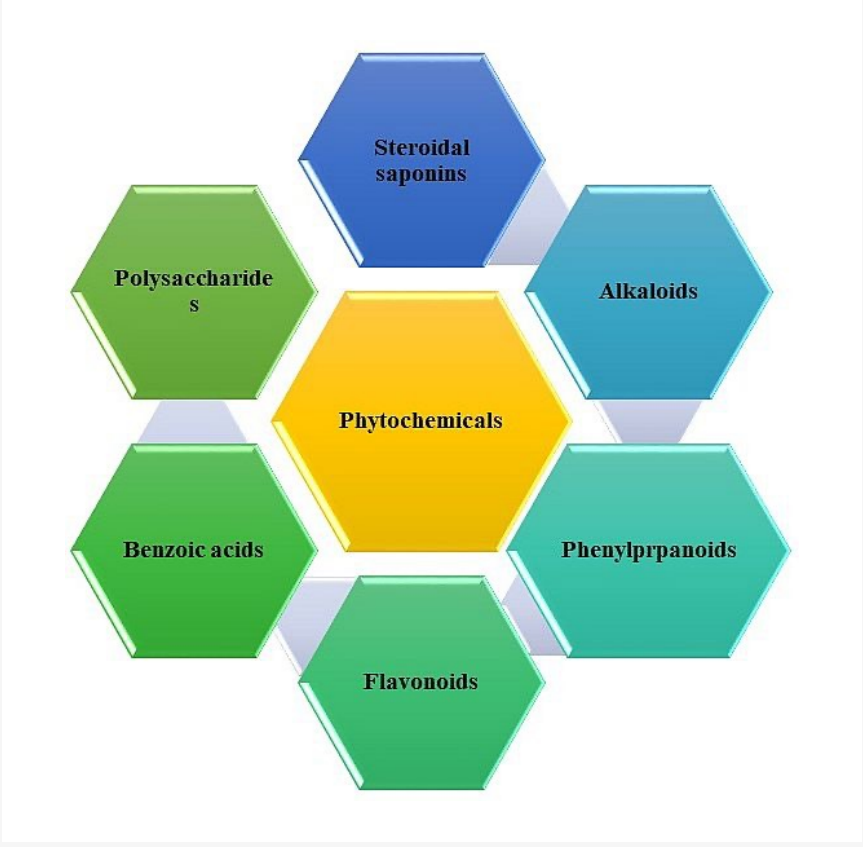


**Figure 2.** Key phytochemicals in *S. nigrum.*

**Slika 2.** Ključne fitokemikalije v *S. nigrum.*

**Table 1.** Phytochemicals identified from *S. nigrum* on the basis of structure.

**Tabela 1.** Fitokemikalije, identificirane iz *S. nigrum* na podlagi strukture.

| Chemical constituents | Molecular formula | CAS | Extracts | References |
|---|---|---|---|---|
| **Steroidal saponins** | | | | |
| Diosgenin | $C_{27}H_{42}O_3$ | 512-04-9 | MeOH | Gao et al. (2021)<br>Tsuyoshi et al. (2003) |
| Degalactotigonin | $C_{50}H_{82}O_{22}$ | 39941-51-0 | EtOH | Wang (2007) |
| Stigmasterol | $C_{29}H_{48}O$ | 83-48-7 | EtOH | Zhao (2010) |
| Pterosterone | $C_{27}H_{44}O_7$ | 18089-44-6 | EtOH | Zhao (2010) |
| 12-keto-porrigenin | $C_{27}H_{42}O_5$ | 189014-45-7 | EtOH | Zhao (2010) |
| 28-*O*-*β*- D-glucopyranosyl betulinic acid 3*β*-*O*-*β*-D glucopyranoside | $C_{42}H6_8O_{13}$ | | EtOH | Yang (2014) |
| *β*-daucosterol | $C_{35}H_{60}O_6$ | 474-58-8 | EtOH | Yang (2014) |
| Uttroside A | $C_{57}H_{96}O_{28}$ | 82003-86-9 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Uttroside B | $C_{56}H_{94}O_{28}$ | 88048-09-3 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Solanigroside J | $C_{61}H_{102}O_{31}$ | 1354759-80-0 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Hypoglaucin H | $C_{39}H_{60}O_{15}$ | 50773-43-8 | EtOH | Zhou (2006) |
| 5α-pregn-16-en-3β-ol-20-one-lycotetraoside | $C_{44}H_{70}O_{21}$ | | EtOH | He et al. (2015),<br>Zhou (2006) |
| Nigrumnin I | $C_{55}H_{90}O_{25}$ | | EtOH | He et al. (2015),<br>Zhou (2006) |
| Nigroside A | $C_{56}H_{94}O_{29}$ | 386747-86-0 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Tigogenin/(25R)-5α-spirostan-3β-ol | $C_{27}H_{44}O_3$ | | EtOH | Wu (2011) |
| (25*R*)-26-*O*-*β*-D-glucopyranosyl-cholest-5(6)-en-3*β*, 26-diol-16,22-dione-3-*O*-*α*-L-rhamnopyranosyl -(1➞2)-[*β*-D-glucopyranosyl-(1➞3)]-*β*-D-galactopyranoside | $C_{51}H_{86}NO_{23}$ | | MeOH | Xiang et al. (2018) |
| Stigmast-5, 22-dien-3β-ol | $C_{29}H_{48}O$ | | EtOH | Sharma et al. (2012) |
| Inunigroside A | $C_{50}H_{82}O_{23}$ | 1427934-51-7 | MeOH | Ohho et al. (2012) |
| Solanigroside Y1 | $C_{51}H_{82}O_{26}$ | 2098576-14-6 | MeOH | Wang et al. (2017) |
| *β*-sitosterol | $C_{29}H_{50}O$ | 83-46-5 | EtOH | Yang (2014) |
| *β*-carotene glycosides | $C_{35}H_{58}O_6$ | | EtOH | Yang (2014) |
| Tigogenin | $C_{27}H_{44}O_3$ | 77-60-1 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Uttronin A | $C_{50}H_{82}O_{22}$ | 39941-51-0 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Uttronin B | $C_{39}H_{62}O_{12}$ | 84955-03-3 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Dumoside | $C_{40}H_{62}O_{16}$ | 221526-58-5 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Cholesterol | $C_{27}H_{46}O$ | 57-88-5 | EtOH | Geng et al. (2020) |
| **Alkaloids** | | | | |
| *β*1-solasonine | $C_{39}H_{63}NO_{11}$ | 73069-18-8 | EtOH | He et al. (2015),<br>Zhou (2006) |
| *β*2-solasonine | $C_{39}H_{63}NO_{12}$ | 73069-19-9 | EtOH | He et al. (2015),<br>Zhou (2006) |
| Solamargine | $C_{45}H_{73}NO_{15}$ | 20311-51-7 | EtOH | He et al. (2015),<br>Zhou (2006),<br>Wang (2007) |

| | | | | |
|---|---|---|---|---|
| (3*β*, 12*β*, 22*α*, 25*R*)-3, 12-dihydroxy-spirosol-5-en-27-oic acid | $C_{27}H_{41}NO_5$ | | EtOH | He et al. (2015), Zhou (2006) |
| Solaoiacid | $C_{44}H_{83}NO_{19}$ | | H2O | Shi et al. (2019) |
| (25*R*)-22*α*N-4-nor-spirosol-5(6)-en-3*β*-ol-6-al-3-*O*-L-rhamnopyranosyl-(1➔2)-[*α*-L-rhamnopyranosyl-(1➔4)]-*β*-D-glucopyranoside | $C_{45}H_{72}NO_{16}$ | | MeOH | Xiang et al. (2019) |
| Solasodine | $C_{27}H_{43}NO_2$ | 126-17-0 | EtOH | He et al. (2015), Zhou (2006) |
| N-methylsolasodine | $C_{28}H_{45}NO_2$ | 7604-92-4 | EtOH | He et al. (2015), Zhou (2006) |
| Tomatidenol | $C_{27}H_{43}NO_2$ | 546-40-7 | EtOH | He et al. (2015), Zhou (2006) |
| Solanocapsine | $C_{27}H_{46}N_2O_2$ | 639-86-1 | EtOH | Liu et al. (2020), Zhou (2006) |
| Solanaviol | $C_{27}H_{43}NO_3$ | 74131-93-4 | EtOH | He et al. (2015), Zhou (2006) |
| Solasodine-3-*O*-*β*-D-glucopyranoside | $C_{33}H_{53}NO_7$ | | EtOH | Chang et al. (2017) |
| 12*β*-hydroxysolasodine *β*-solatrioside | $C_{45}H_{73}NO_{17}$ | | EtOH | He et al. (2015), Zhou (2006) |
| (3*β*, 22*α*, 25*R*)-spirosol-5-en-3yl-*O*-*α*-L-Rhamanopyranosyl-(1-2)-[O-β-D-glucopyranosyl(1–3)]-*O*-*β*-D-galactopyranoside | $C_{39}H_{85}NO_{15}$ | 101009-59-0 | EtOH | Yang (2014) |
| 15*α*-hydroxysolasodine | $C_{27}H_{43}NO_3$ | 10009-88-8 | EtOH | Wu (2011) |
| *α*-Solanine | $C_{45}H_{73}NO_{15}$ | 20562-02-1 | EtOH | Huang et al. (2020) |
| Solasonine | $C_{45}H_{73}NO_{16}$ | 19121-58-5 | EtOH | He et al. (2015), Zhou (2006) |
| Leptinine I | $C_{45}H_{73}NO_{15}$ | | EtOH | Gao et al. (2021) |
| Cannabisin F | $C_{36}H_{36}N_2O_8$ | 163136-19-4 | EtOH | Li et al. (2019a) |
| Adenine | $C_5H_5N_5$ | 73-24-5 | MeOH | Gao et al. (2021) |
| Pyroglutamic acid | $C_5H_7NO_3$ | 98-79-3 | MeOH | Gao et al. (2021) |
| Nicotinic acid | $C_6H_5NO_2$ | 59-67-6 | MeOH | Gao et al. (2021) |
| 9-aminononane-1,3,9-tricarboxylic acid | $C_{12}H_{21}NO_6$ | | MeOH | Gao et al. (2021) |
| Glutarylcarnitine | $C_{12}H_{21}NO_6$ | 102636-82-8 | MeOH | Gao et al. (2021) |
| 6-Hydroxypurine | $C_5H_4N_4O$ | 68-94-0 | MeOH | Gao et al. (2021) |
| Uridine | $C_9H_{12}N_2O_6$ | 58-96-8 | MeOH | Gao et al. (2021) |
| Adenosine | $C_{10}H_{13}N_5O_4$ | 58-61-7 | MeOH | Gao et al. (2021), Wang (2007) |
| Dihydrocapsaicin | $C_{18}H_{29}NO_3$ | 19408-84-5 | MeOH | Gao et al. (2021) |
| Choline | $C_5H_{14}NO$ | 62-49-7 | MeOH | Gao et al. (2021) |
| Allantoin | $C_4H_6N_4O_3$ | 97-59-6 | MeOH | Gao et al. (2021) |
| Uracil | $C_4H_4N_2O_2$ | 66-22-8 | MeOH | Gao et al. (2021) |
| Trigonelline | $C_7H_7NO_2$ | 535-83-1 | MeOH | Gao et al. (2021) |
| Glycyl-L-leucine | $C_8H_{16}N_2O_3$ | | MeOH | Gao et al. (2021) |
| GABA | $C_4H_9NO_2$ | 56-12-2 | MeOH | Gao et al. (2021) |
| FMoc-Asn(Trt)-OPfp | $C_{44}H_{31}F_5N_2O_5$ | | MeOH | Gao et al. (2021) |
| **Phenylpropanoids** | | | | |
| *Trans*-4-Hydroxycinnamic acid | $C_9H_8O_3$ | 501-98-4 | EtOH | Liu et al. (2019b) |
| *Cis*-4-Hydroxycinnamic acid | $C_9H_8O_3$ | 4501-31-9 | EtOH | Liu et al. (2019a) |
| Ethyl 3, 4-dihydroxycinnaMate | $C_{11}H_{12}O_4$ | 102-37-4 | EtOH | Liu et al. (2019c) |
| *Cis*-caffeic acid ethyl ester | $C_{11}H_{12}O_4$ | 74257-25-3 | EtOH | Liu et al. (2019b) |
| *Trans* ferulic acid | $C_{10}H_{10}O_4$ | 537-98-4 | EtOH | Liu et al. (2019a) |

| | | | | |
|---|---|---|---|---|
| *Cis*-ferulic acid | $C_{10}H_{10}O_4$ | 1014-83-1 | EtOH | Liu et al. (2019c) |
| Caffeic acid | $C_9H_8O_4$ | 331-39-5 | MeOH | Gao et al. (2021) |
| 4-(4-hydroxyphenyl)-2-methylene butyrolactone | $C_{11}H_{10}O_3$ | | EtOH | Liu et al. (2019c) |
| Chlorogenic acid | $C_{16}H_{18}O_9$ | 327-97-9 | MeOH | Gao et al. (2021) |
| 3-caffeoylquinic acid methyl ester | $C_{17}H_{20}O_9$ | 123483-19-2 | MeOH | Xiang et al. (2019) |
| Scopoletin | $C_{10}H_8O_4$ | 92-61-5 | EtOH | Zhao (2010), Wang et al. (2007), Wang (2007) |
| Cinnacassoside A | $C_{26}H_{36}O_{12}$ | 1691248-24-4 | MeOH | Xiang et al. (2019) |
| Pinoresinol | $C_{20}H_{22}O_6$ | 81446-29-9 | EtOH | Zhao (2010) |
| Pinoresinol-4-*O*-*β*-D-glucopyranoside | $C_{26}H_{32}O_{11}$ | 69251-96-3 | EtOH | Wang et al. (2007), Wang (2007) |
| Syringaresinol | $C_{22}H_{26}O_8$ | 487-35-4 | EtOH | Zhao (2010) |
| Syringaresinol-4-*O*-*β*-D-glucopyranoside | $C_{28}H_{36}O_{13}$ | 137038-13-2 | EtOH | Wang et al. (2007), Wang (2007) |
| Medioresinol | $C_{21}H_{24}O_7$ | 40957-99-1 | EtOH | Zhao (2010) |
| Acanthoside D | $C_{34}H_{46}O_{18}$ | 573-44-4 | MeOH | Xiang et al. (2019) |
| (+)-medioresonol-di-*O*-*β*-D-glucopyranoside | $C_{33}H_{44}O_{17}$ | | MeOH | Xiang et al. (2019) |
| Quercetin | $C_{15}H_{10}O_7$ | 117-39-5 | EtOH | Yang (2014) |
| Quercitrin | $C_{21}H_{20}O_{11}$ | 522-12-3 | EtOH | Yang (2014) |
| Isoquercitrin | $C_{21}H_{20}O_{12}$ | 21637-25-2 | EtOH | Yang (2014) |
| Quercetin-3-*O*-*β*-D-glucopyranosyl(1-2)-*β*-D-glucopyranoside | $C_{27}H_{30}O_{17}$ | | EtOH | Yang (2014) |
| Quercetin-3-*O*-*β*-D-galactopyranosyl-(1➞6)-*β*-D-glucopyranoside | $C_{28}H_{32}O_{17}$ | | MeOH | Xiang et al. (2019) |
| Quercetin-3-gentiobioside | $C_{27}H_{30}O_{17}$ | 7431-83-6 | EtOH | Wu (2011) |
| Quercetin-3-O-*α*-L-rhaopyranosyl(1➞4)-*O*-*β*-D-glucopyranosyl-(1➞6)-*O*-*β*-D-glucopyranoside | $C_{28}H_{32}O_{17}$ | | EtOH | Li (2010) |
| 6-Hydroxyluteolin 7-sophoroside | $C_{27}H_{30}O_{17}$ | | MeOH | Gao et al. (2021) |
| Kaempferol | $C_{15}H_{10}O_6$ | 520-18-3 | EtOH | Liu et al. (2019a) |
| (8-hydroxy-3′-*β*-D-galactosyl-isoflavone)-2′-8″-(4‴-hydroxy-flavone)-biflavone | $C_{36}H_{28}O_{11}$ | | EtOAc | Sabudak et al. (2017) |
| 2′, 3′, 5-trihydroxy-5″-methoxy-3″-O- *α*-glucosyl-3-4‴-O-biflavone | $C_{36}H_{30}O_{15}$ | | EtOAc | Sabudak et al. (2017) |
| **Benzoic acids** | | | | |
| Gallic acid | $C_7H_6O_5$ | 149-91-7 | MeOH | Gao et al. (2021) |
| 2, 4-Dihydroxybenzoic acid | $C_7H_6O_4$ | 89-86-1 | MeOH | Gao et al. (2021) |
| Protocatechuic acid | $C_7H_6O_4$ | 99-50-3 | EtOH | Zhao (2010) |
| Vanillic acid | $C_8H_8O_4$ | 121-34-6 | EtOH | Zhao (2010) |
| 4-Hydroxybenzoic acid | $C_7H_6O_3$ | 99-96-7 | EtOH | Liu et al. (2019c) |
| Salicylic acid | $C_7H_6O_3$ | 69-72-7 | EtOH | Liu et al. (2019c) |
| 2, 5-Dihydroxybenzoic acid | $C_7H_6O_4$ | 490-79-9 | MeOH | Gao et al. (2021) |
| **Other compounds** | | | | |
| Galacturonic acid | $C_6H_{10}O_7$ | 14982-50-4 | MeOH | Gao et al. (2021) |
| Pyruvic acid | $C_3H_4O_3$ | 127-17-3 | MeOH | Gao et al. (2021) |
| Formic acid | $CH_2O_2$ | 64-18-6 | MeOH | Gao et al. (2021) |
| Succinic acid | $C_4H_6O_4$ | 110-15-6 | MeOH | Gao et al. (2021) |
| Fumaric acid | $C_4H_4O_4$ | 110-17-8 | MeOH | Gao et al. (2021) |
| Ursolic acid | $C_{30}H_{48}O_3$ | 77-52-1 | EtOH | Zhao (2010) |

| | | | | |
|---|---|---|---|---|
| Linolenic acid | $C_{18}H_{30}O_2$ | 463-40-1 | MeOH | Gao et al. (2021) |
| Oleic acid | $C_{18}H_{34}O_2$ | 112-80-1 | EtOH | Wang (2007) |
| Linoleic acid | $C_{18}H_{32}O_2$ | 60-33-3 | EtOH | Wang (2007) |
| Palmitic acid | $C_{16}H_{32}O_2$ | 57-10-3 | EtOH | Wang (2007) |
| 1-monolinolenin | $C_{21}H_{36}O_4$ | 75685-85-7 | EtOH | Zhao (2010) |
| Lignoceric acid | $C_{24}H_{48}O_2$ | 557-59-5 | EtOH | Zhao (2010) |
| (*E*)-docosyl-3-(4-hydroxy-3-methoxyphenyl)acrylate | $C_{32}H_{54}O_4$ | | EtOH | Zhao (2010) |
| *α*-carotene | $C_{40}H_{56}$ | 432-70-2 | EtOH | Wu (2011) |
| *β*-carotene | $C_{40}H_{56}$ | 7235-40-7 | EtOH | Wu (2011) |
| Xanthophyll | $C_{40}H_{56}O_2$ | 127-40-2 | EtOH | Wu (2011) |

A wide spectrum of compounds have been chronicled and culled from various fractions of *S. nigrum*, implying therapeutic properties and potential use in pharmacological treatments for several diseases. Whole plant preparations of *S. nigrum* showcased notable effects concerning biological activities (Gasti et al., 2020). An array of organic acids identified in the entire plant of *S. nigrum* entails citric acid, tartaric acid, malic acid, and acetic acid (Yuan et al., 2018). Citric acid and tartaric acid have a crucial antioxidant role by mitigating oxidative damage and portraying adaptive properties in response to environmental challenges (Han et al., 2021). A multitude of studies have stated the presence of glycoalkaloids in *S. nigrum*, noting that solanine is found in substantial quantities in whole plants and fruits, which may be consumed without negative impacts (Yang et al., 2021). Recent studies show potential novel bioactive compounds in different organic fractions from the *S. nigrum* plant that reveal its key pharmaceutical potential (Nawaz et al., 2022a).

## Antimicrobial (antibacterial)

*S. nigrum* can be used as a drug against infectious agents. Various components of *S. nigrum*, including roots, stems, and leaves, can be deployed against infections by employing numerous solvents and extracts. A number of microbes, such as *Staphylococcus aureus, Pseudomonas putrida, Bacillus megaterium, klebsiella pneumonia, Proteous vulgaris, E. coli* and *Bacillus subtilis* (Figure 3), were tested with extracts of *S. nigrum* and compared with standard drugs (Veerapagu et al., 2018). It has been determined that plants have remarkable antibacterial efficacy against several microorganisms and are more effective than commonly used pharmaceuticals (Abbas et al., 2014). Numerous investigations revealed that the entire plant had better therapeutic potential against various pathogens than leaves or berries alone. It was proposed that the whole plant would be more advantageous for the development of novel medications due to its higher concentration of phytoconstituents that are effective against a wide range of pathogens (Kavishankar et al., 2011; Shin and Eum, 2021). Based on these findings, the whole *S. nigrum* plant is recommended for the preparation of herbal drugs to meet low-cost treatment options (Rani et al., 2017). Different organic extracts (ethanol, methanol, ethyl acetate and n-butanol) of *S. nigrum* have been screened (Nawaz et al., 2021; Nawaz et al., 2022b) particularly for antibacterial activity (Hameed et al., 2017). It was observed that *S. nigrum* has great potential against an array of bacteria.

Methanolic extracts of powdered *S. nigrum* fruit exhibited substantial zones of inhibition (ZOI) against both gram-positive and gram-negative bacteria. Although these concentrations implied considerable ZOI, they are not as effective as the standard antibiotics ampicillin or amphotericin B (Abbas et al., 2014). Extract of black nightshade demonstrated bactericidal efficacy to varied extents, with $MIC_{90}$ values varying from 125 - <500 µg/mL. The extracts from aerial portions exhibited superior antibacterial action against several bacterial strains when compared with extracts from the roots. Typically, extracts from aerial portions have a minimum inhibitory concentration (MIC) between 125 and 250 µg/mL, whereas root extracts typically have a MIC of 500 µg/mL or more. The extracts from the aerial portions impeded the development of endospore-forming bacilli and *E. coli* at MIC of 500 µg/mL (Gębarowska et al., 2022). Solasodine had substantial efficacy against conventional bacteria, with a MIC of 512 µg/mL. Combined with the antibiotics gentamicin and norfloxacin for multidrug-resistant bacteria, it demonstrated a 50% decrease in MIC (da Silva et al., 2022).


**Figure 3.** Antibacterial potential of *S. nigrum.*

**Slika 3.** Antibakterijski potencial *S. nigrum.*

## Antiulcer activity

Fruit and other part of *S. nigrum* are often employed throughout Asia and Africa to manage gastrointestinal problems, as they aid in alleviating different ulcerations, gastritis, gastric inflammatory conditions, and other stomach-related ailments. They provide a safeguarding action against gastritis and have been determined highly efficient in aspirin-induced ulcers in rats. Various extracts of *S. nigrum* were supplied orally and assessed for antiulcerogenic action when compared to the conventional medications ranitidine and sucralfate in the context of gastritis. A significant reduction in Evans blue was noticed in tissues and gastrointestinal contents, signifying less mucosal cell injury and vascular permeability relative to normal medications (Figure 4). Fruits provide a more profound guarding effect than leaves against gastritis, but leaves demonstrate greater protection against the ulcer index (Jainu and Devi, 2004).

The antiulcer action and impact on pro- and anti-inflammatory cytokine levels of *S. tuberosum* L. polysaccharide (STP) in rats demonstrated ulcer healing in 60-80% of instances, compared to 50% in the omeprazole group and 25% in the ranitidine group. Glycan significantly accelerates the wound healing of experimental peptic ulcers in rats. STP seems to regulate pro-inflammatory and anti-inflammatory cytokines. A significant reduction in pro-inflammatory cytokines IL-1b and IFN-γ levels was seen in the polysaccharide group compared to the control group (Generalov et al., 2025). An additional investigation examined the gastro-protective in vitro and in vivo antioxidant properties of *Althaea officinalis* and *S. nigrum* extracts on gastric ulceration induced by pyloric ligation and indomethacin in rats. Pyloric-ligation and indomethacin administration substantially increased the number of ulcers, ulcer index, acid and pepsin production, aggressive factors, and gastric mucosal lipid-peroxide levels. Besides, a decrease in titratable acidity, gastric mucosal nitric oxide, antioxidant levels, and protective factors was noted in conjunction with gastric ulceration (Figure 4). Furthermore, an upsurge in pro-inflammatory cytokine levels and a decline in the expression of cystathionine-β-synthase and heme-oxygenase-1 were observed (Zaghlool et al., 2019).

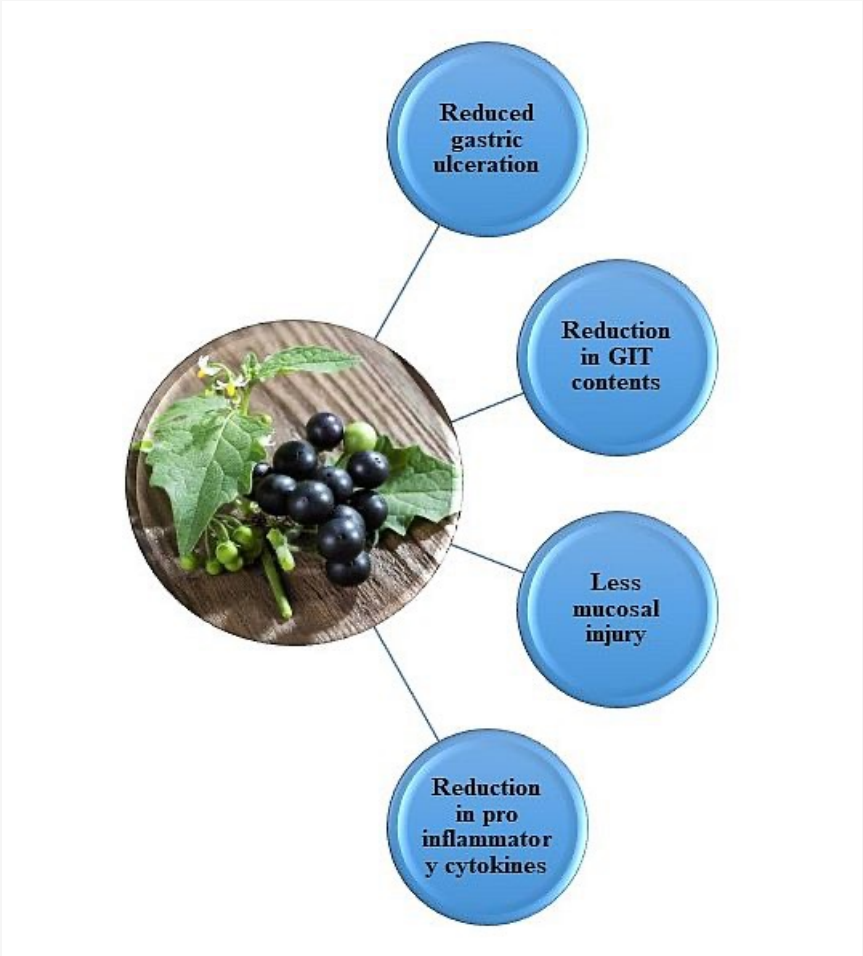


**Figure 4.** Antiulcer activity of *S. nigrum*

**Slika 4.** Protivnetna aktivnost *S. nigrum*

Extracts of *S. nigrum* and certain compounds derived from it have demonstrated anti-inflammatory properties in multiple studies. The impacts might encompass modulation of inflammatory pathways, such as NF-κB, and a reduction in pro-inflammatory cytokines, which can decrease oedema and inflammation in animal models (Boulsmati et al., 2023). Research on *S. nigrum* and ranitidine is limited; however, some studies indicate that *S. nigrum* may offer additional benefits, encompassing anti-inflammatory and antioxidant effects, which do not coincide directly with ranitidine. *S. nigrum* entails several polyphenols, such as flavonoids and phenolic acids, which play a role in its antioxidant capacity. These compounds are inclined to scavenge free radicals and mitigate oxidative stress, thereby protecting cells from damage (Shenbhagan et al., 2022; Saini et al., 2024). Additional research must be conducted to comprehensively assess the potential of *S. nigrum* in conventional medications such as ranitidine.

## Antimycotoxin

There has been a growing interest in reducing the risk of mycotoxins worldwide (Mangalanagasundari et al., 2020). Numerous studies reported the reduction of mycotoxin contamination through the use of *S. nigrum* leaf extract. A substantial quantity of bioactive compounds, including alkaloids, saponins, and tannins, has been determined in *S. nigrum*, demonstrating activity against mycotoxin-synthesizing fungi and inhibiting metabolite production. Various antifungal tests were conducted to evaluate the efficacy and corroborate the findings. Additional research may prove beneficial in identifying natural agents against mycotoxin-producing fungi and in the development of insecticides devoid of harmful effects on various plants and foods (Musto, 2015).

The aqueous extract of *S. nigrum* leaves established antifungal efficacy against mycotoxin-producing fungi

(Musto, 2015; Dar et al., 2017). Some steroidal alkaloids, such as solamargine, solasonine, solanine, and saponin, have been recognised as active compounds with significant antifungal action (Al-Fatimi et al., 2007; Lin et al., 2011). The antifungal efficacy of the extract may also stem from other phytochemical ingredients, including naturally occurring phenol compounds. In many plants, these compounds have been recognised as crucial for curbing development and toxin synthesis by toxigenic fungi (Palumbo et al., 2007; Romero et al., 2009). Keukens et al. (1995) identified that saponins can bind with sterols in fungal cell membranes, resulting in a loss of membrane integrity. Likewise, phenolic compounds are believed to influence cell wall permeability, the release of intracellular components (Figure 5), and the disruption of membrane functionality (Al-Reza et al., 2010). Furthermore, phenolic acids can impede mycotoxin synthesis by mitigating the oxidative stress that initiates or amplifies toxin generation (Mahoney and Molyneux 2004). The exact processes via which the phytochemical components of the extract prevent fungal development remain incompletely elucidated.

## Cardioprotective effect

Myocardial infarction is one of the most significant syndromes associated with elevated death rates worldwide among metabolic disorders. Numerous plants have been documented lately for their cardioprotective properties and may be the preferred therapy option. Many in vivo experiments were performed to ascertain the cardioprotective properties of *S. nigrum*. Several extracts of *S. nigrum* were administered to mice over several days, compared to the conventional medication (Shaik et al., 2015). Additional parameters were assessed, including ECG serum indicators, enzyme levels, body weight, and heart weight. *S. nigrum* extracts exhibited enhanced cardioprotective effects by significantly reducing serum markers SGPT, SGOT, cholesterol, CK, LDH, and triglycerides (Figure 6). *S. nigrum* extracts significantly sustained mice's heart and body weight, which increased owing to isoproterenol production. *S. nigrum* has exhibited significant cardioprotective properties and may be a prophylactic agent in cardiovascular diseases (Elizalde-Romero et al., 2021).

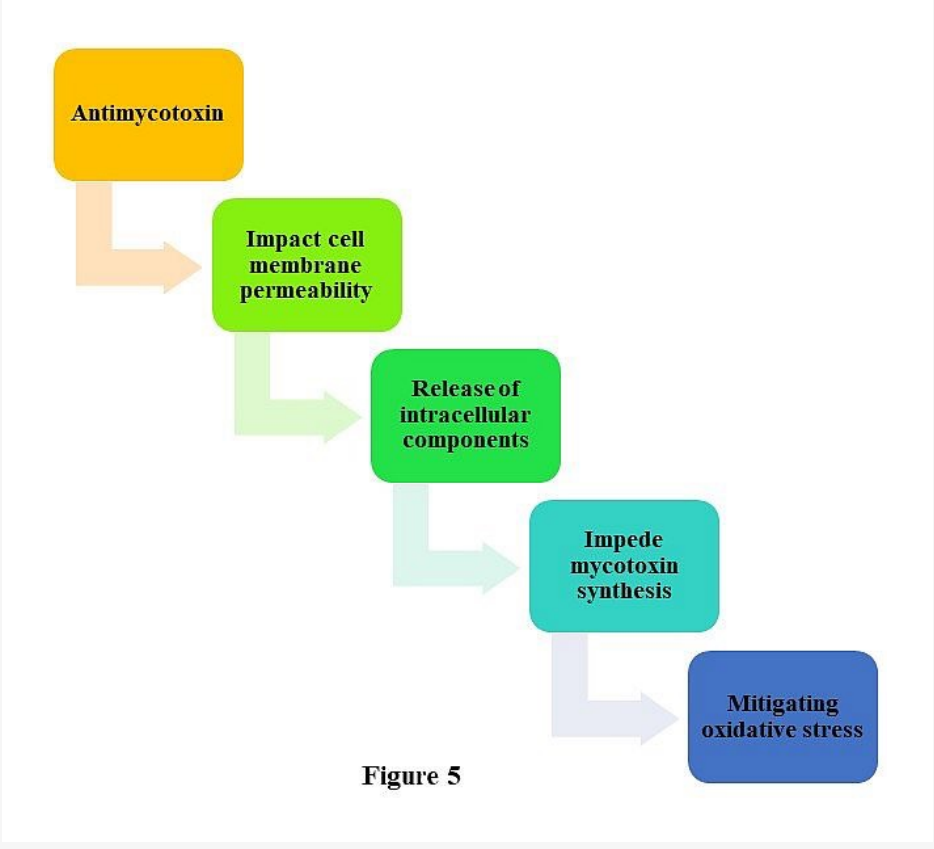


**Figure 5.** Antimycotoxin activity of *S. nigrum.*

**Slika 5.** Antimikotoksinska aktivnost *S. nigrum.*

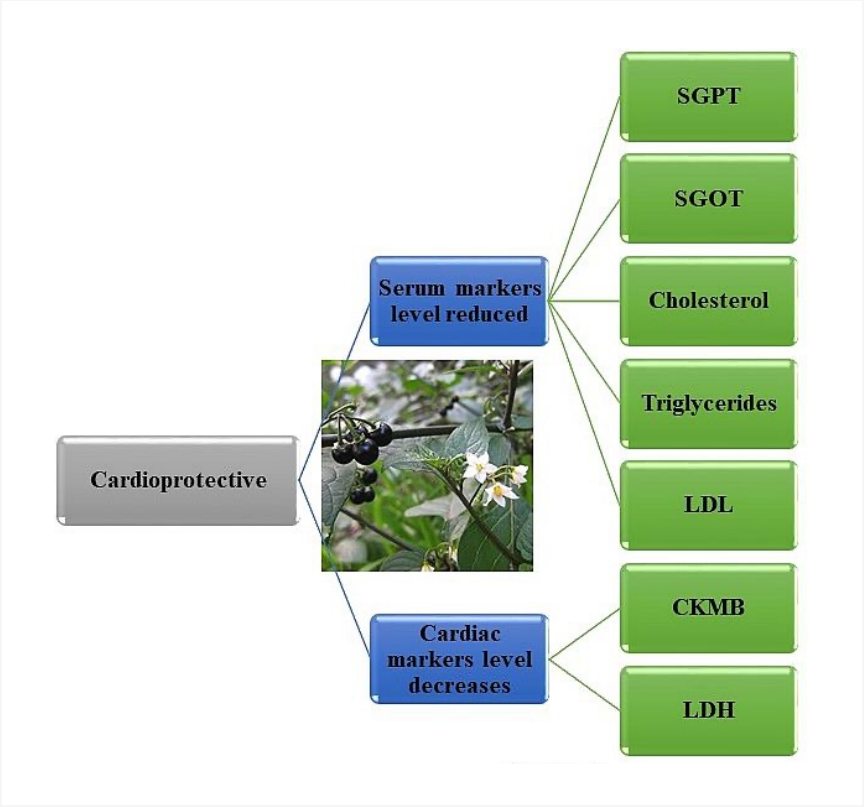


**Figure 6.** Key cardioprotective activity of *S. nigrum* and impact on cardiac markers.

**Slika 6.** Ključna kardioprotektivna aktivnost *S. nigrum* in vpliv na srčne markerje.

In vivo, studies indicated that administration of ethanolic extract of *S. nigrum* fruit (SNE) at doses of 100 mg/kg and 250 mg/kg orally per day for three weeks in fructose-fed rats resulted in a substantial reduction ($P<0.05$) in elevated levels of cholesterol, triglycerides, very low-density lipoprotein, low-density lipoprotein, and atherogenic index, while increasing high-density lipoprotein cholesterol levels and HDL ratio (Paniagua-Zambrana et al., 2024; Ahir et al., 2008). Research demonstrates that *S. nigrum* can profoundly diminish concentrations of cardiac serum markers such as creatinine phosphokinase (CKMB), lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (SGOT), and serum glutamate pyruvate transaminase (SGPT), which serve as indications of myocardial injury. It can safeguard against oxidative stress generated by ischemia-reperfusion, a significant contributor to cardiac injury. Jan et al. (2024), Oner et al. (2023), Varshney et al. (2016).

## Antiviral activity

*S. nigrum* demonstrates diverse antiviral activities and exhibits a heightened capacity to inhibit viral multiplication. Various organic extracts of *S. nigrum* have demonstrated antiviral efficacy with varying IC50 values. These extracts may lessen the viral load by up to 50%, comparable to contemporary therapy methods, particularly for hepatitis (Nyeem et al., 2017). Extracts of *S. nigrum* were also investigated for HCV NS3 protease by the transfection of their plasmid into liver cells. It was noted that with a consistent level of GAPDH, there is a reduction in the function and expression of protease, leading to the conclusion that *S. nigrum* extracts possess an antiviral agent that may serve as a more effective and economical option for the treatment and cure of HIV (Yu, 2004), SARS-CoV-2, SARS-CoV-3 (Choudhury et al., 2020), and HCV (Javed et al., 2011).

Numerous studies have indicated the potential antiviral activities of many phytochemicals, including polyphenols and terpenoids. It highlights the many functions of compounds, including epigallocatechin-3-gallate (EGCG), quercetin, griffithsin (GRFT), resveratrol, linalool, and carvacrol concerning respiratory viral diseases, particularly SARS-CoV-2. Showing their efficacy in regulating immunological responses, altering viral envelopes, and affecting cellular signalling pathways (Goyal et al., 2021). Furthermore,

sugars, saponins, and sterols were also documented. These phytochemicals signify that *S. nigrum* may serve as an effective therapeutic choice for addressing COVID-19 and its subsequent consequences. *S. nigrum* was incorporated into the instructions for Ayurveda practitioners for COVID-19, released by the Ministry of AYUSH, which recommends its consumption as a vegetable in the diet to alleviate COVID-19. Furthermore, in silico investigations of plant phytochemicals targeting numerous SARS-CoV-2 proteins have been beneficial in substantiating the antiviral assertion (Anzoom et al., 2023; Sharma et al., 2023).

## Phytoremediation

Hyperaccumulators provide effective remedies for governing and remediating areas polluted with heavy metals within contemporary environmental plans. It promises cost-effective, environmentally friendly alternatives to sustain a healthy atmosphere with minimal effort and energy conservation (Li et al., 2019b). *S. nigrum* shown a significant capacity to collect cadmium without impacting its height. *S. nigrum* possesses significant potential for remediation and can be cultivated in areas of heavy metal pollution to mitigate ecological damage. It can assist in lessening pollution in industrial cities. Subsequent research may augment its significance for additional heavy metal pollutants and serve as a worldwide environmental modulator (Li et al., 2020).

*S. nigrum* may endure elevated levels of certain metals via several mechanisms, including the augmentation of antioxidant enzyme activity and the sequestration of metals in inactive regions of the plant (Rehman et al., 2017). *S. nigrum*, a prospective cadmium (Cd) hyper-accumulator, exhibits an exceptional capacity for the simultaneous accumulation of Cd, copper (Cu), zinc (Zn), and lead (Pb) in polluted soils. The results from the Cd treatment trials reported increases of 1.66-fold and 1.45-fold in stem and root levels, respectively, coupled with extraction increases of 1.24-fold, 2.17-fold, and 1.61-fold in the leaves, stems, and roots, respectively. The results argue that *S. nigrum* may enhance biomass production and possess a significant capacity to tolerate and accumulate cadmium in soils polluted with lead, zinc, and copper (Yu et al., 2015).

Greenhouse research was done to examine the optimal ratio of organic fertilizer, charcoal, Maifan stone, attapulgite, and phosphate fertilizer combined with *S. nigrum* to achieve maximal phytoremediation effects. The removal rates of heavy metals were highest after the addition of soil amendments, with Zn at 44.0%, followed by Cd at 39.5% and Pb at 36.7%. In conclusion, the outcomes indicate that the efficacy of metal phytostabilization could be enhanced by *S. nigrum*'s synergistic influence (Feng et al., 2020). *S. nigrum*, with the application of biochar/attapulgite as soil amendments, demonstrated enhanced metal removal rates in the following sequence: Cu (39.6%) surpasses Zn (35.0%), followed by Cd (34.1%), Hg (32.1%), Pb (31.8%), and Mn (19.1%) (Li et al., 2019b).

## Nutritional importance

Indigenous vegetables from different countries provide nutritious potential that can aid in minimizing micronutrient deficits in Asian nations. Certain veggies may have antinutritional chemicals. Vegetables from the Solanaceae family are employed as leafy greens and can be consistently utilized to meet nutritional needs across multiple countries (Sangija et al., 2021). These uncommon food crops have not undergone much research regarding their nutritional and antinutritional properties. Various species of vegetables from the Solanaceae family were cultivated in the field, and their leaves were subjected to chemical profiling using LC/ESI-MS (Lakshmi and Kalpana, 2021). Multiple countries excel in fruit cultivation as a primary economic resource. The production capacity is limited owing to inadequate knowledge and insufficient cultivating abilities (Kouadio et al., 2019). Distinct plants generate various fruits, and they differ in size; for instance, *S. nigrum* produces little fruits known as berries. The concentration of hydrogen ions, titratable acidity, soluble solids, proximate composition, mineral content, and assessment of bioactive substances, particularly phenols and antioxidant capacity, were assessed. Furthermore, many assays were conducted to detect reducing and non-reducing carbohydrates. All analyses have demonstrated substantial levels of micronutrients and macronutrients in *S. nigrum* (Reck et al., 2014).

The leaves of *S. nigrum* include substantial quantities of protein and amino acids, minerals such as calcium, iron, and phosphorus, vitamins A and C, fat, and fibre, together with notable levels of methionine, an amino acid rare in other vegetables (Figure 7). The fruit is abundant in iron, calcium, and vitamin B, as well as significant quantities of vitamin C and carotene. The seeds also include vitamin C and carotene. The nutritional levels may fluctuate according to soil fertility, age, and species. The plant's age greatly impacts the protein content of the leaf of *S. nigrum*.

The quantity of ascorbic acid is correlated with both the cooking duration and the volume of water utilized during the cooking process. The species accumulate nitrates and possess oxalates and phenolics (Mohyuddin et al., 2022; Ningthouiam et al., 2024; Asante et al., 2024).

## Anticancer activity

Many plants have been chronicled for their antiproliferative effects across several cancer cell lines (Nawaz et al., 2022b). The cancer-fighting therapeutic activity of *S. nigrum* has been assessed using the MTT test on the hepatoma HepG2 cell line and the cervical HeLa cancer cell line, yielding substantial findings that demonstrate the plant's cytotoxic capability. Various dosages of *S. nigrum* extracts were administered to malignant cells, resulting in the observation of apoptosis across all cell types (Nawaz et al., 2021).

*S. nigrum* exhibits considerable cytotoxic activities since several organic extracts of this particular plant have demonstrated notable antiproliferative capacity against numerous tumour types in diverse global studies. The ethanolic extract of different parts of *S. nigrum* has demonstrated perched cytotoxic activity, with large IC50 values across multiple cell lines (Jabamalairaj et al., 2019). *S. nigrum* has been explored against many malignancies, including lungs, cervical, breast, colon, and hepatic cancer, in multiple trials, demonstrating an antiproliferative activity against all types (Arullappan et al., 2015; Xiang et al., 2018; Campisi et al., 2019; Liu et al., 2019b). Nawaz et al., 2021, highlighted the significant cytotoxic efficacy of *S. nigrum* against HepG2 cells, exhibiting an IC50 value of 7.89 µg/ml, followed by HeLa cells, MCF7 cells, and BHK cells with IC50 values of 19.90 µg/ml, 21.20 µg/ml, and 90.09 µg/ml, respectively. Other fractions had significant anticancer efficacy against HepG2 cells, followed by HeLa cells. In a different investigation, *S. nigrum* extract showed an inhibitory effect on MCF-7 breast cancer cell growth, with an IC50 value of 40.77±4.86 µg/mL, while showing reduced cytotoxicity

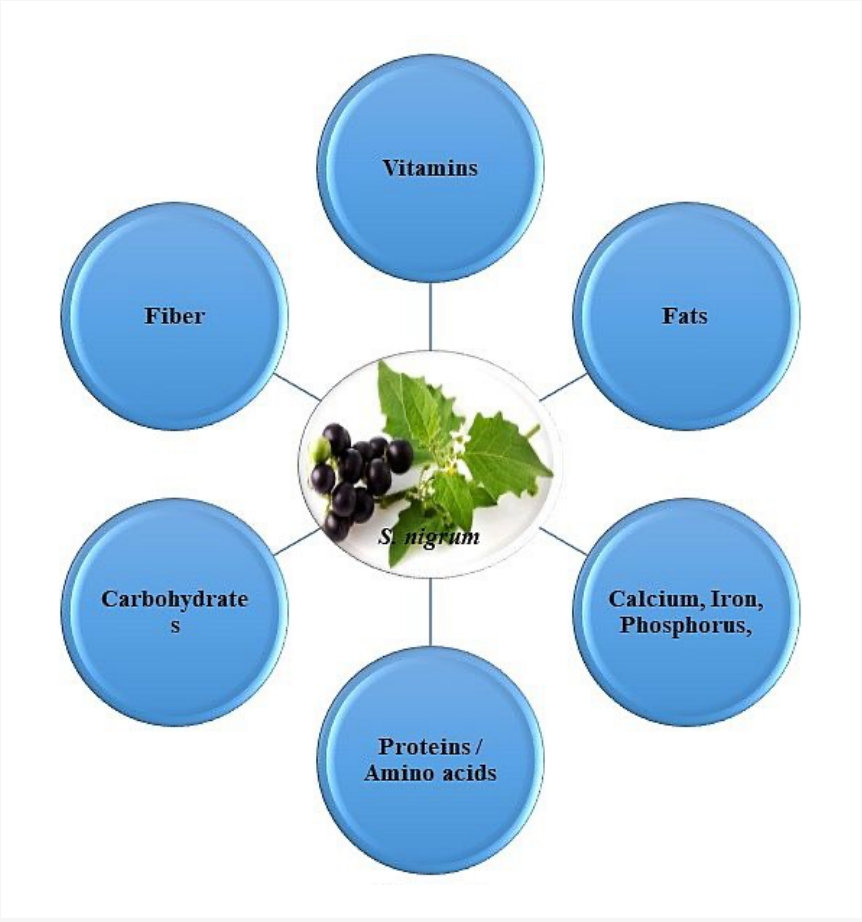


**Figure 7.** Key nutritional elements of *S. nigrum.*

**Slika 7.** Ključni hranilni elementi *S. nigrum.*

towards Vero cells with an IC50 value of 298.96±27.28 µg/mL. The assessment of MCF-7 cell cycles indicated that the extract halted the cell cycle in the S phase and progressed to the G2/M phase at half of the IC50 value (Churiyah et al., 2020). *S. nigrum* suppresses the growth of human gastric cancer cell lines (SNU1/5) with IC50 values of 10 and 12.5 µM, respectively. This may be associated with the upregulation of miR-486-5p by *S. nigrum*, which targets the 3'-UTR region of PI3KR1. Zhang et al. (2020). Li et al. (2022) demonstrated that *S. nigrum* prohibited SGC7901 cell growth in a dose-dependent manner in vitro, with an IC50 of 18 µM, via down-regulating the expression of the essential apoptotic pathway protein Bcl-2/xL, hence triggering apoptosis.

Wang et al. (2020) determined that Degalactotigonin (DGT) is a steroidal glycoside isolated from *S. nigrum*, exhibiting substantial cytotoxicity that mitigated the growth of renal cell carcinoma cell lines (786-O and A498) with IC50 values ranging from 7.52 to 10.21 µM. The anti-tumour action of DGT is connected with the foremost effector protein, yes-associated protein (YAP), inside the Hippo pathway. DGT promotes YAP retention in the cytoplasm of renal cell carcinoma cells by elevating LATS1/2 protein phosphorylation, hence inhibiting YAP-TEAD1 interaction and negatively regulating YAP and its target genes associated with the Hippo pathway. The expression levels of YAP and its target genes AMOTL1, AMOTL2, AXL, CTGF, and CYR61 were diminished in DGT-treated 786-O and A498 cells relative to untreated control cells, therefore impeding cancer cell growth and promoting apoptosis (Wang et al., 2020).

Species of Solanum can modulate the NF-κB pathway, potentially limiting its activation and the related inflammatory processes, which has potential consequences for cancer since the steroidal alkaloid present in *S. nigrum* may inhibit the phosphorylation of ERK1/2, a route capable of activating NF-κB. It may decrease NF-κB activity by obstructing the ERK1/2 and Akt pathways. Solanum may elevate Bax expression, reduce Bcl-2 levels, and lower CD31 expression, thereby limiting tumour growth (Winkeil et al., 2022; Ukwubile et al., 2023). The antiproliferative mechanism of *S. nigrum* relies on its capacity to interfere with the cell membranes of cancer cells by blocking RNA and DNA production. The anticancer efficacy of *S. nigrum* is attributed to its ability to disrupt the structure and function of tumour cell membranes, impede DNA and RNA synthesis, alter cell cycle distribution, hamper the NF-kappa B pathway of apoptosis, and activate caspase cascade reactions (Churiyah et al., 2020).

# Conclusion

*S. nigrum* contains pharmacologically and medicinally significant compounds, including steroids and several alkaloids. *S. nigrum* may be utilized safely for human ailments, as seen by its historical usage in traditional medicine, and it holds promise for contemporary pharmacological advancements due to its diverse therapeutic potential. The plant is widely regarded for several biological activities, including antiviral, hepatoprotective, cardioprotective, anticancer, antibacterial, and anti-ulcer properties. All first investigations were conducted on animals to investigate the pharmacological advantages of the herb. Research on chemicals found in *S. nigrum* will be beneficial for medicinal and industrial purposes.

Multiple considerations are likewise necessary to handle for future views. A substantial volume of research has been conducted on the in vitro and in vivo effects of *S. nigrum* extracts. Crude extracts from *S. nigrum* have medicinal properties, with many components identified, including saponins, alkaloids, polysaccharides, phenolic acids, and flavonoids. The mode of action of the active constituents must be elucidated. Contemporary methods, including efficient preparation, computer virtual screening, and target fishing, can be utilized to trace the active components in *S. nigrum* and elucidate its mode of action. The phytochemical composition and content of the plant should be assessed according to the age of *S. nigrum*. Distinct biosynthetic pathways should be elucidated for *S. nigrum* at various development phases. Toxicological investigations are essential for assessing the safety of herbal medications; nevertheless, knowledge regarding the toxicity of *S. nigrum* remains inadequate. The unripe *S. nigrum* fruit is known to possess significant toxicity, potentially resulting in human poisoning and adverse health effects.

Consequently, it is imperative to conduct systematic toxicity and safety evaluation studies on *S. nigrum* extracts and active constituents to validate the comprehensive use of pharmaceutical resources, adhere to evidence-based medicine standards, and furnish definitive proof for clinical application. By examining the metabolites and metabolic pathways of pharmaceuticals within the body, novel medications with enhanced biological efficacy can be developed. Thus, pharmacokinetic, pharmacodynamic, and toxicological studies are essential in medication development. The processed products of *S. nigrum* necessitate more research, analysis, and utilization.

## Authors contribution

Conceptualization, A.J and A.N.; data curation, A.J and A.N.; writing-original draft preparation, A.J and A.N.; writing-review and editing, A.J and A.N.; supervision, A.A.; M.N.S, data curation. All authors have read and agreed to the published version of the manuscript

## Funding

No government and private organization/institute funded this study.

## Conflict of Interest

All the authors state no conflict of interest.

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ABS

Data Article

# *Noccaea praecox* leaf metabolomes from unpolluted and metal-polluted sites

Matevž Likar [1,*], Valentina Bočaj [2], Paula Pongrac [1,2]

## Abstract

*Noccaea praecox* is a hyperaccumulating plant, i.e. the Cd, Zn, and Pb concentrations in leaves exceed 100-fold the non-toxic levels. In this study, we report the untargeted leaf metabolome of *N. praecox* from an unpolluted site (Lokovec) and a metal-polluted site (Žerjav) in Slovenia. Using UPLC-MS/MS and metabolomic analysis, we identified 7,253 metabolites: 6,659 were non-annotated, and 594 were annotated. Among the identified metabolite classes, carbohydrates, terpenoids, flavonoids, and organic acids were the most dominant. Principal Component Analysis revealed distinct metabolomic profiles between the two *N. praecox* ecotypes, suggesting environmental-related effects in *N. praecox* physiology. This dataset is a valuable resource for studies on plant adaptation, metal accumulation, and metabolite biosynthesis under metal-stress conditions.

## Keywords

Untargeted metabolome; metal hyperaccumulator; metabolomics; metal pollution

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**Citation:** Likar, M., Bočaj, V., Pongrac, P., (2025). *Noccaea praecox* leaf metabolomes from unpolluted and metal-polluted sites. Acta Biologica Slovenica 68 (3)

**Received:** 21.01.2025 / **Accepted:** 11.06.2025 / **Published:** 16.06.2025

**https://doi.org/10.14720/abs.68.3.21668**

## Metabolomi listov *Noccaea praecox* z neonesnaženega in s kovinami onesnaženega območja

**Izvleček**

Rani mošnjak (*Noccaea praecox*) je hiperakumulacijska rastlinska vrsta, t.j. v svojih listih lahko kopiči zelo visoke koncentracije Cd, Zn in Pb. V tej študiji poročamo o netarčnem metabolomu v listih rastlin vrste *N. praecox* iz neonesnaženega (Lokovec) in s kovinami onesnaženega območja (Žerjav) v Sloveniji. Z uporabo UPLC-MS/MS in analize metaboloma smo identificirali 7253 metabolitov: 6659 je bilo neanotiranih, 594 pa anotiranih. Med identificiranimi razredi metabolitov so bili najpogostejši: ogljikovi hidrati, terpenoidi, flavonoidi in organske kisline. Analiza glavnih komponent (PCA) je pokazala razlike v metabolomskem profilu med obema analiziranima ekotipoma vrste *N. praecox*, kar kaže na značilen okoljski vpliv na fiziologijo te vrste. Pridobljen nabor podatkov je dragocen vir za prihodnje študije o prilagajanju rastlin, privzemu kovin in biosintezi metabolitov kot odziv na povečane koncentracije kovin v tleh.

**Ključne besede**

Untargeted metabolome; metal hyperaccumulator; metabolomics; metal pollution

# Objectives

Capturing metabolite composition in leaves of field-collected *N. praecox* from two contrasting habitats, the metal-polluted and the unpolluted one. This dataset (comprising 594 annotated and 7,253 non-annotated metabolites) will support resolving effects of environmental perturbations on the ecology and physiology of *N. praecox*.

# Data description

The dataset represents the untargeted leaf metabolome of *N. praecox* from the unpolluted site in Lokovec (Slovenia) and *N. praecox* from the polluted site in Žerjav (Slovenia). The latter site is characterised by high concentrations of lead (Pb), cadmium (Cd), and zinc (Zn) in the soil (Likar et al. 2009). Feature annotations are presented in a spreadsheet (.xlsx file), which describes metabolites detected in each sample, including their m/z ratios, chemical formulas, ion adducts and molecule identity.

The identified metabolites are representative of various metabolite families of both primary and secondary metabolites, including carbohydrates, terpenoids, flavonoids, alkaloids, organic acids, amino acids, and fatty acyls (Fig. 1).

Principal Component Analysis (PCA) ordination shows distinction in the set of metabolites between *N. praecox* ecotypes from the metal-polluted and the unpolluted site (Fig. 2).

# Value of the Data

- The provided dataset of all metabolites from different ecotypes of *N. praecox* is valuable for researchers interested in studying differences between ecotypes of *N. praecox*.
- The identified metabolites are representative of various metabolite classes, including carbohydrates, terpenoids, flavonoids, alkaloids, organic acids, amino acids, and fatty acyls.
- The metabolite profiles depict differences between the two ecotypes and thus can be of interest to researchers studying metal accumulation in plants with a special focus on hyperaccumulators.

# Experimental Design, Materials and Methods

## Sampling

Three naturally occurring *N. praecox* plants were collected at Lokovec (46°2′39.2706″N, 13°46′8.9934″E) and Žerjav (46°28′26.1258″N, 14°51′56.0118″E). Lokovec is an unpolluted grassland, whereas Žerjav is a metal-polluted grassland site due to centuries-long mining and smelting activity (Likar et al., 2009). A healthy rosette leaf was collected from each plant, flash-frozen in liquid nitrogen for at least 15 minutes,

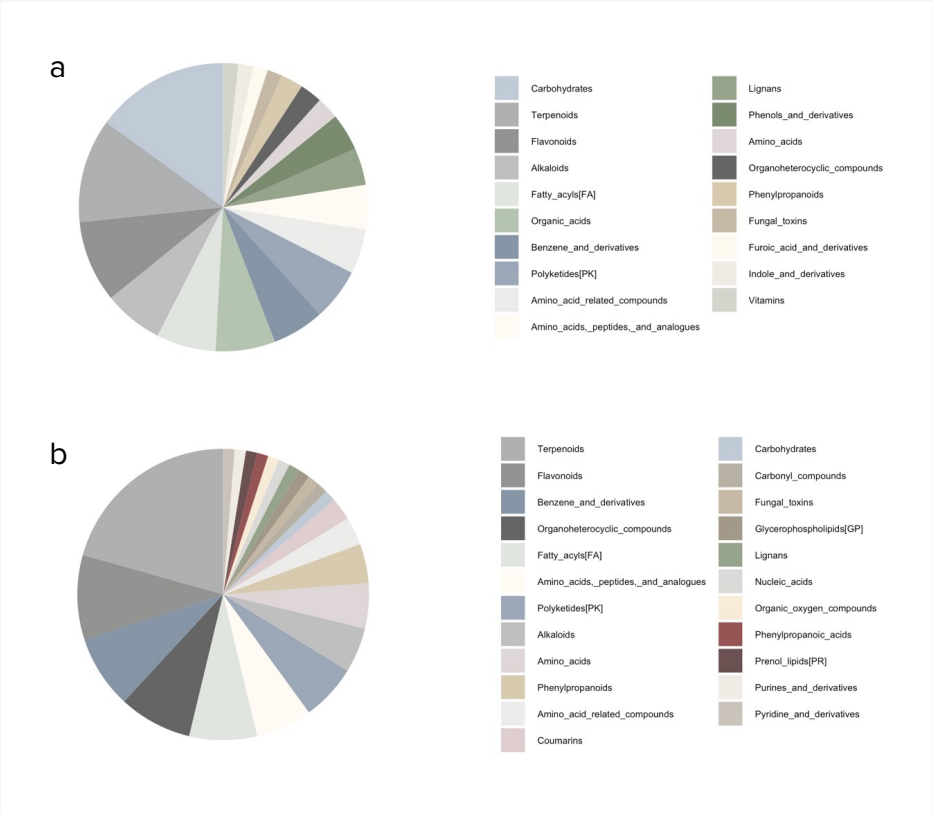


**Figure 1.** Piecharts of metabolite classes in leaves of field-collected *Noccaea praecox* from MS/MS measurement in both a) negative and b) positive ion mode. Only groups with more than one unique metabolite are represented.

**Slika 1.** Diagrami razredov metabolitov vrste *Noccaea praecox* iz meritev MS/MS v a) negativnem in b) pozitivnem ionskem načinu. Predstavljene so samo skupine z več kot enim edinstvenim metabolitom.

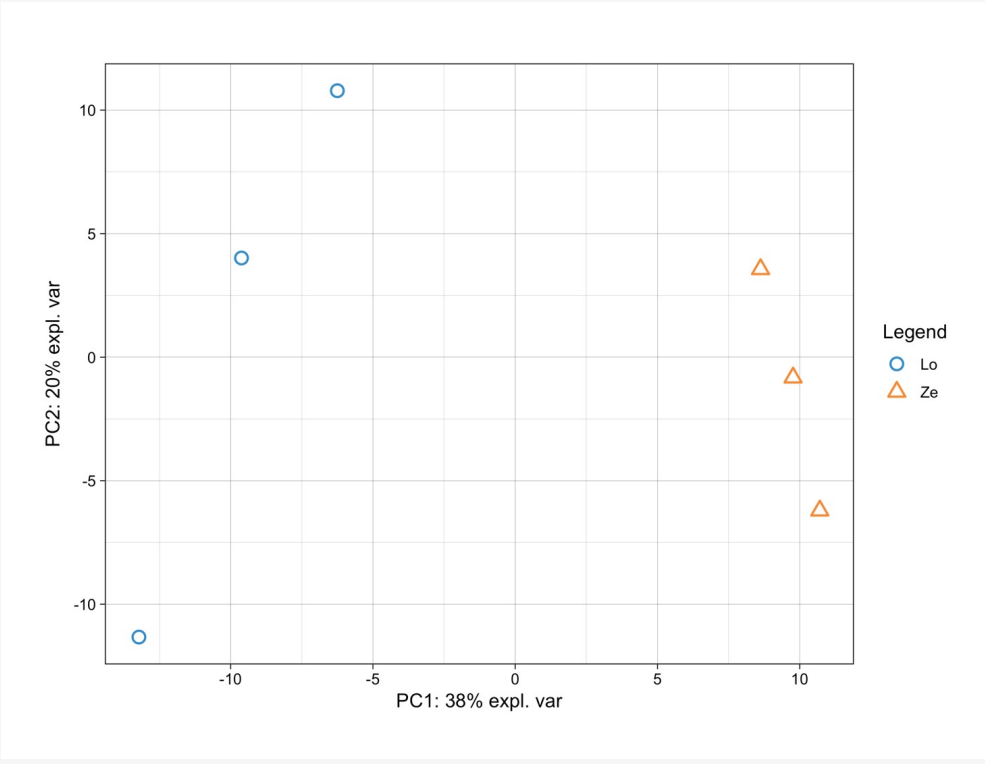


**Figure 2.** PCA ordination for metabolites of leaves of field-collected *Noccaea praecox* from the metal-polluted site Žerjav (Ze) and the unpolluted site Lokovec (Lo).

**Slika 2.** PCA ordinacija za metabolite listov rastlin *Noccaea praecox* z onesnaženega (Ze) in neonesnaženega (Lo) rastišča.

and then crushed into smaller fragments. The samples were freeze-dried for three days at −96 °C and 0.0012 mbar in a Coolsafe (Scanvac) LaboGene system (Allerød, Denmark) and stored at −80 °C before being sent to BGI Tech Solutions (Poland) for untargeted metabolomic analysis.

### Metabolomics

For the analysis, 50 mg of each sample was extracted with 800 µL of a pre-cooled extraction solution (methanol: water = 7:3, v/v) and 20 µL of an internal standard mixture (d3-Leucine, 13C9-Phenylalanine, d5-Tryptophan, and 13C3-Progesterone). The samples were homogenized by grinding and ultrasonication. After incubation at −20 °C for one hour, the extracts were centrifuged at 14,000 rpm for 15 minutes at 4 °C.

Metabolite separation and identification were carried out using a Waters UPLC I-Class Plus system (Waters, Milford, MA, USA) coupled with a Q Exactive high-resolution tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed on a Hypersil GOLD aQ Dim column (1.9 µm, 2.1 × 100 mm; Thermo Fisher Scientific) at a flow rate of 0.3 mL/min and a column temperature of 40 °C. The mobile phase comprised solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The initial mobile phase composition was 5% solvent B, which was held for 2 minutes, then ramped to 95% solvent B over 20 minutes (2–22 min) and maintained for 5 minutes (22–27 min). The injection volume was 5 µL. Mass spectrometry was performed with a scan range of 125–1,500 m/z for positive ions and 100–1,500 m/z for negative ions, using a resolution of 70,000 for MS acquisitions and 30,000 for MS/MS. Fragmentation energies of 20, 40, and 60 eV were applied. The sheath gas flow rate and auxiliary gas flow rate were set at 40 and 10, respectively. Spray voltages for positive and negative ion modes were 3.80 kV and 3.20 kV, respectively. The ion capillary and auxiliary gas heater temperatures were 320 °C and 350 °C, respectively.

Data preprocessing was performed using the metaX library (Wen et al., 2017) in R. Metabolite classification and functional annotation was conducted using the KEGG and HMDB databases, yielding KEGG IDs, HMDB IDs, categories, and associated KEGG pathways.

## Specifications Table

| | |
|---|---|
| Subject | Omics: Metabolomics; Environmental biology |
| Specific subject area | Metabolic differences between ecotypes of N. praecox from polluted and unpolluted sites. |
| Type of data | Excel file |
| How the data were acquired | Metabolite separation and identification were carried out using a Waters UPLC I-Class Plus system (Waters, Milford, MA, USA) coupled with a Q Exactive high-resolution tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). |
| Data format | Excel tables |
| Description of data collection | Leaves of three mature plants were collected per site; 50 mg sample from each plant was extracted with 800 µL of pre-cooled extraction solution (methanol: H2O = 7:3, v/v); chromatographic separation on Hypersil GOLD aQ Dim column on 5-95% water-ACN gradient. The scan range was 125–1,500 m/z for positive ions and 100–1,500 m/z for negative ions, with a resolution of 70,000 for MS acquisitions. For MSMS acquisition, 30,000 was used. Data preprocessing was performed using metaX in R. Classification and functional annotation analysis via KEGG and HMDB databases were performed to obtain KEGG ID, HMDB ID, category, and KEGG Pathway. |
| Data source location | Plant material was collected by Valentina Bočaj at Lokovec (46°2′39.2706″N, 13°46′8.9934″E) and Žerjav (46°28′26.1258″N, 14°51′56.0118″E). |
| Data accessibility | Metabolome data tables were deposited at Zenodo (10.5281/zenodo.10991915). |
| Related research article | Bočaj, V.; Pongrac, P.; Fischer, S.; Likar, M. Species-Specific and Pollution-Induced Changes in Gene Expression and Metabolome of Closely Related Noccaea Species Under Natural Conditions. Plants 2024, 13, 3149. https://doi.org/10.3390/plants13223149 |

## Author Contributions

Conceptualization, M.L. and V.B.; formal analysis, P.P. and V.B.; investigation, V.B., writing—original draft preparation, V.B. and M.L.; writing—review and editing, P.P. and M.L.; visualization, M.L. All authors have read and agreed to the published version of the manuscript.

## Funding

This study was funded by the Slovenian Research and Innovation Agency (research core funding No. P1-0212) and project funding (Lessons from nutrient-use-efficient plants to benefit dietary mineral intake; J4-3091).

## Conflicts of Interest

The authors declare no conflict of interest.

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ABS

Short Communication

# Prevalence and Age-Group Distribution of Malaria in Lokoja and Okene Local Government Areas, Kogi State, Nigeria: A Comparative Study Across Health Centers

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## Abstract

Malaria remains a significant public health challenge, with its prevalence varying across regions. This study assessed the prevalence of malaria in Lokoja and Okene, Kogi State, Nigeria, analyzing its distribution across health centres, age groups, and gender. Out of 322 samples collected, 68.95% tested positive for malaria. Notably, Lokoja Local Government Area (LGA) had a higher prevalence of 38.19%, compared to Okene LGA, which recorded 30.74%. Among individual health centres, Lokoja's Poly Hospital and Body Affairs Medical Laboratory reported positivity rates of 18.94% and 19.25%, respectively, while Okene's General Hospital Okengwe and Tao Hospital had rates of 16.46% and 14.29%, respectively. When examined by age-specific data, Lokoja showed higher prevalence among children aged 4-5 years (17.70%) and those 2-4 years old (13.98%). However, Okene reported the highest prevalence within the 2-4 years age group (17.10%). In terms of gender, the difference in prevalence rates was not significant, with males at 36.65% and females at 32.30%. This study highlights the regional disparities in the malaria burden present within Kogi State and emphasizes the urgent need for targeted intervention strategies, particularly aimed at young children under five. Additionally, further investigation is required to uncover the underlying factors contributing to these regional differences.

## Keywords

Malaria prevalence; Lokoja; Okene; Age-specific distribution; Health center; Gender comparison

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**Citation:** Atawodi, J., Above-Godwin G., Thankgod, A., Wahab, A., Yakubu, S., Yusuf, H., (2025). Prevalence and Age-Group Distribution of Malaria in Lokoja and Okene Local Government Areas, Kogi State, Nigeria: A Comparative Study Across Health Centers. Acta Biologica Slovenica 68 (3)

**Received:** 31.12.2024 / **Accepted:** 28.04.2025 / **Published:** 29.04.2025

**https://doi.org/10.14720/abs.68.3.21543**

## Razširjenost in razporeditev malarije po starostnih skupinah v lokalnih upravnih območjih Lokoja in Okene, zvezna država Kogi, Nigerija: primerjalna študija med zdravstvenimi centri

**Izvleček**

Malarija ostaja pomemben izziv za javno zdravje, njena razširjenost pa se razlikuje med posameznimi regijami. Ta študija je ocenila razširjenost malarije v Lokoji in Okeneju v zvezni državi Kogi v Nigeriji ter analizirala njeno porazdelitev po zdravstvenih centrih, starostnih skupinah in spolu. Od 322 zbranih vzorcev je bilo 68,95 % pozitivnih na malarijo. Zlasti lokalna upravna enota Lokoja (LGA) je imela višjo prevalenco 38,19 % v primerjavi z lokalno upravno enoto Okene, ki je zabeležila 30,74 %. Med posameznimi zdravstvenimi centri so Poly Hospital in Body Affairs Medical Laboratory v Lokoji poročali o pozitivnih rezultatih v 18,94 % in 19,25 % primerih, medtem ko sta General Hospital Okengwe in Tao Hospital v Okene poročala o 16,46 % in 14,29 % primerih. Glede na podatke po starosti je Lokoja pokazala višjo prevalenco med otroki, starimi 4–5 let (17,70 %), in otroki, starimi 2–4 leta (13,98 %). Okene pa je zabeležila najvišjo prevalenco v starostni skupini 2–4 leta (17,10 %). Glede na spol razlika v stopnjah razširjenosti ni bila znatna, saj je bila pri moških 36,65 %, pri ženskah pa 32,30 %. Ta študija poudarja regionalne razlike v bremenu malarije v zvezni državi Kogi in poudarja nujnost ciljnih intervencijskih strategij, zlasti za otroke, mlajše od pet let. Poleg tega so potrebne nadaljnje raziskave, da se odkrijejo temeljni dejavniki, ki prispevajo k tem regionalnim razlikam.

**Ključne besede**

Razširjenost malarije; Lokoja; Okene; Razporeditev po starosti; Zdravstveni center; Primerjava med spoloma

# Introduction

Malaria remains a significant public health challenge in Nigeria, particularly among children under five years of age. Despite ongoing control efforts, the country continues to bear a substantial portion of the global malaria burden. Recent data indicate that Nigeria accounts for approximately 27% of global malaria cases and 31% of malaria deaths, emphasizing the persistent threat of the disease (Report on Malaria in Nigeria, 2022).

Within Nigeria, regional disparities exist, with certain areas exhibiting higher prevalence rates due to factors such as climate, healthcare access, and socioeconomic conditions. Understanding the epidemiology of malaria at local levels is crucial for tailoring effective interventions and reducing the disease burden (Gosling et al., 2020).

Kogi State, located in north-central Nigeria, presents a unique epidemiological landscape for malaria transmission. The state's diverse topography and climate, characterized by numerous water bodies and a tropical environment, create favourable breeding conditions for Anopheles mosquitoes, the primary vectors of malaria (Taiga et al., 2024). Despite this, there is a paucity of recent, detailed data on malaria prevalence within specific Local Government Areas (LGAs) of Kogi State, such as Lokoja and Okene. This lack of localized data poses a challenge to implementing targeted and effective malaria control strategies.

Lokoja and Okene are both Local Government Areas in Kogi State. Lokoja, located at the confluence of the Niger and Benue rivers, presents an ideal ecological niche for *Anopheles* mosquitoes, the vectors of malaria, thereby elevating transmission risk. Okene, on the other hand, although geographically proximate and located within the same state, is a more inland town lacking significant aquatic ecosystems, suggesting a different vector ecology and malaria transmission pattern. The existence of these environmental contrasts is known to influence the prevalence of malaria in some other climes. However, the extent to which these differences affect the occurrence of malaria and its distribution across age groups in these locations is undocumented.

Therefore, this study aimed to determine and compare the prevalence and age-group distribution of malaria

among patients in health centres across Lokoja and Okene LGAs, Kogi State, to assess the influence of local geographic and environmental factors on malaria transmission. A comparative analysis of Lokoja and Okene will also provide critical insights for geographically targeted malaria control strategies that will support evidence-based public health planning.

# Materials and Methods

## Study Area

This research was carried out in the Lokoja and Okene Local Government Areas (LGAs) of Kogi State, Nigeria. Lokoja is situated in North-Central Nigeria at the confluence of the Niger and Benue rivers, with GPS coordinates of latitude 7°48'59.99" N and longitude 6°44'59.99" E. Meanwhile, the Okene LGA is positioned at latitude 7°33'N and longitude 6°14'E. The health facilities chosen for this study included Poly Hospital and Body Affairs Medical Laboratory in Lokoja and Okengwe General Hospital and Tao Hospital in Okene LGA, all within Kogi State.

## Study Population

The study focused on children aged between 0 and 5 years who presented symptoms like fever, headache, muscle and joint pain, cold-like symptoms, abdominal discomfort, diarrhoea, vomiting, and rashes. Those who were referred to the selected hospital laboratories for malaria testing and provided consent were included in the study.

## Sample Collection, Processing and Examination

A total of 322 blood samples were collected from randomly selected children within the age range of 0 to 5 years from both LGAs, with 161 samples sourced from each area. The samples were collected in EDTA vials that were pre-labelled with pertinent patient information, such as name, gender, age, date, and both laboratory and hospital identification numbers.

Thin and thick blood smears were prepared from the collected blood samples on clean, grease-free glass slides. These slides were air-dried with the thin films fixed using absolute methanol. Subsequently, all blood films were stained with a 10% Giemsa solution for 10 minutes and then washed in buffered distilled water with a pH of 7.2. The stained slides were air-dried and examined under high-power magnification (X100 objective) with immersion oil. The number of parasites was assessed by counting them within different fields.

## Data Analysis

Data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software, version 16.0 for Windows. The relationship between infection prevalence and various determining factors was evaluated using the Pearson Chi-square test, with statistical significance determined at a p-value of less than 0.05.

# Results

In this study, the overall prevalence of malaria was found to be 68.95%, with 222 samples out of 322. Specifically, 123 samples (38.19%) tested positive for malaria in the Lokoja Local Government Area, while 99 samples (30.74%) were positive in Okene LGA (Table 1).

Table 2 shows a breakdown by health centres. This table reveals that in Lokoja LGA, Poly Hospital reported 61 positive cases (18.94%), and Body Affairs Medical Laboratory recorded 62 positive cases (19.25%). Conversely, in Okene LGA, General Hospital Okengwe had 53 positive cases (16.46%), and Tao Hospital reported 46 positive cases (14.29%).

Among the 123 positive samples from Lokoja LGA, the age-group analysis showed that children aged 4-5 years exhibited the highest prevalence at 57 cases (17.70%). This was followed by the 2-4 years age group with 45 cases (13.98%), while the 0-2 years age group had the lowest prevalence at 6.52% (21 cases). In Okene LGA, the age-specific positivity rates revealed that children aged 4-5 years had the lowest prevalence at 15 cases (4.70%), followed by the 0-2 years age group with 29 cases (9.00%), and the highest prevalence was found among children aged 2-4 years with 55 cases (17.10%) (Table 3).

Additionally, out of the 166 male participants in the study, 118 (36.65%) tested positive for the malaria parasite, while 104 (32.30%) of the 156 female participants also tested positive (Table 4). However, this difference was not statistically significant.

**Table 1.** Overall Prevalence of Malaria among Children Attending Some Selected Hospitals in Lokoja and Okene Local Government Areas of Kogi State.
**Tabela 1.** Splošna razširjenost malarije med otroki, ki obiskujejo izbrane bolnišnice v lokalnih upravnih območjih Lokoja in Okene v zvezni državi Kogi.

| Sample Location | Positive (+VE) | Negative (-VE) | Total (%) |
|---|---|---|---|
| **Lokoja Local Government** | 123 (38.19%) | 38 (11.80%) | 161 (50.00%) |
| **Okene Local Government** | 99 (30.74%) | 62 (19.25%) | 161 (49.99%) |
| **Total (%)** | 222 (68.95%) | 100 (31.05) | 322 (100%) |

P-value 0.0769

**Table 2.** Prevalence Of Malaria Parasite Among Under Five-Year-Old Children Attending Some Selected Health Centres in Lokoja and Okene LGA.
**Tabela 2.** Razširjenost malarijskih parazitov med otroki, mlajšimi od pet let, ki obiskujejo izbrane zdravstvene centre v Lokoji in Okene LGA.

| Health Centers | Positive (+VE) | Negative (-VE) | Total (%) |
|---|---|---|---|
| **Poly Hospital Lokoja** | 61 (18.94%) | 22 (6.83%) | 83 (25.77%) |
| **Body Affairs Medical Laboratory** | 62 (19.25%) | 16 (4.97%) | 78 (24.22%) |
| **General Hospital Okengwe** | 53 (16.46%) | 30 (9.32%) | 83 (25.78%) |
| **Tao Hospital Okene** | 46 (14.29%) | 32 (9.94%) | 78 (24.23%) |
| **TOTAL(%)** | 222 (68.94%) | 100 (31.06%) | 322 (100%) |

**Table 3.** The distribution of malaria parasites in Lokoja and Okene LGA, Kogi State, is based on age.
**Tabela 3.** Porazdelitev malarijskih parazitov v Lokoji in Okene LGA, zvezna država Kogi, glede na starost.

| Location | Age | Number Examined | Positive (+VE) | Negative (-VE) |
|---|---|---|---|---|
| **Lokoja LGA** | 0-2 | 25 | 21 (6.52%) | 4 (1.24%) |
| | 2-4 | 55 | 45 (13.98%) | 10 (3.11%) |
| | 4-5 | 81 | 57 (17.70%) | 24 (7.45%) |
| **Total** | | 161 | 123 (76.40%) | 38 (11.80%) |
| **Okene LGA** | 0-2 | 45 | 29 (9.00%) | 16 (4.96%) |
| | 2-4 | 83 | 55 (17.10%) | 28 (8.69%) |
| | 4-5 | 33 | 15 (4.70%) | 18 (5.59%) |
| Total | | 161 | 99 (61.5%) | 62 (38.51%) |
| **Total** | | **161** | **99 (30.74%)** | **62 (19.25%)** |

**Table 4.** Distribution of malaria parasite by sex among under-five-year-old children attending some selected health centres in Lokoja and Okene LGA.
**Tabela 4.** Razporeditev malarijskih parazitov po spolu med otroki, mlajšimi od pet let, ki obiskujejo izbrane zdravstvene centre v Lokoji in Okene LGA.

| Sample Location | Male | Female | Total (%) |
|---|---|---|---|
| **Lokoja LGA** | 64 (19.87%) | 59 (18.33%) | 123 (38.20%) |
| **Okene LGA** | 54 (16.78%) | 45 (13.97%) | 99(30.75%) |
| **P-value** | 0.5013 | | |
| **Total** | 118 (36.65%) | 104(32.30%) | 222 (68.95%) |

## Discussion

The observed malaria prevalence of 68.95% in this study is markedly higher than the national average reported in recent surveys. For instance, the 2021 Nigeria Malaria Indicator Survey reported a national prevalence of 22% (Isiko et al., 2024). This significant disparity stresses the need for localized studies to accurately assess malaria burden and tailor interventions accordingly.

The higher prevalence in Lokoja LGA (38.19%) compared to Okene LGA (30.74%) may be attributed to several factors, including environmental conditions conducive to mosquito breeding, such as proximity to water bodies. Lokoja being home to the confluence of Rivers Niger and Benue, would play a crucial role in malaria transmission dynamics by supporting the breeding of mosquito vectors (Taiga et al., 2024).

Age-specific prevalence data reveal that in Lokoja LGA, children aged 4-5 years exhibited the highest prevalence (17.70%), whereas in Okene LGA, the 2-4 years age group had the highest prevalence (17.10%). This aligns with national data indicating that children under five are particularly vulnerable to malaria (Isiko et al., 2024). The slight variations between LGAs could be due to differences in exposure risks, immunity levels, and effectiveness of preventive measures among these age groups.

The disparity in terms of gender-specific analysis revealing a higher prevalence among males (36.65%) compared to females (32.30%) is consistent with other studies that have reported higher malaria prevalence among males, potentially due to increased outdoor activities leading to greater exposure to mosquito bites (Afolabi et al., 2021; Ibrahim et al., 2023). However, cultural and behavioural factors affecting healthcare-seeking behaviours and utilization of preventive measures may also contribute to this disparity.

There appears to be some level of heterogeneity of malaria prevalence across different regions and populations in Nigeria when the results of this study are compared with other studies. For instance, a study in rural Southwestern Nigeria reported a malaria prevalence of 71.1% among adolescents, while another study focusing on children under five reported a prevalence of 36.81% (Shekarau et al., 2024), yet another study conducted by Funwei et al. (2021) reported a malaria prevalence of 73.0% among children under 5 years old in rural areas of south-south, Nigeria. These variations emphasize the importance of localized data in understanding malaria dynamics and implementing effective control strategies.

The high prevalence observed in this study calls for intensified malaria control efforts in the affected LGAs. Interventions should include the distribution and proper use of insecticide-treated nets, indoor residual spraying, community education on malaria prevention, and improved access to prompt diagnostic and treatment services. Additionally, addressing environmental factors that facilitate mosquito breeding, such as stagnant water bodies, is crucial in reducing transmission.

Similar to the current study, other studies in nearby regions, such as Afolabi et al. (2021) in southeastern Nigeria, found that age and gender were strong predictors of malaria prevalence. However, the WHO World Malaria Report (2023) highlights the positive impact of ongoing interventions such as insecticide-treated nets (ITNs) and indoor residual spraying (IRS) in reducing malaria transmission. These interventions could be applied to other communities as part of the control strategies against malaria in the region.

## Conclusions

This study reveals a significantly higher malaria prevalence in Lokoja than in Okene LGA, suggesting that the proximity of Lokoja to the confluence of Rivers Niger and Benue is a major factor affecting the incidence of malaria in Kogi State, Nigeria.

This comparative analysis of Lokoja and Okene LGAs provides critical insights that would be useful for geographically targeted malaria control strategies and supporting evidence-based public health planning because localized malaria control interventions would reduce the disease burden in these communities effectively.

The provision of updated and detailed prevalence data by this study seeks to inform and enhance local malaria control efforts, contributing to the broader national goal of malaria reduction and eventual elimination.

### Author Contributions

Conceptualization, J.A.; methodology, J.A, G.A.; software, J.A, G.A, A.T, S.Y, A.W and H.O.; validation, J.A, G.A; formal analysis, J.A; investigation, J.A, G.A, A.T, S.Y, A.W and H.O.; resources, J.A and G.A.; data curation, J.A.; writing—original draft preparation, A.T, S.Y, A.W and H.O.; writing—review and editing, J.A. and G.A.; visualization, J.A, G.A, A.T, S.Y, A.W and H.O supervision, J.A.; project administration, J.A

funding acquisition, J.A and G.A. All authors have read and agreed to the published version of the manuscript.

## Data Availability

Data will be made available on request.

## Conflicts of Interest

The authors declare no conflict of interest.

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Brief Notes

# Successful purification of DNA from PFGE agarose plugs for whole genome sequencing

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## Abstract

Whole-genome sequencing (WGS) has replaced Pulsed-field gel electrophoresis (PFGE)-based bacterial genotyping as the reference genotyping method. We investigated the suitability of purified genomic DNA extracted from PFGE agarose plugs stored in a laboratory collection for WGS in cases where bacterial isolates are no longer available. Our study has shown that bacterial WGS can be successfully performed on DNA extracted from PFGE agarose plugs.

## Keywords

DNA purification; PFGE plugs; genotyping; bacterial isolates, WGS

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**Citation:** Strašek Smrdel, K., Celar Šturm, A., Triglav, T., Pirs, M., (2025). Successful purification of DNA from PFGE agarose plugs for whole genome sequencing. Acta Biologica Slovenica 68 (3)

**Received:** 18.02.2025 / **Accepted:** 08.04.2025 / **Published:** 25.04.2025

**https://doi.org/10.14720/abs.68.3.21898**

## Uspešno čiščenje DNK iz agaroznih čepkov PFGE za sekvenciranje celotnega genoma

**Izvleček**

Sekvenciranje celotnega genoma (WGS) je nadomestilo tipizacijo bakterij na osnovi gelske pulzne elektroforeze (PFGE) kot referenčne metode genotipizacije. Raziskali smo primernost genomske DNA, očiščene iz agaroznih čepkov PFGE, shranjenih v laboratorijski zbirki, za WGS v primerih, ko bakterijski izolati niso več na voljo. Naša študija je pokazala, da je bakterijski WGS mogoče uspešno izvesti na DNA, očiščeni iz agaroznih čepkov PFGE.

**Ključne besede**

čiščenje DNA, čepki PFGE, genotipizacija, bakterijski izolati, WGS

# Introduction

For decades, pulsed-field gel electrophoresis (PFGE) genotyping has been central to bacterial genome fingerprinting analysis, providing invaluable insights into microbial epidemiology, transmission routes and outbreak investigations. However, the widespread adoption of whole-genome sequencing (WGS) has significantly diminished the relevance of classical genotyping methods such as PFGE, rendering them more or less obsolete. WGS has revolutionised the approach to genotyping bacterial isolates by providing comprehensive genetic information in a single analysis.

If the WGS genotyping includes isolates over an extended period of time, older isolates may no longer be available for various reasons (e.g., equipment failure planned removal of old frozen bacterial samples to make room for newer isolates in the laboratory freezers). In cases where older bacterial isolates are no longer available but have undergone PFGE genotyping, their DNA incorporated into PFGE agarose plugs may still be stored at 4°C.

The sample preparation procedure for both PFGE and WGS genotyping begins with a pure bacterial culture obtained by subculturing a single colony. PFGE agarose plugs contain total bacterial DNA, but different methods have to be used to analyse chromosomal and plasmid DNA separately using the PFGE genotyping method (Barton et al., 1995; Goering, 2010; Matushek et al., 1996). Classical molecular techniques (e.g. polymerase chain reaction, PCR) frequently involve the use of agarose gel electrophoresis to separate DNA fragments (one or more) by their molecular mass and visualise them as band(s) under ultraviolet light by staining the DNA with fluorescent dyes (Hamelin & Yelle, 1990). Various methods for extracting DNA from agarose gels have been described, although purification of DNA from the agarose gel is not always necessary for some PCR reactions (Gao et al., 2021). To the best of our knowledge, there is no publication on the use of preserved purified bacterial DNA from PFGE agarose plugs for WGS.

The aim of our study was to perform WGS directly from bacterial DNA incorporated into PFGE agarose plugs of a number of old *Acinetobacter baumannii* strains. The agarose plugs were between seven and eleven years old, and the original isolates were no longer available.

# Materials and Methods

## PFGE agarose plugs

Forty-five samples of *A. baumannii* genomic DNA stored in the PFGE agarose in a TE buffer at 4°C between seven and 11 years were used. Originally, the stored PFGE agarose plugs were prepared using the following procedure: Using a sterile swab, a standardised suspension of each *A. baumannii* isolate in a buffer (e.g., SE) was prepared. To ensure a sufficient amount of DNA in the agarose plug, the cell density was measured using a spectrophotometer (typically 0.5 and 1.5 A610) or a nephelometer (typically McFarland standard ≥3). A small volume of the cell suspension was mixed with an equal volume of low melting point agarose (typically at 50–55°C) and immediately pipetted into the plug moulds and allowed to solidify. The bacterial cells incorporated into the agarose plugs were lysed using cell lysis buffer with proteinase K (20 mg/ml) at 54 to 55°C with shaking (150 to 175 rpm), usually for 2 to 4 hours. After

lysis, the plugs were first washed with molecular biology grade water and then with TE buffer (5-6 times in total) to remove contaminant compounds. Plugs containing purified whole DNA were transferred to fresh TE buffer and stored at 4°C (Goering, 2010; Leber, 2016; Matushek et al., 1996).

### Purification of DNA from stored PFGE agarose plugs for WGS

For DNA purification, different numbers of agarose plugs were used: protocol 1 used three agarose plugs, protocol 2 used five agarose plugs and protocol three was performed with three agarose plugs.

The DNeasy Blood and Tissue Kit (Qiagen) with slight modifications was used for DNA purification. Three to five agarose plugs per sample were placed in a sterile tube (Table 1); 200 µl AL buffer (Qiagen) was added and incubated at 56°C to melt the agarose gel. After the agarose was completely dissolved, purification was performed with ethanol precipitation and washing according to the manufacturer's instructions. The DNA was eluted in 200 µl (eluted twice with 100 µl, protocol 1) or in 100 µl (reloading of the eluat on the membrane, protocols 2 and 3) of buffer AE (Qiagen). After purification, the concentration and purity of eluted DNA were measured with Qubit dsDNA HS Assay Kit on Qubit 3.0 Fluorometer (Thermo Fischer Scientific) and Nanodrop 2000/2000c Spectrophotometer (Thermo Fischer Scientific), respectively.

### WGS protocol

Before sequencing, the size of the fragments was evaluated using a high-sensitivity DNA kit (Agilent). Short-read sequencing genomic libraries were prepared using Nextera XT Library Preparation Kit (Illumina). Isolates were sequenced on the NextSeq 550 System (Illumina) using 2×149bp paired-end reads chemistry. Fastp v0.23.2 was used (Chen et al., 2018) to trim raw reads of adapter sequences and low-quality reads using the parameters »--correction –cut_right –length_required 30«. The quality of both raw and trimmed reads was assessed using FastQC v0.11.9 (Andrews, 2010). Assembly of trimmed reads into contigs was done with SPAdes v3.15.3 (Bankevich et al., 2012) using the default Kmer values and the "--careful" parameters. Quast v5.2.0 (Mikheenko et al., 2018) and BUSCO (Manni et al., 2021) were used for the quality assessment of the assemblies.

### Reference genome

Reference strain for *A. baumannii* K09-14 (accession number - GCF_008632635.1) was used for WGS analysis (chromosome size 3.972.439 bp).

### Quality control

After sequencing, quality control parameters were checked according to the EURGenRefLabCap protocol »Agreed common WGS-based genome analysis methods and standard protocols for national CCRE surveillance and integrated outbreak investigations« (EURGen-RefLabCap, 2022), including phred quality score Q30, average read size, number of contigs (<500), N50 (>15.000) and genome size.

## Results

DNA was purified from 45 bacterial genomes incorporated into PFGE agarose plugs using three different protocols - protocol 1 (23 samples), protocol 2 (7 samples) and protocol 3 (15 samples). After whole-genome sequencing (WGS), the quality parameters were evaluated (Table 1). The number of agarose plugs has no influence on the purity of the eluted DNA. Therefore, the agarose is efficiently removed. When using a higher number of plugs and a correspondingly higher amount of DNA, as well as lower elution volume, the DNA concentration and some NGS quality parameters (average number of contigs and N50) are higher.

## Discussion

WGS provides comprehensive bacterial genotyping information in a single analysis and is often required for molecular biology research and outbreak investigations. When performing genotyping over an extended period of time, bacterial isolates are sometimes no longer available. If PFGE genotyping has been performed in the past, the DNA agarose plugs of the isolates may still be stored. To determine whether bacterial DNA preserved in PFGE agarose plugs is suitable for WGS, we purified genomic DNA from the plugs using three different approaches varying the number of plugs per sample and the elution volumes. The quality control of the sequencing parameters was consistent for all three purification protocols, and the

**Table 1.** WGS quality control parameters follow the purification of DNA from PFGE agarose plugs according to three different protocols.

**Tabela 1.** Parametri kontrole kvalitete WGS po čiščenju DNK iz agaroznih čepkov glede na tri različne protokole.

| Quality control parameters | Protocol 1 | Protocol 2 | Protocol 3 |
|---|---|---|---|
| N of agarose plugs | 3 | 5 | 3 |
| Volume of elution (µl) | 200 | 100 | 100 |
| N of samples | 23 | 7 | 15 |
| Average concentration (ng/µl) | 1,0 | 6,4 | 2,5 |
| Average purity (A260/280) | 1,8 | 1,9 | 1,9 |
| Average fragment size (bp) | 524 | 549 | 517 |
| Reads passing filter (%) | 92.9 | 93.9 | 92.9 |
| Q30 after filtering (%) | ~94 | ~94 | ~94 |
| Average read size before filtering (bp) | 133 | 134,72 | 135 |
| Average read size after filtering (bp) | ~128 | ~130 | ~130 |
| Genome size compared to reference⁺ genome (bp) | 99.6 | 99.2 | 100 |
| Average number of contigs | 277 | 297 | 275 |
| N50 (bp) | 95.950 | 106.394 | 76.738 |
| BUSCO completeness (%) | 99.7 | 99.7 | 99.7 |

Legend: ⁺ reference strain *Acinetobacter baumannii* K09-14 (accession number - GCF_008632635.1)

purified DNA was of high quality and suitable for effective genome assembly (Table 1).

In summary, all three protocols showed high efficiency in terms of read quality and genome assembly, with minor differences in read size, contig number and N50 values. Protocol 2 yielded the highest N50 value, while Protocol 3 achieved the full expected genome size (EURGen-RefLab-Cap, 2022). All protocols had excellent BUSCO completeness scores, indicating high-quality assemblies.

# Conclusions

Our study has demonstrated that WGS can be successfully performed on bacterial genomic DNA preserved in PFGE agarose plugs, provided an appropriate purification protocol is used to remove inhibitory compounds. This finding expands the possibilities for studying older bacterial isolates, even when viable cultures are no longer available.

## Author Contributions

KSS, TT and MP participated in the conception and design of the study; KSS performed DNA extraction, writing and revision of the manuscript; ACŠ performed sequencing and analysis of sequencing data, revision and editing of the manuscript; MP contributed bacterial genomes in PFGE agarose plugs, writing, revision and editing of the manuscript; TT contributed revision and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

## Funding

This research was supported by the Slovenian Research and Innovation Agency (P3-0083 Host-parasite relationship).

## Conflicts of Interest

Authors declare no competing interest.

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