EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

Evaluation of antioxidant and anti-inflammatory activities of *Bacillus* sp. CZ-Rh4, CZ-Rh7, and CZ-L11 extracts

Linh Chi Tran, Chong Kim Thien Duc, Thanh Tan Nguyen, Duy Toan Pham, Dinh Kim Phan, Trang Thi Xuan Dai, Tuan Trong Nguyen

PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Evaluation of antioxidant and anti-inflammatory activities of *Bacillus* sp. CZ-Rh4, CZ-Rh7, and CZ-L11 extracts

Linh Chi Tran[1,2], Chong Kim Thien Duc[3], Thanh Tan Nguyen[3], Duy Toan Pham[3], Dinh Kim Phan[3], Trang Thi Xuan Dai[3], Tuan Trong Nguyen[3]

[1]*Institute of Food and Biotechnology, Can Tho University, Can Tho City 94000, Vietnam*
[2]*Faculty of Medicine, Nam Can Tho University, Can Tho City 94000, Vietnam*
[3]*College of Natural Sciences, Can Tho University, Can Tho City 94000, Vietnam*

Bacteria with medicinal potential are receiving research attention from scientists. This study was conducted to explore the *in vitro* antioxidant and anti-inflammatory potential of extracts from *Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7, and *Bacillus* sp. CZ-L11. Four methods including: 2,2-diphenyl-1-picryl-hydrazyl free radical neutralization, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) free radical neutralization, total antioxidant capacity, and ferric reducing-antioxidant power were used to evaluate antioxidant activity. Besides, four methods such as: nitric oxide formation inhibition, red blood cell membrane protection, inhibition of bovine serum albumin and egg white albumin denaturation were used to evaluate anti-inflammatory activity. From the results obtained, extracts from endophytic bacterial strains showed good *in vitro* antioxidant and anti-inflammatory activities. Besides, the *in vitro* antioxidant and anti-inflammatory activities of the extracts depended on the total polyphenol and flavonoid content. *Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7, and *Bacillus* sp. CZ-L11 were able to produce secondary metabolites as natural sources of antioxidants and anti-inflammatory materials.

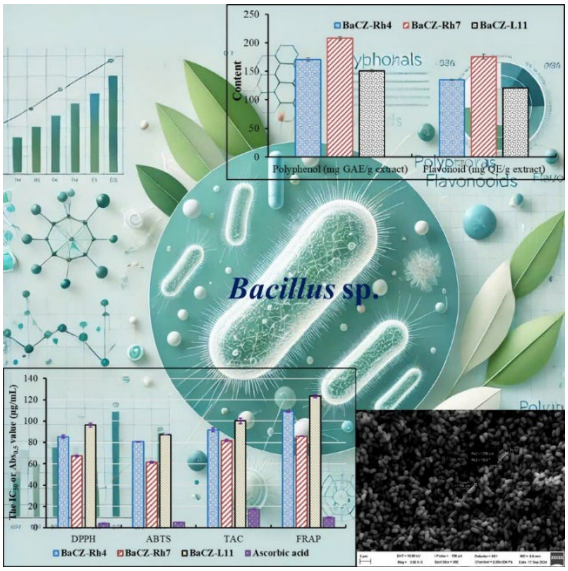
Keywords: antioxidant, anti-inflammatory, *Bacillus* sp., endophytic bacteria, flavonoid, polyphenol, secondary metabolites

ARTICLE HISTORY
Submitted: February 18, 2025
Accepted: March 21, 2025

CORRESPONDENCE TO
Tuan Trong Nguyen,
College of Natural Sciences,
Can Tho University,
Can Tho City 94000, Vietnam
Email: trongtuan@ctu.edu.vn
DOI: 10.21608/ejbo.2025.360059.3191

EDITED BY: K. Ghanem

GRAPHICAL ABSTRACT



INTRODUCTION

Oxidative stress is characterized by the excessive generation of free radicals in cells and tissues that cannot be regulated by the antioxidant system, and this is where oxidative stress is described (Jomova et al., 2023). The presence of an excessive amount of free radicals in the body can result in the destruction of biological components including DNA, proteins, and lipids (Chandimali et al., 2025). Free radicals are a contributor to the development of chronic illnesses such as cancer, diabetes, cardiovascular disease, and neurodegeneration. These diseases are caused by the excessive production of free radicals during the metabolic process (Zaric et al., 2023). In addition to oxidative stress, chronic inflammation is not only a defensive mechanism, but it also promotes metabolic and autoimmune illnesses, which present a threat to the health of people all over the world (Zuo et al., 2019). Flavonoids and polyphenols have been shown to have anti-inflammatory and anti-free radical properties. Plants, animals, fungi, and bacteria all naturally contain polyphenols and flavonoids, which have a variety of pharmacological properties that are crucial to human health (Marchut-Mikołajczyk et al., 2023; Intharuksa et al., 2024; Yu et al., 2025). Plants are the source of most of the chemicals in the polyphenol and flavonoid categories that are utilized as human medications. There is a risk of depletion since many medicinal plants have been overused for therapeutic purposes. The current study discovered that a fresh supply of raw materials with comparable pharmacological properties is a good way to lessen the exploitation of medicinal plants.

Endophytic bacteria in plants have the potential to produce secondary metabolites for therapeutic application. More and more scientific data suggests that endophytic bacteria residing inside medicinal plants have therapeutic properties like the host plant, owing to the synthesis of secondary metabolites. Endophytic bacteria produce metabolites such as

pterocidin (Igarashi et al., 2006), javanicin (Kharwar et al., 2009), cajaninstilbene acid (Zhao et al., 2012), kakadumycin (Christina et al., 2013), and compounds belonging to the group of phenols, tannins, flavonoids, ascorbic acid, and carotenes (Kumaresan et al., 2015). Besides, endophytic bacteria's secondary metabolites contain antibacterial, antifungal, antiviral, antioxidant, anticancer, and anti-inflammatory effects (Lata & Gond, 2025). Numerous biological compounds with a wide range of actions, such as antibacterial, antifungal, antiviral, anticancer, and plant growth stimulants, are found in bacteria belonging to the genus *Bacillus* (Nisa et al., 2022; Ramirez-Olea et al., 2022). As a result, *Bacillus* products are widely employed in business (food processing enzymes, textiles), medicine (antibiotics, anti-cancer medications), and agriculture (biopesticides) (Dame et al., 2021). Future study focuses on identifying new species and developing strategies for extracting and using secondary metabolic components from *Bacillus* bacteria to create therapeutic herbs and functional support items disease therapy. The purpose of this study is to assess the *in vitro* antioxidant and anti-inflammatory activities of extracts from three strains of *Bacillus* sp. CZ-Rh4, CZ-Rh7, and CZ-L11 that live endogenously in medicinal plants, with the goal of developing biological products for health care and disease prevention related to inflammation and oxidative stress.

MATERIALS AND METHODS

Materials

The study used endophytic bacterial strains (*Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7 and *Bacillus* sp. CZ-L11) isolated from *Curcuma zedoaria*. They were stored and cultured at the Faculty of Medicine, Nam Can Tho University to prepare the extract. Information about these endophytic bacterial strains (*Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7 and *Bacillus* sp. CZ-L11) were published on the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI) with the numbers PQ525292.1, PQ533188.1 and PQ533185.1, respectively as described in Table 1. *Bacillus* sp. CZ-Rh4 strain cultured on potato dextrose agar medium after 24 hours will develop into round colonies, size 2.5 mm, ivory color. *Bacillus* sp. CZ-Rh4 strain has rod-shaped cells (0.41 × 1.16 µm) and Gram-positive. *Bacillus* sp. CZ-Rh7 strain cultured on potato dextrose agar medium after 24 hours will develop into round colonies, size 2.5 mm, milk-white color. *Bacillus* sp. CZ-Rh7 strain has rod-shaped cells (0.35 × 1.02 µm) and Gram-positive. *Bacillus* sp. CZ-L11 cultured on potato dextrose agar medium after 24 hours will develop into round colonies, 2 mm in size, milk-white in color, and tissue-like appearance. *Bacillus* sp. CZ-L11 has rod-shaped cells (0.62 × 1.79 µm) and Gram-positive. The morphological characteristics of the colonies and cells of *Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7 and *Bacillus* sp. CZ-L11 are shown in Figure 1 (Tran et al., 2025).

Methods

Extracts preparation from endophytic bacterial strains

In potato dextrose broth medium, endophytic bacteria were cultivated for 24 hours at 30°C with an initial pH of 7. The enrichment culture of the bacterial strain was then modified to achieve an optical density of 0.5 at a wavelength of 600 nm ($OD_{600\ nm}$=0.5). Then, these endophytic bacterial strains were inoculated (2% inoculum) into a conical flask containing potato dextrose broth medium (250 mL), initial pH 7, cultured for 24 hours, temperature 30°C, shaking at 200 rpm. The culture was centrifuged (3000 rpm), the cell-free supernatant was extracted using ethyl acetate solvent at a 1:1 (v/v) ratio. A rotating vacuum evaporator (Heidolph, Germany) was used to evaporate the solvent under low pressure to obtain ethyl acetate extract from *Bacillus* sp. CZ-Rh4 (abbreviated as BaCZ-Rh4 extract), *Bacillus* sp. CZ-Rh7 (abbreviated as BaCZ-Rh7 extract), and *Bacillus* sp. CZ-L11 (abbreviated as BaCZ-L11 extract), the remaining extract was then incubated at -18°C for 24 hours, and finally freeze-dried for 48 hours using a BK-FD10PT device (Biobase, China), which eliminated the water added during the liquid-liquid extraction process. As directed by the manufacturer, a Kern DAB 100-3 moisture analyzer from Kern & Sohn (Germany), was used to measure the extracts' moisture content. For further experiments, these extracts were kept in glass vials at 4°C.

Qualitative and quantitative compound compositions

Determination of compositions of bacterial extracts

The components of bacterial extracts including reducing sugars, polyphenols, alkaloids, flavonoids, steroids, saponins, and tannins, were qualitatively determined as described by Dubale et al. (2023), with some modifications.

Determination of total polyphenol content (TPC)

Total Polyphenol Content (TPC) was calculated using the method of Deb et al. (2021). 50 µL of the extract, 50 µL of deionized water, and 50 µL of Folin-Ciocalteu

Table 1. The NCBI's GenBank nucleotide database contains details on the endophytic bacterial strains CZ-Rh4, CZ-Rh7, and CZ-L11.

Samples	GenBank	Link
Bacillus sp. CZ-Rh7	PQ533188.1	https://www.ncbi.nlm.nih.gov/nuccore/PQ533188
Bacillus sp. CZ-Rh4	PQ525292.1	https://www.ncbi.nlm.nih.gov/nuccore/PQ525292
Bacillus sp. CZ-L11	PQ533185.1	https://www.ncbi.nlm.nih.gov/nuccore/PQ533185

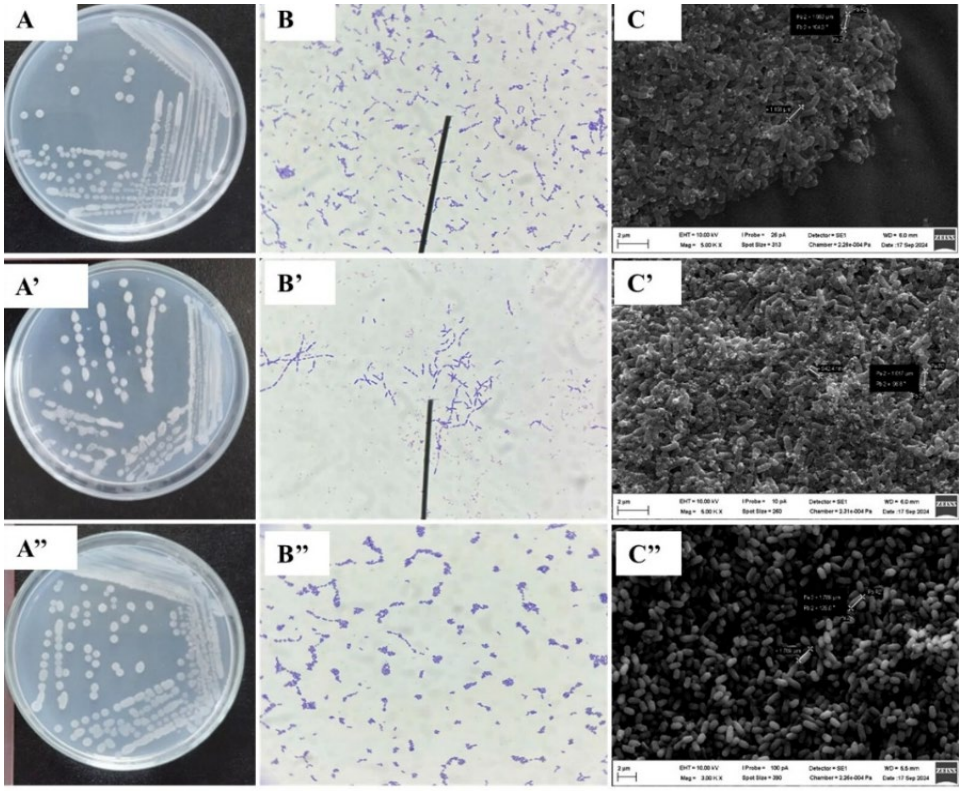


Figure 1. The colony and cell morphology of endophytic bacteria. *Note: A, B, C represent the colony morphology of Bacillus* sp. CZ-Rh4*, Bacillus* sp. CZ-Rh7 *and Bacillus* sp. CZ-L11. *A', B', C' are the Gram characteristics of Bacillus* sp. CZ-Rh4*, Bacillus* sp. CZ-Rh7 *and Bacillus* sp. CZ-L11. *A", B", C" are the cell morphology of Bacillus* sp. CZ-Rh4*, Bacillus* sp. CZ-Rh7 *and Bacillus* sp. CZ-L11.

reagent was combined and well mixed. Next, add 50 µL of 10% sodium carbonate, and incubate in a water bath at 40°C for 30 min. At 765 nm, the reaction mixture's spectral absorbance was measured. The extract's total polyphenol content (mg GAE/g extract) was calculated using the gallic acid standard curve equation, which has the formula y=0.0027x + 0.0886.

Determination of total flavonoid content (TFC)

The aluminum chloride colorimetric method was used to estimate the total flavonoid content, with some modifications (Masoko, 2017). 50 µL of extract was combined with 50 µL of deionized water to create the reaction mixture, which was then well shaken. After 5 min, ten microliters of 5% sodium nitrite were added to the reaction mixture, followed by ten microliters of 10% aluminum chloride hexahydrate and a thorough shake. The reaction mixture was incubated for 6 min before 100 µL of 1 M sodium hydroxide were added. Lastly, 30 µL of water were added, and the spectral absorbance at 510 nm was measured. The extract's total flavonoid content (mg QE/g extract) was calculated using the quercetin standard curve equation, which has the formula y=0.0053x + 0.0125.

Evaluating *in vitro* antioxidant activities of bacterial extracts

The *in vitro* antioxidant activities of the extracts were investigated using methods including ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) free radical neutralization, and total antioxidant capacity (TAC), as reported by Duc et al. (2024) with some modifications. Dimethyl sulfoxide (DMSO 10%) served as the negative control, whereas ascorbic acid served

as the positive control. The concentration (µg/mL) at which ascorbic acid or extract decreased or neutralized 50% of the free radicals was used to compare the antioxidant activity of the extracts with ascorbic acid; this is known as the 50% inhibitory concentration, or IC_{50} value. The IC_{50} values of the extracts and ascorbic acid were determined based on the efficiency of neutralizing or inhibiting free radical formation for $ABTS^{\bullet+}$, DPPH methods and based on the spectral absorption of metal complexes for FRAP, TAC methods. The process for investigation *in vitro* antioxidant activity assays is presented specifically as follows: Total antioxidant activity (TAC) was determined by adding 150 µL of extract to eppendorf, then adding 450 µL of TAC solution, incubating for 90 min at 95 °C. After incubation, the absorbance was measured at 695 nm. The ability to neutralize the free radical 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was determined by reacting $ABTS^{\bullet+}$ (990 µL) with 10 µL of extract for 6 min at room temperature. The reaction mixture was measured for absorbance at 734 nm. Ferric reducing-antioxidant power (FRAP) was determined by reacting 10 µL of extract with FRAP solution (990 µL) for 30 min in the dark. The absorbance of the test solution was determined at 593 nm. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical neutralization capacity was determined by reacting 10 µL of DPPH (2.5 mM) with 240 µL of extract, incubating in the dark at 30 °C for 30 min. The absorbance of DPPH free radical was determined at 517 nm.

Evaluating *in vitro* anti-imflammatory activities of bacterial extracts

The study's chosen methods included preventing the production of nitric oxide ($NO^{\bullet}$), protecting red blood cells (RBCs), and preventing the denaturation of egg white albumin (EWA) and bovine serum albumin (BSA), as reported by Inkanuwat et al. (2019) and Warsidah et al. (2020), with some modifications. By analyzing several inflammatory response mechanisms, such as protein structure stabilization, cell membrane protection, and free radical neutralization, these mmethods help to provide a more comprehensive view of the research subjects' anti-inflammatory effectiveness. The negative control was dimethyl sulfoxide (DMSO 10%), whereas the positive controls were ascorbic acid and diclofenac. Based on the inhibitory efficiency (%) as well as 50% inhibitory concentration (IC_{50}), as reported by Inkanuwat et al. (2019) and Warsidah et al. (2020), the extracts' capacity to prevent $NO^{\bullet}$ production, safeguard RBCs, and prevent BSA and EWA denaturation was assessed. Furthermore, based on IC_{50} values, the study examined the extracts' capacity to prevent $NO^{\bullet}$ production, preserve RBCs, and prevent BSA and EWA denaturation with ascorbic acid standards and diclofenac. The process for investigation *in vitro* anti-inflammatory activity assays is presented specifically as follows: Bovine serum albumin (BSA) denaturation inhibition assay was performed by adding 100 µL of extract and 100 µL of BSA solution (0.5%) to an eppendorf tube. The reaction mixture was then incubated at 37 °C for 15 min. BSA denaturation was induced by keeping the reaction mixture at 70 °C for 10 min. After cooling, the absorbance spectrum was measured at 660 nm at room temperature. Egg white albumin (EWA) denaturation inhibition assay was performed by adding 100 µL of EWA (fresh egg), 1.4 mL of phosphate buffer (pH=6.4) and 1 mL of extract to an eppendorf tube. The mixture was then incubated at 37 °C for 15 min. Protein denaturation was induced by keeping the reaction mixture at 70 °C for 5 min. After cooling, the absorbance was measured at 660 nm. Protection of red blood cells (RBCs) from heat-induced hemolysis: Mouse blood was centrifuged at 3000 rpm for 10 min, the clear liquid was removed and washed three times with saline. RBCs were diluted with saline to a concentration of 10%. Then, 1 mL of the extract was reacted with 1 mL of RBCs (10%). The mixture was incubated at 56 °C for 30 min and cooled. Then, the reaction mixture was centrifuged at 3000 rpm for 5 min. The absorbance was determined at 560 nm, at room temperature. The inhibition of nitric oxide ($NO^{\bullet}$) formation was determined by adding 200 µL of the extract and 400 µL of sodium nitroprusside (5 mM) to an eppendorf tube. The reaction mixture was incubated for 60 min at 25 °C, followed by centrifugation at 11000 rpm for 15 min. The centrifuge was supplemented with 600 µL of Griess reagent. The sample was then incubated for another 5 min and the absorbance was determined spectrophotometrically at 546 nm at room temperature.

Statistical analysis and data processing

The study's data was repeated three times, kept on Microsoft Excel 2016, and statistically analyzed using Minitab 16.0. The data were shown as mean (Mean) ± standard error (Stdev). The Tukey's test (ANOVA) was used to examine differences at the 5% level.

RESULTS AND DISCUSSION

Extract preparation, qualitative and quantitative

Bacillus strains including *Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7, and *Bacillus* sp. CZ-L11. The researchers used solvent evaporation to get 2.35 g of BaCZ-Rh4 extract, 2.04 g of BaCZ-Rh7 extract, and 2.58 g of BaCZ-L11 extract. The resulting extracts were thick, copper-yellow in color, and have a distinct scent. BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract all contain moisture content of 4.95, 4.99, and 4.93%, respectively. The analyses of BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract revealed the presence of alkaloids, polyphenols, flavonoids, triterpenoids, steroids, tannins, saponins, and glycosides (Table 2). Polyphenols and flavonoids in BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract were quantified in this investigation. According to our research findings, the BaCZ-Rh7 extract has a higher quantity of flavonoids (175.63±4.25 mg QE/g extract) and polyphenols (208.00±2.96 mg GAE/g extract) than the other extracts. This difference is statistically significant ($p<0.05$) (Figure 2). Natural compounds from the polyphenol and flavonoid families have been isolated from the cell-free supernatant of *Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7, and *Priestia* sp. CZ-L11 using the solvent ethyl acetate. Because ethyl acetate's medium polarity solvents with the molecular characteristics of flavonoids and polyphenols, it extracts these molecules efficiently. For many biological extraction and medical research purposes, this makes it the perfect solvent.

In vitro antioxidant activities of BaCZ-Rh7, BaCZ-Rh4, BaCZ-L11 extracts

The human body's oxidation-reduction processes are intimately linked to anti-aging and disease resistance (Asejeje and Ogunro, 2024). In order to identify efficient antioxidants for use in food, medicine, and healthcare, researchers are now focusing on natural active substances that can control oxidation-reduction processes in the human body (Rahaman et al., 2023). Figure 3 illustrates the concentration (µg/mL) at which the extract or ascorbic acid reached 50% effectiveness (IC_{50} or $Abs_{0.5}$), which was used to compare the antioxidant capacity of BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract with ascorbic acid. Free radical oxidation can cause cell membrane disruption, membrane protein damage, and DNA mutations, all of which contribute to the aging process and can lead to the development of a variety of diseases including diabetes, arteriosclerosis, cancer, liver damage, inflammation, skin damage, cardiovascular disease, and arthritis (Curieses Andrés et al., 2023). As a result, regulated free radicals can either prevent radical chain reactions or prevent reactive oxidants from forming at the outset, hence avoiding illness (Di Meo and Venditti, 2020).

DPPH, ABTS, TAC, and FRAP were the four methods used in our investigation to evaluate the antioxidant activities of BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract. The research by Duc et al. (2024) states that a sample is considered extremely strong if its IC_{50} value is less than 50 µg/mL, strong if it is between 50 and 100 µg/mL, and moderate if it is between 101 and 150 µg/mL. Weak antioxidants, on the other hand, have an IC_{50} value more than 150 µg/mL. The study's findings demonstrated that BaCZ-Rh7 extract had a significant antioxidant capacity in every technique examined ($IC_{50, DPPH}$=67.32±0.91 µg/mL; $IC_{50, ABTS^{\bullet+}}$=61.24±0.73 µg/mL; $IC_{50, TAC}$=81.65±0.98 µg/mL and $IC_{50, FRAP}$=85.69±0.22 µg/mL). BaCZ-Rh4 extract was shown to have a moderate antioxidant capacity in the FRAP technique (IC_{50}=108.93±0.68 µg/mL), a high antioxidant capacity in DPPH method (IC_{50}=85.40±1.28 µg/mL), ABTS method (IC_{50}=80.69±0.25 µg/mL), and TAC method (IC_{50}=91.78±1.80 µg/mL). The BaCZ-L11 extract has a moderate antioxidant capacity in the TAC (IC_{50}= 100.31±2.59 µg/mL) and FRAP (IC_{50}=123.34± 1.42 µg/mL) methods but is assessed to have significant antioxidant activity by the DPPH method (IC_{50}=96.20± 1.89 µg/mL) and ABTS method (IC_{50}=87.25±0.14 µg/mL). According to DPPH, ABTS, TAC, and FRAP tests, ascorbic acid exhibits more antioxidant qualities than BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract. Besides, it has been demonstrated that other endophytic bacterial strains found in various medicinal plants may neutralize the free radicals DPPH and $ABTS^{\bullet+}$. For instance, the bacterial strain *Pseudomonas aeruginosa* CP43328.1 living endophytically in the leaves of *Anredera cordifolia* has been shown to have the ability to neutralize $ABTS^{\bullet+}$ and DPPH free radicals with IC_{50} values of 300 µg/mL and 650 µg/mL, respectively (Nxumalo et al., 2020). Therefore, it was shown that the endophytic bacterial strains (*Bacillus* sp. CZ-Rh4, *Bacillus* sp. CZ-Rh7, *Bacillus* sp. and CZ-L11) in the present investigation were more effective at neutralizing DPPH and $ABTS^{\bullet+}$ free radicals than the endophytic bacterial strain *Pseudomonas aeruginosa* (CP43328.1) in the study of Nxumalo et al. (2020).

Table 2. The analyses of BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract

Chemicals	Test	Results		
		BaCZ-Rh4 extract	BaCZ-Rh7 extract	BaCZ-L11extract
Alkaloids	Dragendroff	+	+	+
	Wagner	+	+	+
Polyphenols	Folin-Ciocalteu	+	+	+
Flavonoids	$FeCl_3$ 5%	+	+	+
	1% NaOH/ethanol	+	+	+
Triterpenoids	Rosenthaler	+	+	+
Steroids	Salkowski	+	+	+
Tannins	Saturated $Pb(CH_3COO)_2$	+	+	+
Saponins	NaOH (pH=13)	-	-	-
	HCl (pH=1)	-	-	-
Glycosides	Fehling	+	+	+

Note: "+" is the presence of the compound group in the extract. "–"is the presence of the compound group in the extract has not been detected.

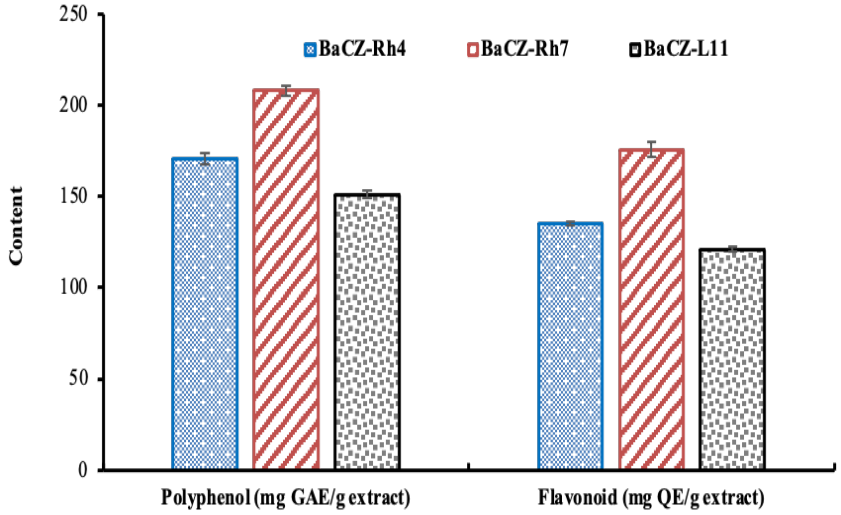


Figure 2. Total polyphenol and flavonoid content in the extracts

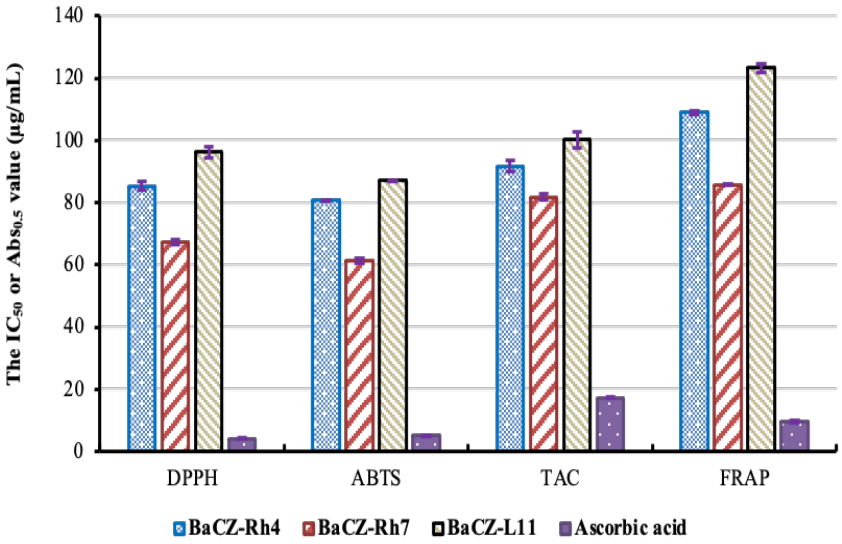


Figure 3. *In vitro* antioxidant activities of extracts and ascorbic acid

In vitro anti-inflammatory activities of BaCZ-Rh7, BaCZ-Rh4, BaCZ-L11 extracts

Anti-inflammatory activity is essential for protecting the body from excessive inflammatory responses, which are the root cause of many chronic illnesses such as arthritis, diabetes, cardiovascular disease, and cancer (Liaqat and Eltem, 2018). Natural anti-inflammatory compounds reduce tissue damage, ease pain, and prevent inflammation (Hahn et al., 2020).

In vitro testing methods are crucial for assessing the anti-inflammatory effectiveness of natural extracts (Gunathilake et al., 2018). In the current study, the methods used included: inhibition of nitric oxide production (nitric oxide is a key mediator in the inflammatory response, inhibition of nitric oxide production in macrophages is an important indicator for evaluating the anti-inflammatory activity of the test sample); protection of red blood cells (this method helps determine the ability to protect red blood cell membranes from inflammatory lysis, a phenomenon that occurs when there is tissue damage and increased oxidative stress); inhibition of denaturation of bovine serum albumin and egg white albumin (protein denaturation is a typical sign of the inflammatory response, the ability to prevent or reduce protein denaturation reflects the anti-inflammatory potential of natural extracts). A more thorough understanding of the anti-inflammatory activity of BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract is made possible by the simultaneous application of these *in vitro* anti-inflammatory methods, which enable the analysis of numerous inflammatory response mechanisms, ranging from preventing the production of free radicals, shielding cell membranes, and stabilizing protein structure.

The results presented in Table 3 show that BaCZ-Rh4 extract, BaCZ-Rh7 extract and BaCZ-L11 extract all have anti-inflammatory ability in vitro in NO•, BSA, EWA and RBCs methods with IC_{50} values ranging from 48.77±1.16 to 99.63±1.59 µg/mL. In which, BaCZ-Rh7 extract has the ability to inhibit NO• production (IC_{50}=79.69±1.32 µg/mL), inhibit BSA denaturation (IC_{50}=48.77±1.16 µg/mL), inhibit EWA denaturation (IC_{50}=57.75±3.48 µg/mL) and protect RBCs (IC_{50}=60.09±0.79 µg/mL) stronger than BaCZ-Rh4 extract ($IC_{50,\ NO•}$=95.44±1.43 µg/mL; $IC_{50,\ BSA}$=61.61±1.02 µg/mL; $IC_{50,\ EWA}$=66.75±0.60 µg/mL; $IC_{50,\ RCBs}$=75.22±0.56 µg/mL) were 1.20, 1.26, 1.16 and 1.25 times, respectively. The inhibitory effects of BaCZ-Rh7 extract on NO• production, BSA denaturation, EWA denaturation and RBCs protection were 1.20, 1.26, 1.16 and 1.25 times stronger than BaCZ-L11 extract ($IC_{50,\ NO•}$=99.63±1.59 µg/mL; $IC_{50,\ BSA}$=67.21±0.42 µg/mL; $IC_{50,\ EWA}$=82.03±1.37 µg/mL; $IC_{50,\ RBCs}$=83.21±2.38 µg/mL). However, BaCZ-Rh7 extract still had weaker anti-inflammatory ability than diclofenac in all survey methods.

Our study's findings further demonstrate that the anti-inflammatory properties of BaCZ-Rh4 extract, BaCZ-Rh7 extract, and BaCZ-L11 extract are strongly correlated with their TPC, TFC, and antioxidant activity. This has been explained in studies on the relationship between total polyphenol and flavonoid content with antioxidant and anti-inflammatory activities in plant extract samples (Hu et al., 2017; Ruiz-Ruiz et al., 2017; Derouich et al., 2020). However, there hasn't been much discussion of research elucidating the connection between endophytic bacterial extracts' total polyphenol and flavonoid content and their anti-inflammatory and antioxidant properties. Since polyphenols and flavonoids may neutralize free radicals and prevent the oxidation of lipids in cell membranes, polyphenols and flavonoids are two classes of compounds with potent antioxidant qualities that shield cells from oxidative stress, a major contributor to inflammation (El Oirdi, 2024; Zahra et al., 2024).

Table 3. The IC_{50} values of extracts and standard substance

Methods	IC_{50} values of extracts and standard substance			
	BaCZ-Rh4 extract	BaCZ-Rh7 extract	BaCZ-L11 extract	Standard substance
NO•	95.44^{b}±1.43	79.69^{c}±1.32	99.63^{a}±1.59	21.64^{d}±0.13
RBCs	75.22^{b}±0.56	60.09^{c}±0.79	83.21^{a}±2.38	17.23^{d}±0.65
BSA	61.61^{b}±1.02	48.77^{c}±1.16	67.21^{a}±0.42	21.47^{d}±0.05
EWA	66.75^{b}±0.60	57.75^{c}±3.48	82.03^{a}±1.37	24.35^{d}±1.07

*Note: There is no significant difference (p>0.05) between values in the same row that are followed by the same letters (a, b, c, and d). * Ascorbic acid is the standard substance used in the NO method, whereas diclofenac is the standard substance used in the RBCs, BSA, and EWA procedures.*

Numerous studies have shown that polyphenols and flavonoids not only increase antioxidant capacity but also directly decrease the inflammatory response by blocking inflammatory mediators and enzymes including cyclooxygenase (COX), lipoxygenase (LOX), and nitric oxide ($NO^{\bullet}$) (Chagas et al., 2022; Fakhar et al., 2024). Furthermore, secondary metabolites belonging to the polyphenol and flavonoid groups help stabilize cell membranes and prevent protein denaturation (two of important mechanisms in the inflammatory process) (Ullah et al., 2020; Bolat et al., 2024). As a result, the higher the polyphenol and flavonoid concentration, the greater the anti-inflammatory and antioxidant activity. This connection, however, is not always perfectly proportionate and is also determined by the substances' compound structure and bioavailability. In conclusion, TPC and TFC are critical in the antioxidant and anti-inflammatory properties of bacterial extracts. As a result, finding and improving these compounds is a significant research avenue for developing pharmaceutical medicines and functional foods to aid in the treatment of disorders involving inflammation and oxidative stress.

CONCLUSIONS

The study found that extracts from *Bacillus* sp. CZ-Rh4*, Bacillus* sp. CZ-Rh7 and *Bacillus* sp. CZ-L11 have anti-inflammatory and antioxidant properties *in vitro*. According to research findings, *Bacillus* sp. CZ-Rh7 extracts exhibit greater antioxidant and anti-inflammatory properties in vitro than *Bacillus* sp. CZ-Rh4 and CZ-L11 extracts. This has to do with how much total polyphenol and flavonoid content in each extract. The extracts' levels of flavonoids and polyphenols progressively rose from BaCZ-L11 extract to BaCZ-Rh4 extract to BaCZ-Rh7 extract. These *in vitro* biological activities help to establish a solid foundation for the use and exploitation of bacterial extracts in medical research to aid in the management of illnesses brought on by inflammation and oxidative stress.

HIGHLIGHTS

- The study identified three strains of *Bacillus* sp. CZ-Rh4, CZ-Rh7 and CZ-L11 with outstanding ability to produce bioactive compounds.
- *Bacillus* sp. CZ-Rh4, CZ-Rh7 and CZ-L11 strains could produce alkaloids, polyphenols, flavonoids, triterpenoids, steroids, tannins, saponins, and glycosides.
- *Bacillus* sp. CZ-Rh4, CZ-Rh7 and CZ-L11 strains had high antioxidant and anti-inflammatory abilities.
- Polyphenol and flavonoid content - important biological compounds contributing to the antioxidant and anti-inflammatory activities of extracts from *Bacillus* sp. CZ-Rh4, CZ-Rh7 and CZ-L11.
- *Bacillus* sp. proposed as a natural raw material for the development of antioxidant and anti-inflammatory products that are environmentally friendly and sustainable.

REFERENCES

Asejeje, FO, & Ogunro, OB (2024) Deciphering the mechanisms, biochemistry, physiology, and social habits in the process of aging. *Archives of Gerontology and Geriatrics Plus*, 1, 100003.

Bolat, E, Sarıtaş, S, Duman, H, Eker, F, Akdaşçi, E, Karav, S, & Witkowska, AM (2024) Polyphenols: Secondary metabolites with a biological impression. *Nutrients*, 16, 2550.

Chagas, MSS, Behrens, MD, Moragas-Tellis, CJ, Penedo, GXM, Silva, AR, & Gonçalves-de-Albuquerque, CF (2022) Flavonols and flavones as potential anti-inflammatory, antioxidant, and antibacterial compounds. *Oxidative Medicine and Cellular Longevity*, 9966750.

Chandimali, N, Bak, SG, Park, EH, Lim, HJ, Won, YS, Kim, EK, Park, SI, & Lee, SJ (2025) Free radicals and their impact on health and antioxidant defenses: A review. *Cell Death Discovery*, 11, 1-17.

Christina, A, Christapher, V, & Bhore, SJ (2013) Endophytic bacteria as a source of novel antibiotics: An overview. *Pharmacognosy Reviews*, 7, 11-16.

Curieses Andrés, CM, Pérez de la Lastra, JM, Andrés Juan, C, Plou, FJ, & Pérez-Lebeña, E (2023) From reactive species to disease development: Effect of oxidants and antioxidants on the cellular biomarkers. *Journal of Biochemical and Molecular Toxicology*, 37, e23455.

Dame, ZT, Rahman, M, & Islam, T (2021) Bacilli as sources of agrobiotechnology: Recent advances and future directions. *Green Chemistry Letters and Reviews*, 14, 246-271.

Deb, PK, Khound, P, Bhattacharjee, S, Choudhury, P, Sarma, H, Devi, R, & Sarkar, B (2021) Variation in chemical constituents, *in-vitro* bioactivity and toxicity profile among different parts of *Clerodendrum glandulosum* Lindl. (*C. colebrookianum* Walp.). *South African Journal of Botany*, 140, 50-61.

Derouich, M, Bouhlali, EDT, Hmidani, A, Bammou, M, Bourkhis, B, Sellam, K, & Alem, C (2020) Assessment of total polyphenols, flavonoids and anti-inflammatory potential of three *Apiaceae* species grown in the Southeast of Morocco. *Scientific African*, 9, e00507.

Di Meo, S, & Venditti, P (2020) Evolution of the knowledge of free radicals and other oxidants. *Oxidative Medicine and Cellular Longevity*, 9829176.

Dubale, S, Kebebe, D, Zeynudin, A, Abdissa, N, & Suleman, S (2023) Phytochemical screening and antimicrobial activity evaluation of selected medicinal plants in Ethiopia. *Journal of Experimental Pharmacology*, 15, 51-62.

Duc, CKT, Linh, TC, Thanh, NQC, Nhien, PQ, Trang, DTX, Danh, LT, & Tuan, NT (2024) Isolation and evaluation of the antioxidant capacity of compounds from *Ehretia asperula* Zoll. & Moritzi. *Indonesian Journal of Chemistry*, 24, 1206.

El Oirdi, M (2024) Harnessing the power of polyphenols: a new frontier in disease prevention and therapy. *Pharmaceuticals*, 17.

Fakhar, F, Mohammadian, K, Keramat, S, & Stanek, A (2024) The potential role of dietary polyphenols in the prevention and treatment of *Acute leukemia*. *Nutrients*, 16.

Gunathilake, KDPP, Ranaweera, KKDS, & Rupasinghe, HPV (2018) *In vitro* anti-inflammatory properties of selected green leafy vegetables. *Biomedicines*, 6, 107.

Hahn, D, Shin, SH, & Bae, JS (2020) Natural antioxidant and anti-Inflammatory compounds in foodstuff or medicinal herbs inducing heme oxygenase-1 expression. *Antioxidants*, 9.

Hu, J, Ma, W, Li, N, Wang, KJ, Hu, J, Ma, W, Li, N, & Wang, KJ (2017) Antioxidant and Anti-Inflammatory Flavonoids from the Flowers of Chuju, a Medical Cultivar of Chrysanthemum Morifolim Ramat. *Journal of the Mexican Chemical Society*, 61, 282-289.

Igarashi, Y, Miura, SS, Fujita, T, & Furumai, T (2006) Pterocidin, a cytotoxic compound from the endophytic *Streptomyces hygroscopicus*. *The Journal of Antibiotics*, 59, 193-195.

Inkanuwat, A, Sukaboon, R, Reamtong, O, Asawanonda, P, Pattaratanakun, A, Saisavoey, T, Sangtanoo, P, & Karnchanatat, A (2019) Nitric oxide synthesis inhibition and anti-inflammatory effectof polypeptide isolated from chicken feather mealin lipopolysaccharide-stimulated RAW 264.7 Macrophages. *Food Technology and Biotechnology*, 57, 200-212.

Intharuksa, A, Kuljarusnont, S, Sasaki, Y, & Tungmunnithum, D (2024) Flavonoids and other polyphenols: bioactive molecules from traditional medicine recipes/medicinal plants and their potential for phytopharmaceutical and medical application. *Molecules*, 29.

Jomova, K, Raptova, R, Alomar, SY, Alwasel, SH, Nepovimova, E, Kuca, K, & Valko, M (2023) Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. *Archives of Toxicology*, 97, 2499-2574.

Kharwar, RN, Verma, VC, Kumar, A, Gond, SK, Harper, JK, Hess, WM, Lobkovosky, E, Ma, C, Ren, Y, & Strobel, GA (2009) Javanicin, an antibacterial naphthaquinone from an Endophytic Fungus of *Neem*, *Chloridium* sp. *Current Microbiology*, 58, 233-238.

Kumaresan, S, Karthi, V, Senthilkumar, V, & Stephen, A (2015) Biochemical Constituents and Antioxidant Potential of Endophytic Fungi isolated from the Leaves of *Azadirachta indica A. Juss* (Neem) from Chennai, India. *Journal of Academia and Industrial Research*, 3.

Lata, R, & Gond, SK (2025) Antibacterial and antioxidant potentials, detection of host origin compounds, and metabolic profiling of endophytic *Bacillus* spp. Isolated from *Rauvolfia serpentina* (L.) Benth. Ex Kurz. *Scientific Reports*, 15, 2094.

Liaqat, F, & Eltem, R (2018) Chitooligosaccharides and their biological activities: a comprehensive review. *Carbohydrate Polymers*, 184, 243-259.

Marchut-Mikołajczyk, O, Chlebicz, M, Kawecka, M, Michalak, A, Prucnal, F, Nielipinski, M, Filipek, J, Jankowska, M, Perek, Z, Drożdżyński, P, Rutkowska, N, & Otlewska, A (2023) Endophytic bacteria isolated from *Urtica dioica* L.- preliminary screening for enzyme and polyphenols production. *Microbial Cell Factories*, 22, 169.

Masoko, P (2017) Phytochemical analysis, antioxidant and antibacterial properties of *Spilanthes mauritiana* used traditionally in Limpopo Province, South Africa. *Journal of Evidence-Based Complementary & Alternative Medicine*, 22, 936-943.

Nisa, S, Shoukat, M, Bibi, Y, Al Ayoubi, S, Shah, W, Masood, S, Sabir, M, Asma Bano, S, & Qayyum, A (2022) Therapeutic prospects of endophytic *Bacillus* species from Berberis lycium against oxidative stress and microbial pathogens. *Saudi Journal of Biological Sciences*, 29, 287-295.

Nxumalo, CI, Ngidi, LS, Shandu, JSE, & Maliehe, TS (2020) Isolation of endophytic bacteria from the leaves of *Anredera cordifolia* CIX1 for metabolites and their biological activities. *BMC Complementary Medicine and Therapies*, 20, 300.

Rahaman, MM, Hossain, R, Herrera-Bravo, J, Islam, MT, Atolani, O, Adeyemi, OS, Owolodun, OA, Kambizi, L, Daştan, SD, Calina, D, & Sharifi-Rad, J (2023) Natural antioxidants from some fruits, seeds, foods, natural products, and associated health benefits: an update. *Food Science & Nutrition*, 11, 1657-1670.

Ramirez-Olea, H, Reyes-Ballesteros, B, & Chavez-Santoscoy, RA (2022) Potential application of the probiotic *Bacillus licheniformis* as an adjuvant in the treatment of diseases in humans and animals: a systematic review. *Frontiers in Microbiology*, 13, 993451.

Ruiz-Ruiz, JC, Matus-Basto, AJ, Acereto-Escoffié, P, & Segura-Campos, MR (2017) Antioxidant and anti-inflammatory activities of phenolic compounds isolated from *Melipona beecheii* honey. *Food and Agricultural Immunology*, 28, 1424-1437.

Tran, L, Duc, C, Pham, D., Dai, T, & Tuan, N (2025) Bioprospecting endophytic bacteria in Curcuma zedoaria for in vitro antioxidant and anti-inflammatory potentials. Tropical Journal of Natural Product Research, 9(5), 2299-2306.

Ullah, A, Munir, S, Badshah, SL, Khan, N, Ghani, L, Poulson, BG, Emwas, AH, & Jaremko, M (2020) Important flavonoids and their role as a therapeutic agent. *Molecules*, 25, 5243.

Warsidah, Fadly, D, & Bohari, B (2020) Antibacterial and anti-inflammatory activities of ethanol extract obtained from the hooks of *Uncaria tomentosa* (Wild. Ex Schult) DC originated Kalimantan, Indonesia. *Systematic Reviews in Pharmacy*.

Yu, W, Zhang, Y, Lu, Y, Ouyang, Z, Peng, J, Tu, Y, & He, B (2025) Recent research on the bioactivity of polyphenols derived from edible fungi and their potential in chronic disease prevention. *Journal of Functional Foods*, 124, 106627.

Zahra, M, Abrahamse, H, & George, BP (2024) Flavonoids: antioxidant powerhouses and their role in nanomedicine. *Antioxidants*, 13, 922.

Zaric, BL, Macvanin, MT, & Isenovic, ER (2023) Free radicals: relationship to human diseases and potential therapeutic applications. *The International Journal of Biochemistry & Cell Biology*, 154, 106346.

Zhao, J, Fu, Y, Luo, M, Zu, Y, Wang, W, Zhao, C, & Gu, C (2012) Endophytic fungi from pigeon pea *Cajanus cajan* (L.) Millsp. produce antioxidant Cajaninstilbene acid. *Journal of Agricultural and Food Chemistry*, 60, 4314-4319.

Zuo, L, Prather, ER, Stetskiv, M, Garrison, DE, Meade, JR, Peace, TI, & Zhou, T (2019) Inflammaging and oxidative stress in human diseases: from molecular mechanisms to novel treatments. *International Journal of Molecular Sciences*, 20.