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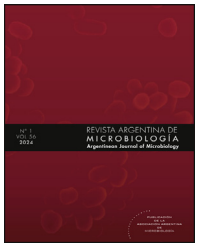

ORIGINAL ARTICLE

Endophytic and rhizospheric bacteria of medicinal plants as sources of bioactive compounds: Antimicrobial and antioxidant potential

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Abstract Endophytes, microorganisms that inhabit internal plant tissues without causing harm, are increasingly recognized as valuable sources of bioactive metabolites with industrial, agricultural, and pharmaceutical relevance. This study aimed to isolate, identify, and evaluate the antimicrobial and antioxidant activities of endophytic and rhizospheric bacteria associated with medicinal plants from North Shewa, Ethiopia. This study hypothesized that endophytic and rhizospheric bacteria associated with medicinal plants from North Shewa, Ethiopia, produce bioactive compounds with significant antimicrobial and antioxidant potential. We hypothesized that these bacterial communities synthesize metabolites capable of inhibiting test microorganisms and scavenging free radicals. Isolation was carried out from sterilized leaves and roots, as well as from root washes, on nutrient agar at 30 °C for 72 h. The isolates were first screened via the perpendicular method and then via the disc diffusion method. Fermented broths were extracted via solvents. Among the 104 isolated microbes, including 89 endophytic and 15 rhizospheric microbes, 29 showed antibacterial activity. The main bioactive-producing isolates were *Pseudomonas oleovorans*, *Ligilactobacillus salivarius*, *Bacillus subtilis*, and *Pseudomonas fluorescens*. Notably, *P. oleovorans* was effective against *Streptococcus epidermidis* (13.7 mm) and *Staphylococcus aureus* (12 mm), with inhibition zones ranging from 8 to 13.7 mm

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for all the tested organisms. The antioxidant activity ranged from 15.42% to 22.57%, with the highest value observed for *P. oleovorans* (22.57%). These findings highlight the potential of endophytic and rhizospheric bacteria as promising and sustainable sources of bioactive compounds.

PALABRAS CLAVE
Endófito;
Rizosfera;
Planta medicinal;
Compuestos bioactivos;
Antioxidante

Bacterias endofíticas y rizosféricas de plantas medicinales como fuentes de compuestos bioactivos: potencial antimicrobiano y antioxidante

Resumen Los endófitos, microorganismos que habitan los tejidos internos de las plantas sin causar daño, son cada vez más reconocidos como valiosas fuentes de metabolitos bioactivos con relevancia industrial, agrícola y farmacéutica. Este estudio tuvo como objetivo aislar, identificar y evaluar las actividades antimicrobianas y antioxidantes de bacterias endófitas y rizosféricas asociadas con plantas medicinales de Shewa del Norte, Etiopía. Este estudio planteó la hipótesis de que estas bacterias producen compuestos bioactivos con un importante potencial antimicrobiano y antioxidante. Planteamos la hipótesis de que estas comunidades bacterianas sintetizan metabolitos capaces de inhibir a los microorganismos de prueba y eliminar radicales libres. El aislamiento se realizó a partir de hojas y raíces esterilizadas, así como de lavados radiculares, en agar nutritivo a 30 °C durante 72 horas. Los aislados se analizaron primero mediante el método perpendicular y luego mediante el método de difusión en disco. Los caldos fermentados se extrajeron con disolventes. De los 104 microbios aislados, incluidos 89 endófitos y 15 rizosféricos, 29 mostraron actividad antibacteriana. Los principales aislados productores de bioactivos fueron *Pseudomonas oleovorans*, *Ligilactobacillus salivarius*, *Bacillus subtilis* y *Pseudomonas fluorescens*. Cabe destacar que *P. oleovorans* fue eficaz contra *Streptococcus epidermidis* (13,7 mm) y *Staphylococcus aureus* (12 mm), con zonas de inhibición de entre 8 mm y 13,7 mm para todos los organismos analizados. La actividad antioxidante osciló entre el 15,42% y el 22,57%, siendo la más altas la de *P. oleovorans* (22,57%). Estos hallazgos resaltan el potencial de las bacterias endofíticas y rizosféricas como fuentes prometedoras y sostenibles de compuestos bioactivos.

Introduction

The escalating global public health crisis of antimicrobial resistance (AMR), driven by the overuse and misuse of antibiotics, necessitates the urgent discovery of novel chemotherapeutic agents. The rate of new antibiotic development has failed to keep pace with the emergence of multidrug-resistant pathogens, often referred to as 'superbugs', creating a critical need to explore unconventional sources for biologically active molecules[36]. Plants and their associated partners in various symbiotic relationships are potential sources of novel antimicrobial compounds[5,34].

Aromatic and medicinal plants are crucial in global healthcare, with more than 80% of people using them for medicinal purposes[56]. Medicinal plants are key sources of bioactive compounds essential for survival and potential drug production. These plants produce a diverse range of bioactive secondary metabolites that are vital for drug development and plant adaptation to unique environments. However, the production of these metabolites typically requires a large amount of botanical material, which risks the rarity and conservation of these plants[19]. Many medicinal plants are currently threatened by human activity, ecosystem degradation, and overuse, particularly those whose roots are harvested[17].

Plants participate in ecosystems through relationships with microorganisms, including endophyte, bacterial, fungal, rhizosphere, and epiphytic interactions[47]. Endophytes are microorganisms that naturally inhabit the internal tissues of plants and are found in all plant species. These microorganisms synthesize therapeutic bioactive metabolites and thrive in plant tissues, such as roots, stems, seeds, fruits, and leaves. Endophytes increase plant growth and produce metabolites that can be used to improve the health of humans, animals, and plants, as well as to control diseases. Endophytes produce various antimicrobial substances, such as phenols and peptides, which offer promising solutions to the growing crisis of drug resistance[45]. The relationship between the host plant and endophyte is often symbiotic, in which the plant provides nutrients and shelter, and the microbe provides essential compounds for defense against pathogens, competition, and environmental stress. This intimate interaction makes endophytes a rich source of novel chemical compounds, as they often mimic or increase the production of the host plant's own medicinal compounds[39].

Endophytes offer a promising and sustainable alternative to whole-plant harvesting for metabolite production. They can independently produce plant-like bioactive compounds[4] while controlling a plant's genetic functions, preventing oxidative stress, synthesizing biocontrol substances, removing heavy metals, and improving the quality and quantity of plant-derived metabolites[49]. Therefore, targeting the culture of endophytes for compound production is a preferable approach for both drug discovery and plant conservation.

Despite their importance, limited research has been conducted on the endophytes associated with medicinal plants in Ethiopia[33]. This study was conducted in North Shewa, Ethiopia, a region known for its rich biodiversity. In general, North Shewa, Amhara, is a hotspot for medicinal plant diversity and traditional healing knowledge, with communities relying on a wide range of species for health care[7,31,32]. The plants selected for this study, including *Rumex nepalensis*, *Echinops kebericho* Mesfin, *Sisymbrium orientale*, *Urtica simensis*, and others, are well-known traditional medicinal species in the region[8,31], which increases the probability of isolating microbes with potent bioactive compounds. A key selection criterion for these plants is their extensive use in traditional medicine, particularly for treating infectious and inflammatory diseases, suggesting that the associated microorganisms have coevolved to produce compounds effective against the pathogens the plant is used to combat[11].

The root of *R. nepalensis* is traditionally used in Ethiopian folk medicine to treat ailments such as ascariasis (intestinal worms) and peptic ulceration and is often administered as an oral root decoction[32]. *E. kebericho* Mesfin is a highly valued, endemic, and threatened medicinal plant in Ethiopia that is often cited in ethnobotanical studies across the country. The root of this plant is widely used in traditional medicine[53]. Oral administration is specifically used for stomachache, tonsillitis, and abdominal bleeding[3]. It is often used for fumigation to treat conditions such as headache, cough, stomachache, febrile illness, and the common cold[46]. *U. simensis* is a crucial wild edible green vegetable[43]. Various parts of *U. simensis* are employed in traditional medicine to treat conditions such as gastritis, heart disease, diabetes, gonorrhea, and malaria[46].

In addition to human alignment, *Rumex nepalensis*[7], *Sideroxylon oxyacanthum*, and *Inula confertiflora*[48] are used to treat livestock diseases. Leaves and roots are the most commonly used plant parts, with remedies typically prepared by crushing, pounding, or powdering and administered orally or dermally. The focus on North Shewa is significant because local traditional knowledge guides plant-part selection, ensuring that the isolation process targets the specific tissues used medicinally.

This investigation was based on the central hypothesis that endophytic and rhizospheric bacteria associated with medicinal plants in North Shewa, Ethiopia, represent valuable reservoirs of bioactive microorganisms. Specifically, it was postulated that these bacterial isolates synthesize secondary metabolites with pronounced antimicrobial activity against a defined panel of test microorganisms and that their crude extracts exhibit measurable antioxidant properties, as evidenced by their capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The overarching expectation was that certain isolates would be identified as producers of broad-spectrum bioactive compounds with strong antimicrobial and antioxidant activities, thereby positioning them as promising and sustainable candidates for drug discovery. In line with these premises, this study aimed to (1) isolate and identify culturable endophytic and rhizospheric bacteria from selected medicinal plant species; (2) screen the isolates for antagonistic activity via the perpendicular cross-streak method; (3) determine the antimicrobial activity of crude extracts from the most active isolates via the disc diffusion assay; and (4) evaluate the antioxidant activity of their culture supernatants via the DPPH radical scavenging assay.

Materials and methods

Collection, identification, and preparation of plants

In total, 18 medicinal plants were collected from the three districts (*Thymus schimperi*, *Rumex nervosus*, *Echinops kebericho*, and *Inula confertiflora* from Ankober district; *Rumex nepalensis*, *Malva verticillate*, *Artemisia abyssinica*, *Sisymbrium oriental*, *Datura stramonium*, *Dodonea angustifólia*, *Urtica simensis*, *Plantago lanceolata*, and *Solanum marginarum* from Debre Berhan district; *Cucumis ficifolius*, *Carissa spinarum*, and *Foeniculum vulgare* from Meniz Mama Midir district). Healthy plant samples were collected from each district, and the plants were taxonomically identified by a botanist at Debre Berhan University. The medicinal plants were selected based on direct consultation with people with indigenous wisdom, observation of the knee, and the response of traditional medicine practitioners[8], as well as a review of the literature. The appropriate plant parts were collected, placed in sterile polyethylene bags and stored at 4 °C during transportation to the laboratory. The plants were processed and tested for endophytic and rhizospheric bacteria within 48 h after collection.

Isolation, purification, and maintenance of the bacterial isolates

The plant samples were thoroughly washed under running tap water to remove any remaining debris, washed with distilled water, and cut into 2-cm-long pieces using sterile scissors. Surface sterilization was performed by repeatedly rinsing five times with sterile water, 70% ethanol for 3 min, and 2.5% sodium hypochlorite for 4 min, after which the samples were aseptically rinsed again with sterile water[33]. After the last wash of the plant material, a portion of the spent water was transferred to solidified nutrient agar medium and used as a negative control.

The surface-sterilized plant species were placed on nutrient agar to isolate the target microorganisms. To isolate rhizosphere bacteria, 0.1 mL of the root wash suspension was spread onto nutrient agar plates. Root wash is the liquid suspension obtained by washing plant roots to remove rhizospheric soil and microorganisms. Each sample was plated in triplicate, and the plates were incubated at 30 °C for 72 h. Purification was performed by streaking on nutrient agar until pure colonies were obtained. The purified bacterial iso-

lates were maintained on nutrient agar slants and stored at 4 °C for further characterization.

Test microorganisms and their inoculum preparation

Eight human pathogenic bacteria, namely, Gram-positive bacteria (*Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25923, *Streptococcus pyogenes* ATCC19615, *Streptococcus epidermidis* ATCC12228), Gram-negative bacteria (*Escherichia coli* ATCC25922, *Klebsiella pneumonia* ATCC700603, *Salmonella serotype Typhimurium* ATCC13311), and yeasts (*Candida albicans*), which were obtained from the Ethiopian Institute of Health and Nutrition Research Institute, were used as test microorganisms to evaluate the antimicrobial activity of secondary metabolites of endophytic and rhizospheric bacterial isolates.

Pure colonies of the test microorganisms were inoculated into test tubes containing 5 ml of sterile nutrient broth until the turbidity of the 0.5 McFarland standard (a mixture of 0.5 ml of 1.17% w/v BaCl solution into 99.5 ml of 1% v/v solution) was achieved. The turbidity of the actively growing cultures was attained with sterile nutrient broth to obtain 0.5 McFarland and a suspension equivalent to 1.5×10^8 CFU/ml[28].

Primary screening of the isolate for antimicrobial activity

Primary screening was performed via the perpendicular cross-streak method[44]. The isolates were streaked across the center of the nutrient agar plates and incubated at 30 °C for 72 h. Later, the test microorganisms (*S. aureus*, *L. monocytogenes*, *S. epidermidis*, *E. coli*, *K. pneumonia*, *Salmonella ser. Typhimurium*, *S. pyogenes*, and *C. albicans*) were individually streaked perpendicularly to the bacterial isolates without touching them and incubated at 37 °C for 24 h. Antagonism was measured by measuring the extent of inhibition.

Secondary screening of isolates for antimicrobial activities

Isolates that were positive in the primary screening were selected for secondary screening. Secondary screening of the isolates was performed via disk diffusion. A single colony of each selected bacterial isolate was harvested and inoculated in 50 ml of nutrient broth medium in 250 ml Erlenmeyer flasks, followed by incubation with continuous shaking at 30 °C for 72 h at 200 rpm. The supernatants and sediments were separated after the culture broths were centrifuged at 4500 rpm for 20 min. The supernatants were collected for the antimicrobial activity test. A 0.1 ml aliquot of the test microorganisms was spread on Mueller–Hinton agar media until the aliquot was uniformly distributed on the plates. A 6 mm diameter sterile paper disk was impregnated with the extract and loaded onto the inoculated Muller–Hinton agar medium, with a negative control nutrient broth and positive control gentamicin. The plates were incubated at 37 °C for 24 h, and the inhibition zone was measured[22].

Characterization and identification of selected isolates

The characterization and identification of the selected isolates were carried out via biochemical and physiological tests, including cell shape, motility, catalase, oxidase, citrate utilization, triple iron sugar, and carbohydrate fermentation tests[24]. The purified isolates were subjected to Gram staining and microscopic observation.

MALDI-TOF for the identification of bacteria

Endophytic and root wash isolates were identified via laser-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Pure colonies of the isolates were picked and placed on 96 microscout plates (Bruker Daltonics). The wells of the plate were coated with a thin layer of colonies and allowed to dry. Then, 1 μl of α-cyano-4-hydroxycinnamic acid solution (12.5 mg per 1 ml of a mixture of 50% acetonitrile and 2.5% trifluoroacetic acid) was added. After drying, the MALDI 96 microscout plates were inserted into the MALDI-TOF MS device. A mass between 2000 and 20 000 Da in linear positive ion mode was selected for the device. The ion source was a 337 nm wavelength nitrogen laser operating at 60 Hz. To measure each colony, 40 packets of 240 laser pulses were delivered[13].

Fermentation and extraction of bioactive compounds

Bioactive compounds are produced via submerged fermentation. The bacterial isolates were cultured in nutrient broth and incubated at 30 °C and 200 rpm for 96 h. Separation was carried out via centrifugation at 4500 rpm for 20 min, and a cell-free supernatant was collected in sterile Erlenmeyer flasks. The mixture was subsequently macerated with ethyl acetate, hexane, and a mixture of chloroform and hexane (1:1, v/v) for three days with agitation. The ratio of the fermented broth to the solvent was 1:2 (v/v). These mixtures were then allowed to evaporate in a rotary evaporator operating at 40 °C and 110 rpm until the dried residue was collected as a crude extract. The crude extracts were preserved at 4 °C until further analysis, and the extract yields were calculated. Bioactivity was tested via disc diffusion.

Antimicrobial assay

A concentration of 50 mg/ml of each extract was used to test the antimicrobial activity via the disc diffusion assay. The solution for the antimicrobial assay was prepared by dissolving 50 mg of the crude extract in 1 ml of dimethyl sulfoxide (DMSO). Then, 0.1 ml of 0.5 McFarland turbidity standard of the test microorganisms (*L. monocytogenes*, *S. aureus*, *S. pyogenes*, *S. epidermidis*, *E. coli*, *K. pneumonia*, *Salmonella ser. Typhimurium*, and *C. albicans*) was swabbed on Mueller–Hinton agar.

Sterilized 6 mm diameter Whatman paper discs were impregnated with 20 μl of crude extract and placed on the surface of inoculated Muller–Hinton agar plates. Standard gentamicin antibiotic discs were placed on media as a pos-

itive control, and DMSO was used as a negative control. Finally, all the plates were incubated at 37 °C for 24 h. The clear zone of inhibition around the disc was measured in millimeters. The experiment was performed in triplicate, and the mean value of each test isolate was recorded.

Antioxidant activity of the metabolites of the isolates

The antioxidant activity of the metabolites was measured via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The DPPH radical scavenging activity was assayed according to the method described by Shimada[42] with some modifications. One milliliter of DPPH solution (96 mg/l DPPH in methanol) was mixed with 1 ml of the microbial extract. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 1 h. The absorbance of the mixture was measured at 517 nm. Ascorbic acid and DPPH without any samples were used as standards and control references during the absorbance measurements. The percentage of inhibition of DPPH radical scavenging activity was calculated via the following formula:

$$\%\,\text{of inhibition} = \frac{\text{Absorbance of DPPH} - \text{Absorbance of sample}}{\text{Absorbance of DPPH}} \times 100$$

Data analysis

Process optimizations for bioactive compounds and isolate growth were computed via descriptive statistics, and the results are displayed in tables and graphs. Each experiment was performed in triplicate, and the results are expressed as the means ± SDs via Microsoft Excel 2019. The significance of the differences in antimicrobial and antioxidant activities between the isolates and treatments was determined via analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Endophytic and rhizosphere bacterial isolates

A total of 104 bacterial isolates were obtained from the 18 medicinal plant samples collected, comprising 89 endophytic and 15 rhizospheric isolates. The observed diversity aligns with prior reports that plant-associated microbes exhibit ecological and metabolic variations that are reflective of host plant diversity[37]. Recent metagenomic studies have further confirmed that the microbiome of medicinal plants is uniquely structured by host phylogeny and environmental factors, which in turn shape their biosynthetic potential for novel antimicrobial agents[20]. These findings suggest that the rich biodiversity of medicinal plants in the North Shewa region of Ethiopia is a promising source for isolating microbes with potential bioactive compounds.

Primary screening of the bacteria for antimicrobial activity

Among the 104 bacterial isolates, 29 (27.88% of the endophytic isolates and 2.88% of the rhizosphere isolates) exhibited antagonistic activity in primary cross-streak screening, inhibiting at least one of the eight test microorganisms. The extent of inhibition varied substantially among the isolates, with inhibition distances ranging from 5 to 27.7 mm (Table 1).

Primary screening of the isolates revealed substantial variability in their antimicrobial capacity. Some isolates (AK51, AK2, DBSM4, and DBSK) strongly inhibited *S. aureus*, suggesting the presence of potent antistaphylococcal metabolites. Similarly, the inhibition of *S. epidermidis* was restricted to a minority of the isolates (GER4 and DBSM4), indicating that antagonistic activity against coagulase-negative staphylococci is less common among the tested strains. These findings are consistent with broader observations that bacterial natural products remain a rich source of antibacterials, especially against Gram-positive pathogens[35]. Notably, endophytic *Bacillus* and *Pseudomonas* spp. are prolific producers of antistaphylococcal lipopeptides and cyclic peptides, which are promising candidates for treating skin and soft-tissue infections[52].

Some isolates also inhibited Gram-negative bacteria such as *E. coli* and *Salmonella ser. Typhimurium* and, in fewer cases, also *K. pneumoniae*. For example, AT21, DbTR1, DBSM4, and DBSK inhibited *E. coli* and *Salmonella ser. Typhimurium*, whereas AK51, AT31, and DBSM4 inhibited *K. pneumoniae*. Given the intrinsic permeability barrier posed by the outer membrane (OM) of Gram-negative bacteria, these results are encouraging, suggesting that certain isolates may produce compounds capable of crossing or disrupting the OM. Recent reviews highlight renewed efforts to target the Gram-negative outer membrane barrier or develop compounds (e.g., novel peptides, lipopeptides) that can overcome it[29].

The antifungal activity against *C. albicans* was more limited, with only GER4, AK51, and DBSM4 showing noticeable inhibition. This pattern mirrors reports in the recent literature showing that while bacteria remain a major reservoir for antibacterials, fewer bacterial metabolites show antifungal activity, and the discovery of such compounds remains more sporadic[16]. However, recent research has shown that endophytic *Lactobacillus* species can produce antifungal biosurfactants and cyclic dipeptides that are effective against *Candida* biofilms, suggesting untapped potential in lactic acid bacteria from plants[25]. Overall, isolates such as AK51, DBSM4, and DbTR1, which show broad-spectrum activity against Gram positive, Gram negative, and fungal targets, stand out as promising candidates for further study, fractionation, and chemical characterization.

Biochemical characterization and identification of the isolate

The results of the biochemical characterization and identification of the isolates are presented in Table 2. The isolates SM4, AK51, SK, and DbTR1 were classified as *Ligilactobacillus salivarius*, *P. fluorescens*, *B. subtilis*, and *P.*

Table 1 In situ microbial susceptibility tests were performed using perpendicular screening methods against test microorganisms, as indicated by the inhibition distance in mm (mean ± standard deviation), where NA refers to no activity. Values sharing different superscript letters in a column are significantly different at $p < 0.05$.

Isolate code	Isolated from	Microbial test organisms							
		S. aureus	*E. coli*	*Salmonella ser. Typhimurium*	*L. monocytogenes*	*S. epidermidis*	*S. pyogenes*	*C. albicans*	*K. pneumonia*
AK2	Root of *E. kebercho*	23.33 ± 1.52^{b}	NA	NA	NA	19 ± 1^{a}	NA	NA	NA
AK51	Root of *E. kebercho*	27.67 ± 2.51^{a}	NA	NA	NA	19 ± 1^{a}	NA	NA	19.33 ± 3.21^{a}
$AT2_1$	Root of *T. schimperi*	NA	11 ± 2.16^{a}	8.67 ± 0.47^{b}	11 ± 0.47^{c}	6.33 ± 0.47^{c}	NA	7.67 ± 2.05^{b}	9.33 ± 0.47^{c}
$AT3_1$	Root of *T. schimperi*	8.67 ± 0.573^{e}	7.67 ± 1.15^{b}	5 ± 0^{c}	5 ± 0^{e}	NA	NA	5 ± 0^{c}	8 ± 2.64^{d}
AT3	Root of *T. schimperi*	5 ± 0^{f}	5 ± 0^{c}	5 ± 0^{c}	5 ± 0^{e}	NA	NA	5 ± 0^{c}	5 ± 0^{f}
DBCH1	Leaf of *A. abyssinica*	$5 \pm \pm 0^{f}$	5 ± 0^{c}	NA	5.67 ± 0.57^{de}	NA	NA	NA	8.67 ± 0.57^{cd}
$DBCH1_1$	Leaf of *A. abyssinica*	5 ± 0^{f}	5 ± 0^{c}	NA	5.67 ± 0.57^{de}	5 ± 0^{d}	NA	8.33 ± 1.52^{b}	9.33 ± 1.52^{c}
DbTR1	Root wash of *R. nepalensis*	17.67 ± 2.08^{c}	10.33 ± 0.57^{a}	10.66 ± 1.15^{a}	13.33 ± 2.88^{b}	NA	NA	4 ± 1^{d}	7 ± 2.64^{e}
DBSM4	Leaf of *U. simensis*	23.33 ± 1.52^{b}	10.33 ± 0.57^{a}	6.67 ± 2.08^{c}	10 ± 0^{c}	9 ± 1^{b}	NA	5 ± 0^{c}	13 ± 2^{b}
GER4	Root wash of *S. marginatum*	NA	5 ± 0^{c}	5.33 ± 0.47^{d}	5 ± 0^{e}	9.66 ± 0.47^{b}	NA	13 ± 1.63^{a}	12.33 ± 2.05^{b}
Ke	Root of *V. Sinaiticus*	14.67 ± 0.57^{d}	NA	5.33 ± 0.57^{d}	NA	NA	NA	NA	NA
DBSK	Leaf of *S. oriental*	22.33 ± 3.05^{b}	7.33 ± 1.15^{b}	7 ± 1^{c}	6 ± 0^{d}	NA	NA	NA	NA

Table 2 Biochemical tests and identification of isolates by MALDI-TOF MS.

Isolate code	Host plant	MALDI-TOF MS-based identification of the isolates		Catalase	Oxidase	Gram staining	SIM motility	Citrate utilization	Carbohydrate fermentation			
		Species	Score*						Glucose	Lactose	Sucrose	Fructose
DbTR1	*Rumex nepalensis*	*Pseudomonas oleovorans*	2.09	+	+	− rod	Motile	+	−	−	−	−
AK51	*Echnobes kebercho*	*Pseudomonas fluorescens*	2.26	+	+	− rod	Motile	+	−	−	−	−
DBSK	*Sisymbrium oriental*	*Bacillus subtilis*	2.53	+	−	+ rod	Motile	+	+	−	+	+
DBSM4	*Urtica simensis*	*Ligilactobacillus salivarius*	2.18	−	−	+ rod	Nonmotile	−	+	+	+	+

Where − = negative, has no activity and + = positive, has activity.
* Scoring criteria: score <1.7 unreliable; 1.7–2.0 probable genus; ≥2.0 probable species.

oleovorans, respectively, on the basis of matrix-assisted laser desorption/ionization-time of flight mass spectrometry. All four isolates tested positive for both catalase and oxidase. In aerobic environments, most aerobic and bioactive compound-producing bacteria synthesize catalase to decompose H_2O_2. Bacteria produce antioxidant enzymes such as catalase to facilitate cell detoxification, mitigate oxidative stress, and counteract the bactericidal effects of H_2O_2. Consequently, the isolate may produce cytochrome c oxidase and utilize oxygen as the terminal electron acceptor during respiration. Cytochrome c oxidase is the primary enzyme in the respiratory chain of most aerobic organisms. Microscopic examination of the bacterial isolates via the Gram-staining technique revealed that they were Gram-positive rods organized into single, pair, and chain formations.

The motility test conducted on the isolates demonstrated that, in addition to the rhizospheric isolate (*P. fluorescens*), the endophytic isolates, including *P. oleovorans*, *L. salivarius*, and *B. subtilis*, exhibited motility. Notably, most endophytic bacteria are motile, enabling them to disseminate within internal systems and penetrate the interior tissues of the plant[38].

Numerous studies have identified *P. oleovorans*, *P. fluorescens*, and *B. subtilis* as prevalent constituents of plant endophytes[10,26,50,54,55]. Lactic acid bacteria are significant microorganisms because of their ability to produce metabolites that can dissolve biofilms. Recent studies have highlighted *P. oleovorans* as an emerging biocontrol agent capable of producing rhamnolipids and polyhydroxyalkanoates with dual antimicrobial and plant growth-promoting activities[15]. While these bacteria are frequently associated with fermented foods and beverages, as well as the normal biota of animals, they are also present in plants[1]. In this study, *L. salivarius*, an endophytic lactic acid bacterium, was detected in the leaves of stinging nettle (*Urtica simensis*). A plant-derived *L. salivarius* strain produced bacteriocin-like inhibitory substances (BLISs) with potent activity against antibiotic-resistant enterococci, underscoring their pharmaceutical potential beyond traditional probiotic roles[41].

Yield of extract

In this study, ethyl acetate, hexane, and a chloroform:hexane mixture (1:1) were employed for extraction, and the yields of the fermented extracts are presented in Figure 1. The highest yield was achieved using ethyl acetate, followed by the chloroform:hexane mixture and hexane. Ethyl acetate is characterized by its medium polarity and minimal toxicity to test strains, which enables the extraction of a broad spectrum of biological substances, both polar and nonpolar[2]. These findings indicate that the isolates produced polar metabolites. Therefore, the extraction of secondary metabolites from microbial fermentation is contingent upon the selection of an appropriate solvent[27]. Recent research supports the use of ethyl acetate for extracting antimicrobial peptides and phenolic compounds from bacterial fermentation, owing to its optimal balance between polarity and low cytotoxicity[14].

Antimicrobial activity of the crude extract against the test microorganisms

Crude extracts of bioactive compounds in ethyl acetate and a chloroform:hexane (1:1) mixture demonstrated antimicrobial activity. In contrast, the hexane extracts exhibited no antimicrobial properties. The bacterial strains *K. pneumoniae* and *S. epidermidis* were susceptible to the crude ethyl acetate extracts across all the isolates; however, *Salmonella ser. Typhimurium* displayed resistance to the ethyl acetate extracts from the four bacterial sources (Table 3). The bacterial isolates *P. fluorescens*, *P. oleovorans*, *L. salivarius*, and *B. subtilis* exhibited antimicrobial activity against one or more selected microbial test organisms. A notable inhibition zone diameter from the ethyl acetate crude extract was observed in *P. oleovorans*, which exhibited greater efficacy against *S. epidermidis* (13.7 ± 1.5), followed by *S. aureus* (12 ± 1). Consistent with this study, *P. oleovorans*, which was isolated from the roots of *Ralstonia solanacearum*-susceptible and resistant tomato seedlings, produced significant levels of antimicrobial substances that are effective against bacterial wilt disease[50]. Consequently, the extract of *P. oleovorans* had potential as a biocontrol agent for managing wilt diseases in various crops. Additionally, an endophytic *B. subtilis* isolated from maize has demonstrated efficacy against fungal pathogens[10]. Similarly, volatile compounds produced by *B. subtilis* are effective against *Curvularia lunata*[54]. *B. subtilis* endophytes from medicinal plants produce surfactin and fengycin analogs with strong anti-MRSA and antibiofilm activities, highlighting their importance in combating persistent infections[23,30].

S. pyogenes and *S. epidermidis* were susceptible to the chloroform:hexane (1:1) crude extracts from all the isolates. Like the ethyl acetate extracts, *Salmonella ser. Typhimurium* demonstrated resistance to the chloroform:hexane (1:1) crude extracts of the four bacterial isolates (Table 4). This phenomenon may be attributed to the polar nature of the bioactive compounds produced by the isolates, as polar solvents preferentially extract polar compounds with antimicrobial properties. Hexane extracts alone did not show any biological activity. *P. fluorescens* is capable of producing well-established secondary metabolites such as siderophores, hydrogen cyanide (HCN), and proteases, all of which exhibit antagonistic functions. Additionally, it has been confirmed to exert effective biological control effects on multiple soil-borne phytopathogenic fungi[26]. *L. salivarius*, the lactic acid bacterium isolated from stinging nettle leaves in this study, is efficacious against bacterial and fungal pathogens[21,51]. *L. salivarius* from medicinal plants produces exopolysaccharides with synergistic antioxidant and antimicrobial effects, which may explain its broad-spectrum activity[12].

A substantial zone of inhibition was observed for crude microbiological material derived from *P. oleovorans* via a chloroform:hexane extraction method against *S. epidermidis* (12 ± 1) (Table 4). Variations in microbial activity are primarily attributed to the unique chemical architectures of compounds from target microbes, their capacity to synthesize diverse secondary metabolites, or their disparate inhibitory mechanisms[40]. The findings of the current investigation reveal that endophytic and rhizosphere bacterial

Table 3 Antimicrobial activity of the crude extract of ethyl acetate by the disc diffusion method (inhibition diameter in mm, mean ± standard deviation); NA refers to no activity. Values sharing different superscript letters in a column are significantly different at $p < 0.05$.

Isolate	Microbial test organisms							
	S. aureus	*E. coli*	*S. ser. Typhimurium*	*L. monocytogenes*	*S. epidermidis*	*S. pyogenes*	*C. albicans*	*K. pneumonia*
P. fluorescens	NA	6.7 ± 0.6^{a}	NA	6.8 ± 0.2^{a}	6.7 ± 0.3^{a}	8.7 ± 0.6^{ab}	NA	6.3 ± 0.3^{a}
P. oleovorans	12.0 ± 1.0^{a}	6.7 ± 0.3^{a}	NA	NA	13.7 ± 1.5^{c}	9.5 ± 0.5^{b}	6.7 ± 0.3^{a}	6.8 ± 0.3^{a}
L. salivarius	NA	NA	NA	6.5 ± 0.0^{a}	8.3 ± 0.6^{b}	8.3 ± 0.3^{a}	7.2 ± 0.3^{a}	6.7 ± 0.3^{a}
B. subtilis	NA	6.8 ± 0.2^{a}	NA	NA	8.5 ± 0.4^{b}	NA	NA	6.7 ± 0.2^{a}
Gentamicin	23.0 ± 1.0^{b}	16.0 ± 1^{b}	14.0 ± 1.0	16.3 ± 0.6^{b}	21.3 ± 0.6^{d}	12.7 ± 0.6^{c}	10.0 ± 0.0^{b}	10.3 ± 0.6^{b}
DMSO	NA	NA	NA	NA	NA	NA	NA	NA

Table 4 Antimicrobial activity of chloroform:hexane (1:1) crude extract via the disc diffusion method (inhibition diameter in mm, mean ± standard deviation); NA refers to no activity. Values sharing different superscript letters in a column are significantly different at $p < 0.05$.

Isolates	Microbial test organisms							
	S. aureus	*E. coli*	*S. ser. Typhimurium*	*L. monocytogenes*	*S. epidermidis*	*S. pyogenes*	*C. albicans*	*K. pneumonia*
P. fluorescens	NA	NA	NA	6.5 ± 0.0^{b}	7.3 ± 0.5^{c}	8.0 ± 0.0^{b}	NA	NA
P. oleovorans	8.0 ± 0.0^{b}	6.7 ± 0.2^{c}	NA	NA	12.0 ± 0.2^{b}	7.5 ± 0.4^{bc}	NA	6.5 ± 0.0^{b}
L. salivarius	6.5 ± 0.0^{c}	7.8 ± 0.2^{b}	NA	NA	7.8 ± 0.2^{d}	7.0 ± 0.0^{c}	6.5 ± 0.0^{b}	6.5 ± 0.0^{b}
B. subtilis	NA	NA	NA	NA	8.8 ± 0.2^{c}	7.0 ± 0.0^{c}	NA	6.5 ± 0.0^{b}
Gentamicin	23.0 ± 1.0^{a}	16.0 ± 1.0^{a}	14.0 ± 1.0	16.3 ± 0.6^{a}	21.3 ± 0.6^{a}	12.7 ± 0.6^{a}	10.0 ± 0.0^{a}	10.3 ± 0.6^{a}
DMSO	NA	NA	NA	NA	NA	NA	NA	NA

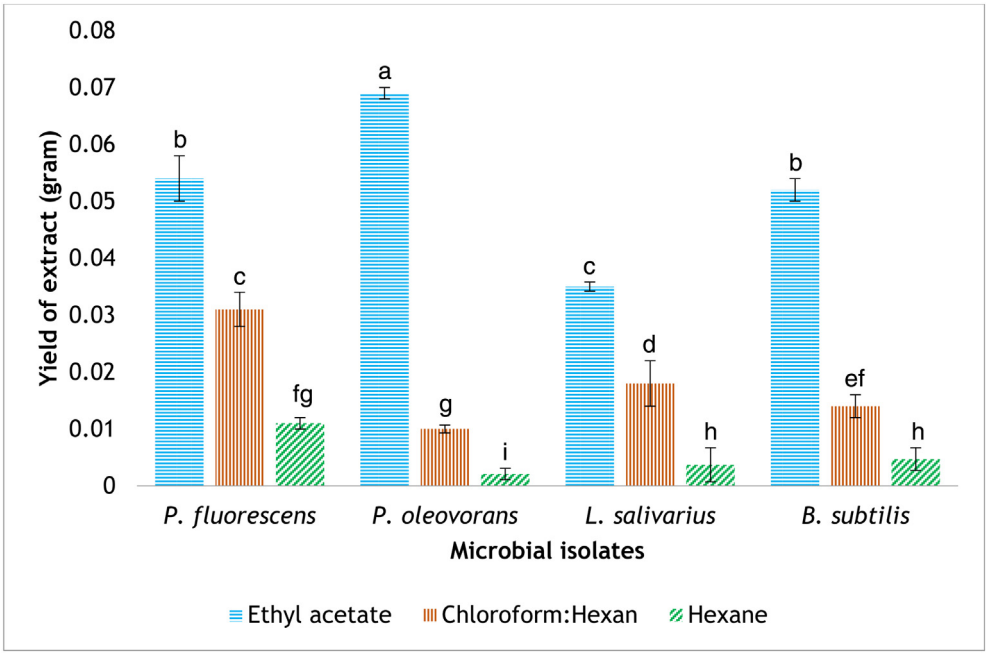

Figure 1 Yields of bacterial isolates extracted by ethyl acetate, chloroform:hexane (1:1), and hexane. The error bars represent standard deviations to show the dispersion between the triplicates. Different letters on the bars indicate statistically significant differences at $p < 0.05$.

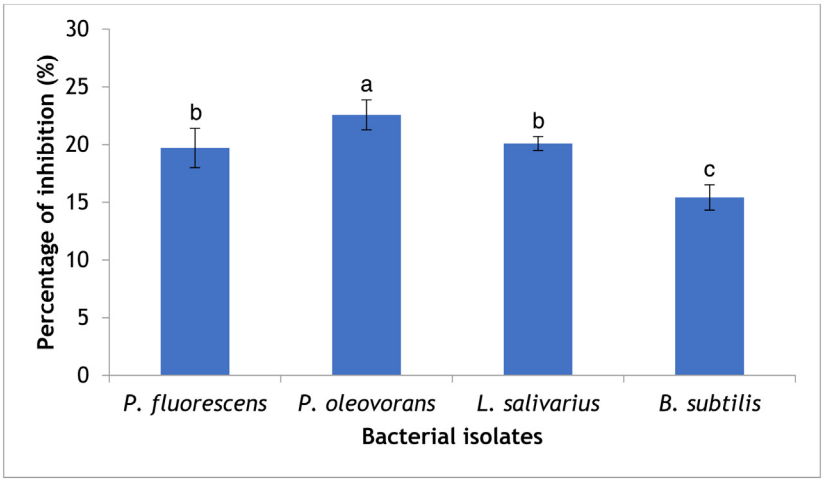

Figure 2 Antioxidant activity of bacterial isolates via DPPH free radicals. The error bars represent standard deviations to show the dispersion between the triplicates. Different letters on the bars indicate statistically significant differences at $p < 0.05$.

isolates have the potential to produce bioactive compounds with extensive antagonistic activities against test microorganisms.

Antioxidant activity of the supernatants of bacteria grown in nutrient broth

The antioxidant properties of the supernatants derived from the four bacterial isolates were evaluated, revealing the capacity of bacterial metabolites within the supernatants to neutralize DPPH radicals (Fig. 2). *P. oleovorans* presented the highest level of antioxidant activity, at 22.57%, whereas *B. subtilis* presented the lowest degree (15.42%). Alkaloid and flavonoid metabolites produced by medicinal plants, along with associated bacteria, constitute one of the primary sources of natural antioxidants[18]. Endophytic *Pseudomonas* spp. can produce significant amounts of phenolic compounds and glutathione analogs, which contribute to their high DPPH scavenging activity and potential use in oxidative stress-related therapies[6]. Furthermore, lactic acid bacteria such as *L. salivarius* produce antioxidant metabolites, such as γ-aminobutyric acid (GABA) and superoxide dismutase, which may increase the host plant's resilience to environmental stress[9].

Conclusions

The crude extracts of *P. fluorescens* (from the root wash of *Rumex nepalensis*), *P. oleovorans* (from the root of *Echinops kebericho* Mesfin), *L. salivarius* (from the leaf of *Sisymbrium orientale*), and *B. subtilis* (from the leaf of *Urtica simensis*) had antimicrobial activities against one or more of the test microorganisms. Extracts of endophytic and rhizospheric isolates from plants have demonstrated antimicrobial activity. Moreover, these findings lead to the conclusion that microbes present within or in association with the plant might have a direct or indirect role in the synthesis of bioactive compounds in the plant. In addition to their antimicrobial activity, they also exhibited antioxidant activity. This study recommends further investigations on the identification of endophytes and medicinal plant compounds. It is better to produce microbial substances from endophytes rather than plants if the antimicrobial substances produced by both endophytes and medicinal plants are identical, so that we can conserve the diminishing number of medicinal plants, especially root-based medicinal plants.

Authors' contributions

Conceptualization, S.H.K. and W.J.K.; methodology, S.H.K., W.J.K., and B.T.; validation, J.R.; formal analysis, S.H.K., W.J.K. and J.R.; investigation, B.T., K.B., and M.G.; resources, A.T.; data curation, K.A.R., B.T., and K.B.; writing – original draft preparation, B.T.; writing – review and editing, K.A.R. and A.T.; visualization, M.G.; supervision, A.T.; fund acquisition, S.H.K. and W.J.K.; project administration, A.T. All authors agree to be accountable for all aspects of the work.

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Conflict of interest

The authors declare no conflict of interests

Data availability

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All data generated or analyzed during this study are included in this published article.

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