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# Bioprospecting Endophytic Bacteria in *Curcuma zedoaria* for *In Vitro* Antioxidant and Anti-inflammatory Potentials

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ABSTRACT

Endophytic bacterial strains in medicinal plants have many valuable pharmacological effects similar to the host plant. This study was conducted to screen endophytic bacteria in *Curcuma zedoaria* that are capable of producing polyphenols, flavonoids, antioxidants and anti-inflammatories *in vitro*. Root, rhizome and leaf samples of *Curcuma zedoaria* were used as raw materials to isolate endophytic bacteria. 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. 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. Research results show that, bacterial strains CZ-R5, CZ-Rh4, CZ-Rh7 and CZ-L11 were able to produce polyphenols, flavonoids, antioxidants and anti-inflammatories *in vitro* more effectively than the remaining bacterial strains. The results of *16S rRNA* gene sequencing showed that the bacterial strains CZ-R5, CZ-Rh4, CZ-Rh7 and CZ-L11 belong to the *Enterobacter* and *Bacillus* genera. Endophytic bacteria in *Curcuma zedoaria* can become a source of biologically active bacteria to support the treatment of diseases related to oxidative stress and inflammation.

***Keywords***: anti-inflammatory, antioxidant, *Curcuma zedoaria*, endophytic bacteria, polyphenols

## Introduction

These days, diseases related to human aging such as cancer, neurological diseases (Alzheimer, Parkinson), cardiovascular problems, lipid metabolism disorders, diabetes, gout, hepatitis or kidney failure are increasingly on the rise. They are not only related to the deterioration of the functions of organs in the body or the immune system but also reflect complex interactions between lifestyle, living environment and genetics. This startling rise presents problems for modern medicine, necessitating early management of many illnesses using cutting-edge diagnostic techniques with the goal of prevention and therapy to enhance lifespan in the face of complicated population aging. The above mentioned diseases are all characterized by some common features such as protein imbalance, oxidative stress, inflammatory response disorder, causing serious damage to the body. In which, protein disorder is a condition in which the body loses control of the process of protein synthesis, folding or degradation, leading to abnormal accumulation of protein, from which cell or tissue damage will occur. When free radicals exceed the permitted amount, oxidative stress occurs, which has a detrimental impact on DNA, proteins, and other critical biological components, resulting in the onset of many hazardous illnesses. Furthermore, prolonged or uncontrolled inflammation causes tissue damage, disrupts the body's metabolic equilibrium, impairs cell function, and damages tissues or muscles. These processes not only affect the body's normal biological functioning, but also worsen illness situations, making it important to discover effective and durable remedies.[1,2] Age-related diseases and metabolic syndrome often have no effective treatment. Medical research shows that supplementary foods such as polyphenols as well as flavonoids is a potential method to mitigate the risk of oxidative stress and inflammation. Compounds in the polyphenol and flavonoid groups interfere with many signaling pathways related to the balance of free radical activity, protein homeostasis, regulation of inflammatory responses and regulation of metabolism.[3] They can manage unstable factors that damage cells, decrease the aging process, neutralize free radicals without producing new dangerous free radicals, and so reduce age-related illnesses including cancer, diabetes, and heart disease. The anti-inflammatory effect of polyphenol and flavonoid compounds is also noteworthy, particularly in chronic inflammatory situations, which contributes to better health by modulating cell signaling pathways. It is possible to infer that polyphenols and flavonoids play an indispensable role not only in overall health, but also in mitigating the detrimental effects of aging and the environment.[4–6] Therefore, these compounds are proposed as a useful solution for the prevention or treatment of aging-related diseases and metabolic syndrome.

Our investigation was conducted to find a new source of natural materials containing secondary metabolites such as polyphenols, flavonoids, and so on, which have antioxidant and anti-inflammatory properties. The subjects chosen for the study were medicinal endophytic bacteria, which are bacteria that reside in plant tissues without causing negative effects on the host, and are especially capable of producing natural compounds similar to the host plant, thereby bringing many new applications in the pharmaceutical industry in particular and medicine in general. In recent years, endophytic bacteria have. been a potential component and have gotten a lot of attention from researchers because of their unique capabilities, making many new discoveries in science.[7–11] Moreover, the study of plant endophytic bacteria is employed to

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establish a novel approach to mitigate the overexploitation of medicinal plants. Besides, plant endophytic bacteria are cultivated in an artificial environment to facilitate timely initiatives, minimize manufacturing costs, and lower product pricing.

In this study, *Curcuma zedoaria* was used as a source of endophytic bacteria. Because of its many therapeutic benefits, *C. zedoaria*, a rhizome plant that is a member of the Ginger family (Zingiberaceae), is frequently utilized in traditional medicine. Tropical regions, particularly those in Southeast Asia, are home to this species. Therefore, *C. zedoaria* is a suitable place for many types of endophytic bacteria because of its tropical environment with high temperature, high humidity, and abundance, which enable the variety of endophytic bacteria. This environment encourages the creation and maintenance of symbiotic partnerships among plants and microbes, helping plants adapt to harsh conditions such as drought, waterlogging, and pests. At the same time, the humid climate and warm temperature also promote bacterial metabolism, increasing the ability to produce beneficial secondary metabolites. In folk remedies, *C. zedoaria* is often used to treat diseases such as diarrhea, stomach pain, flatulence, indigestion, blood circulation and prevention of blood clotting during menstruation, even support cancer treatment.[12,13] Scientific research publications have shown that *C. zedoaria* has antioxidant,[13] antifungal,[14] antibacterial,[15] anticancer, [16] and liver-protective.[17] In addition, *C. zedoaria* has also been shown to contain secondary metabolic compounds belonging to the polyphenols, flavonoids, terpenoids, and so on.[13,16,18–21]. Bioactive compounds found in *C. zedoaria* have the ability to affect the symbiotic microbiota. As a result, the bacteria that were separated from *C. zedoaria* have a great potential for producing bioactive compounds or promoting plant development. Additionally, *C. zedoaria* can serve as a natural biofilter, aiding in the selection of advantageous bacteria with the capacity to adapt to unique living environments and prospective uses in bioindustry, agriculture, and medicine. From there, it is clear that *C. zedoaria* is a potential source of endophytic bacteria strains capable of producing secondary metabolic compounds (polyphenols, flavonoids) and many other valuable biological activities. As far as we are aware, no published scientific publications on the investigation of *C. zedoaria* endophytic bacteria strains have been reported. Thus, in addition to assessing its properties in reducing oxidative stress as well as inflammation, our research will help to diversify knowledge on endophytic bacteria in medicinal plants and provide a scientific foundation on this topic.

## Materials and Methods

*Materials*

*C. zedoaria* (Figure 1) (about 12 months old) was collected in Thanh Phu district, Ben Tre province in September 2024. Potato dextrose broth and potato dextrose agar media from Himadia, India were used to isolate bacteria. Gram staining kit (Nam Khoa Service & Trading Company Limited) was used to determine Gram of bacterial strains according to the manufacturer's instructions.

**Figure 1**. *C. zedoaria*

The equipment used in the study includes: drying oven BE 200, incubation tank (manufactured by Memmert), and refrigerated centrifuge Mikro 12-24 (manufactured by Hettich) were from Germany. Mettler Toledo of Switzerland provided the analytical balance AB104-S. Velp of Italy provided the Vortex Mixer ZX3. The Thermo Scientific Multiskan GO spectrophotometer came from Finland, while Sturdy of Taiwan provided the autoclave steriliser SA-300VF. Jeiotech of Korea provided the biological safety cabinet BC-11B.

*Methods*

*Method of isolating endophytic bacteria and extracting cell-free supernatant*

When harvested, *C. zedoaria* is washed under tap water and divided into roots, rhizomes and leaves. Then, each part is washed again with sterile distilled water, cut into short pieces of 3-4 cm and continued to be sterilized with 70% ethanol for 30 seconds, rinsed with sterile distilled water 3-4 times. Finally, the sample is sterilized with 3% hydrogen peroxide for 4 min and thereafter washed with sterile distilled water 4 times to eliminate residual chemicals. The roots, rhizomes and leaves of *C. zedoaria* after sterilization are put into separate porcelain mortars and crushed. Sterile distilled water with a volume of 3 mL is added to the porcelain mortar, mixed well and left for 10 min to let the sediment settle. 100 µL of the supernatant is pipetted into a test tube containing 10 mL of semi-solid potato dextrose agar medium (0.15% agar) that was prepared earlier. The tubes containing bacteria were incubated for 48 hours at 30 °C. If a thin white opaque film (pellicle) was observed at 2-5 mm below the surface of the medium, it was a sign of endophytic bacteria. The pellicle (100 µL) was collected, spread evenly on the surface of potato dextrose agar, incubated at 30 °C and monitored for colony growth after 48 hours. Isolation and separation of endophytic bacterial strains until pure. The pure endophytic bacterial strains were observed and recorded for colony characteristics (shape, size, color, cover shape, buoyancy), bacterial cell shape and Gram characteristics after 24 hours of culture on potato dextrose agar medium. Pure endophytic bacterial strains were cultured in potato dextrose broth medium at 30 °C, 24 hours, then the suspension of the endophytic bacterial strains was adjusted so that the optical density at 600 nm was 0.5 ($OD_{600nm}$ = 0.5). Then, these endophytic bacterial strains were inoculated at 2% into a conical flask containing potato dextrose broth medium (250 mL), initial pH was 7, cultured for 24 hours, temperature was 30 °C, shaking at 200 rpm. The enrichment culture of the endophytic bacterial strains was collected and centrifuged at 3000 rpm, obtaining the cell-free supernatant. The cell-free supernatant of the endophytic bacterial strains was used for subsequent investigations.

*Method for quantifying total polyphenol and flavonoid content*

The total polyphenol content (TPC) and total flavonoid content (TFC) produced by endophytic bacterial strains were determined as described by Dibacto,[22] with some modifications. The polyphenol (mg GAE/mL cell-free supernatant) and flavonoid (mg QE/mL cell-free supernatant) contents in cell-free supernatant were established using the gallic acid (GAE) standard curve equation in the form y=0.0027x + 0.0886 and quercetin (QE) in the form y=0.0053x + 0.0125. In which, for TPC (cell-free supernatant concentration used for quantification is 250 µL/mL) and this figure for TFC is 200 µL/mL.

*Determination of in vitro antioxidant capacity of endophytic bacterial strains*

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) free radical neutralization method, ferric reducing-antioxidant power (FRAP) and total antioxidant capacity (TAC), with some modifications, as described by previous studies were used to investigate the antioxidant activity.[23–25] The antioxidant activity of endophytic bacterial strains in *C. zedoaria* is determined based on the antioxidant content in the cell-free supernatant, which is conventionally equivalent to the ascorbic acid standard (mg AAE/mL cell-free supernatant). Specifically, the Guenane method was used, with some modifications, to assess the cell-free supernatant of endophytic bacterial strains's DPPH radical neutralizing activity. Briefly, 480 µL of test sample at varying concentrations was combined with 20 µL of DPPH (1000 µg/mL) and

incubated for 30 min at 37°C in the dark. A wavelength of 517 nm was used to test the mixture's optical absorbance after the process.[23]

The Duc method was used [24], with some modifications, to assess the cell-free supernatant of endophytic bacterial strains's $ABTS^{\bullet+}$ radical neutralizing activity. To do this, mix 495 µL of $ABTS^{\bullet+}$ with 5 µL of the sample and incubate at ambient temperature for 15 min. Following the process, the mixture's optical absorbance was measured at 734 nm.[24]

The Kačániová method [25] was used, with some modifications, to assess the cell-free supernatant of endophytic bacterial strains's the ferric reducing-antioxidant power. To begin, 495 µL of FRAP solution was combined with 5 µL of test sample in the absence of light for 30 min. The combined optical absorbance was then measured at 593 nm.[25]

The Guenane method was used [23] , with some modifications, to assess the cell-free supernatant of endophytic bacterial strains's total antioxidant capacity. Combine 450 µL of 0.6 M $H_2SO_4$, 28 mM sodium phosphate, and 4 mM ammonium molybdate with 150 µL of the sample. For 90 min, incubate at 90 to 95°C. After the reaction, the mixture's optical absorbance was measured at 695 nm.[23]

The antioxidant activity in the cell-free supernatant of endophytic bacterial strains will be determined based on the standard curve equation of ascorbic acid for each antioxidant method, specifically as follows: DPPH (y = -0.1374x + 1.0975), $ABTS^{\bullet+}$ (y = -0.0593x + 0.694), FRAP (y = 0.0462x + 0.0206), and TAC (y = 0.0267x + 0.0243).

*Determination of in vitro anti-inflammatory ability of endophytic bacterial strains*

The method of inhibiting nitric oxide ($NO^{\bullet}$) formation, protecting red blood cells (RBCs), inhibiting the denaturation of bovine serum albumin (BSA) and egg white albumin (EWA), with modifications, as described by previous studies, applied to explore the inflammation-reducing activity.[26–29] *In vitro* anti-inflammatory activity of endophytic bacterial strains in *C. zedoaria* was determined based on the anti-inflammatory substance content in the cell-free supernatant, which was conventionally equivalent to the ascorbic acid standard (mg AAE/mL cell-free supernatant) for the $NO^{\bullet}$ method or equivalent to diclofenac standard (mg DE/mL cell-free supernatant) for RBCs, BSA and EWA methods.

Specifically, the [26] was used (RBCs method), with some modifications. After centrifuging the mice blood for ten min at 3000 rpm, the clear liquid was extracted and given three saline washes. The RBC solution was then obtained by diluting the blood with saline to a 10% concentration. Then, 1 mL of 10% RBC was reacted with 1 mL of extracellular fluid. Following 30 min of incubation at 56°C, the mixture was cooled. The reaction mixture was subsequently centrifuged at 3000 rpm for 5 min. Absorbance at 560 nm was then recorded at room temperature.[26]

The Das method was used [27] (BSA method), with some modifications. First, 1 mL of 5% bovine serum albumin (BSA) solution was combined with 1 mL of sample solution (at various concentrations). Next, the mixture was incubated at 30°C for 20 min. To induce protein denaturation, the reaction mixture was maintained at 80°C for 10 min. Optical absorbance was measured at 660 nm after cooling.[27]

The Samaraweera[28] method was used (EWA method), with some modifications. The reaction mixture consisted of 1 mL of cell-free supernatant, 1.5 mL of phosphate buffer (pH=6.4), and 100 microliters of EWA (fresh egg). After that, the mixture was incubated for 15 min at 37°C. The reaction mixture was maintained at 70°C for five min in order to produce protein denaturation. The absorbance was determined using spectrophotometry at 660 nm after cooling.[28]

The Fonseca [29] method was used ($NO^{\bullet}$ method), with some modifications. To conduct the survey, 200 µL of 5 mM sodium nitroprusside was mixed with 100 µL of cell-free supernatant, and the mixture was incubated for 60 min at 25 °C. After that, 300 µL of Griess reagent was added. At 546 nm, the spectral absorbance was measured.[29]

The anti-inflammatory content in the cell-free supernatant of the endophytic bacterial strains will be determined based on the standard curve equation of ascorbic acid as well as diclofenac for each anti-inflammatory method, specifically as follows: $NO^{\bullet}$ (y = -0.0216x + 0.9481), RBCs (y = -0.029x + 1.0057), BSA (y = -0.0245x + 1.0687) or EWA (y = -0.0336x + 0.9997).

*Identification of endophytic bacteria in C. zedoaria*

Endophytic bacterial strains in *C. zedoaria* that can produce polyphenols, flavonoids, and have antioxidants as well as anti-inflammatory *in vitro* abilities will be chosen to define the scientific name using *16S rRNA* gene sequencing methods. Combined with physical traits, the bacterial genus was determined by comparing it to the *16S RNA* database on the Gene bank. The morphology of bacterial cells was examined using an optical microscope (CX21FS1, Olympus, Japan) and a scanning electron microscope (Carl Zeiss, Germany). Endophytic bacterial strains were isolated and sequenced for the *16S rRNA* gene at DNA SEQUENCING COMPANY LIMITED (Cai Rang district, Can Tho city, Vietnam). Primer sequences 1492R (5'-TACGGTTACCTTGTTACGACTT-3') and 27F (5' AGAGTTTGA TCCTGGCTCAG-3') were used in the investiagtion.[30] The sequences were derived using BLAST findings of several endophytic bacterial strains in the research against the *16S rRNA* gene database of bacteria and archaea. The control sample (outgroup) was chosen as *Escherichia coli* and *Lactiplantibacillus plantarum*. Multiple sequence alignment was carried out using Bioedit software version 7.2.1, and the sequence length after alignment was 1309 nucleotides. The Maximum Likelihood approach was used to build the phylogenetic diagram, which was then validated for reliability using the bootstrap method with 1000 replications. The investigations were done out using the MEGA X program.[31]

*Data processing and analysis*

The study's data was repeated three times, recorded on Microsoft Excel 2016, and statistically analyzed using Minitab (Minitab, Limited Liability Company, version: 16.0, year of release: 2010, United States). The Tukey's test (ANOVA) was used to examine differences at the 5% level. The data is shown as mean (Mean) ± standard error (SE). The number of times each experiment is conducted is three.

## Results and Discussion

*Endophytic bacteria strains present in C. zedoaria*

Eleven endophytic bacterial strains were obtained from different sections of *C. zedoaria* (3 roots, 6 rhizomes, 2 leaves). The endophytic bacterial strains in *C. zedoaria* are designated as follows: CZ-Rx, CZ-Rhx, and CZ-Lx where CZ is *C. zedoaria*, R is root, Rh is rhizome, L is leaf, and x is the bacterial strain number. These bacteria can grow on PDA and PDB media at 30 °C, pH = 7. The isolated bacterial strains formed colonies that were mostly round (11/11 bacterial strains), opaque white (6/11 bacterial strains), ivory white (1/11 bacterial strains), milky white (3/11 bacterial strains) or yellow (1/11 bacterial strains), raised (8/11 bacterial strains) or flat (3/11 bacterial strains), intact (11/11 bacterial strains), with diameters ranging from 0.2 to 3 mm. The endophytic bacterial strains in *C. zedoaria* were rod-shaped (7/11 bacterial strains) or spherical (4/11 bacterial strains), with both Gram-negative bacteria (4/11 bacterial strains) and Gram-positive bacteria (7/11 bacterial strains). The endophytic bacteria's morphological traits that were separated from *C. zedoaria*'s roots, rhizomes, and leaves are shown in (Table 1).

*Total polyphenol and flavonoid content*

Due to their potent biological activity, polyphenols as well as flavonoids are significant classes of secondary metabolites that are crucial for both prevention and therapy, as well as for safeguarding human health. According to many previous publications, polyphenols and flavonoids have good antioxidant and anti-inflammatory properties.[32–35] These two kinds of them have also been demonstrated to be produced by endophytic bacteria.[36,37] From there, our study surveyed the capacity of endophytic bacterial strains in *C. zedoaria* to create polyphenols and flavonoids in order to advance scientific understanding of this problem. It has been demonstrated that the medicinal species *C. zedoaria* is abundant in secondary metabolites, such as flavonoids and polyphenols.[13,16,18–21] Figure 2 shows TPC and TFC in 11 plant endophytic bacterial strains, which were isolated from parts such as roots, rhizomes and leaves of *C. zedoaria*.

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**Table 1:** Colony and cell morphological characteristics of endophytic bacterial strains

| No. | Bacterial strains | Colony shape | Colony color | Elevation | Margin | Colony size (mm) | Cell shape | Gram |
|---|---|---|---|---|---|---|---|---|
| 1 | CZ-Rh1 | Circular | Ivory | Convex | Entire | 1 | Spherical | Positive |
| 2 | CZ-Rh2 | Circular | Ivory | Flat | Entire | 2 | Spherical | Negative |
| 3 | CZ-Rh3 | Circular | Ivory | Flat | Entire | 1.2 | Rod-shaped | Negative |
| 4 | CZ-Rh4 | Circular | Ivory | Convex | Entire | 2.5 | Rod-shaped | Positive |
| 5 | CZ-Rh5 | Circular | Off white | Convex | Entire | 0.8 | Spherical | Positive |
| 6 | CZ-Rh7 | Circular | Milk-white | Convex | Entire | 2.5 | Rod-shaped | Positive |
| 7 | CZ-R1 | Circular | Milk-white | Convex | Entire | 0.5 | Rod-shaped | Positive |
| 8 | CZ-R3 | Circular | Light yellow | Convex | Entire | 0.2 | Spherical | Positive |
| 9 | CZ-R5 | Circular | Ivory | Convex | Entire | 1.7 | Rod-shaped | Positive |
| 10 | CZ-L6 | Circular | Ivory | Flat | Entire | 3 | Rod-shaped | Positive |
| 11 | CZ-L11 | Circular | Milk-white | Convex | Entire | 2 | Rod-shaped | Positive |

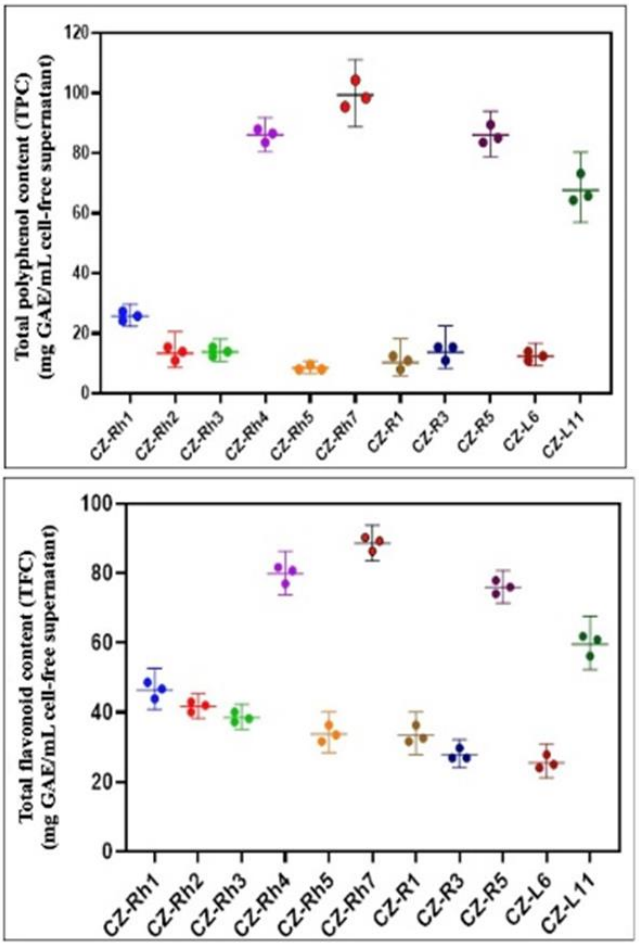


**Figure 2**: TPC and TFC in cell-free supernatant of endophytic bacterial strains

The findings of this investigation not only advance knowledge of the biological potential of *C. zedoaria* endophytic strains, but also promote research and application of natural compounds from plant endophytic bacteria, thereby opening up many new research directions for science. According to research findings, endophytic bacterial strains isolated from *C. zedoaria* can produce polyphenols and flavonoids. The bacterial strains generated polyphenols with contents ranging from 8.49±0.86 to 99.36±4.53 mg GAE/mL cell-free supernatant. Besides, they also generate flavonoids ranging from 25.63±1.96 to 88.21±0.94 mg QE/mL cell-free supernatant. Among these, the CZ-Rh7 bacterial strain was shown to generate the most polyphenols and flavonoids. The lines CZ-Rh5 and CZ-L6 have the lowest polyphenol and flavonoid production capacity. Thus, our work found that endophytic bacterial strains isolated from *C. zedoaria* may create polyphenols and flavonoids. This is also the first time surveying the TPC and TFC of cell-free supernatant of endophytic bacterial strains from *C. zedoaria* has been carried out.

*Results of in vitro antioxidant capacity*

Plant endophytic bacterial strains are affected by oxidative stress throughout their survival and development. Oxidizing chemicals in the extracellular and intracellular environment, including reactive oxygen species (ROS) and so on, have a deleterious impact on the survival of bacterial strains.[38] To survive and adapt to the severe circumstances induced by oxidative stress, plant endophytic bacterial strains have developed defensive mechanisms such as producing compounds with antioxidant qualities such as polyphenols and flavonoids.[36,37] As a result, the study has assessed the antioxidant capacity of endophytic bacterial strains in *C. zedoaria* using methods such as DPPH, $ABTS^{\bullet+}$, TAC, and FRAP. Our study, which sought to evaluate the possible antioxidant activity of endophytic bacterial strains, was the first to report these findings. Figure 3 depicts the ascorbic acid-equivalent antioxidant capacity of endophytic bacterial strains isolatedd from *C. zedoaria*. The amount of compounds in endophytic bacterial strains that could neutralize the free radicals in the DPPH and $ABTS^{\bullet+}$ methods varied from 14.95±0.49 to 200.67±6.75 mg AAE/mL cell-free supernatant, respectively. Meanwhile, the values of the content of compounds in endophytic bacteria isolated from *C. zedoaria* with antioxidant capacity according to two methods (TAC and FRAP) range from 76.36±0.40 to 168.34±3.52 mg AAE/mL cell-free supernatant. The CZ-Rh7 endophytic bacterial strain has a statistically significant difference ($p<0.05$) in its ability to produce higher antioxidants compared to the remaining endophytic bacterial strains. The amount of substances in CZ-Rh7 that have antioxidant properties endophytic bacterial strain in $ABTS^{\bullet+}$, DPPH, TAC, FRAP methods was 10.20, 2.80, 1.68 and 1.67 times higher, respectively, than that of the CZ-Rh5 endophytic bacterial strain.

Numerous investigations have demonstrated a substantial relationship between the antioxidant capability of a sample and its TPC, TFC. In particular, a test sample's resistance to oxidation improves with increasing TPC and TFC.[39–41] Therefore, endophytic bacterial strains that are capable of producing secondary metabolites will also have antioxidant activity. In 2020, [42] investiagted the antioxidant capacity of endophytic bacteria that were separated from *Emilia sonchifolia*. According to this study, the best antioxidant activity was exhibited by

the endophytic bacterial strains that contained the most total polyphenol and flavonoid content.[42] The previous research of [43], determined that the bacterial strains that generated the most phenolic compounds had the highest antioxidant activity using three techniques ($ABTS^{\bullet+}$, DPPH, and FRAP) in 2023 on endophytic bacterial strains derived from *Pyrrosia piloselloides*. [43] Our study's findings on the close connection between TPC and TFC and the test sample's antioxidant capacity are entirely in line with the findings of the previously published research. Out of the eleven endophytic bacterial strains examined in this study, CZ-Rh7 had the greatest TPC and TFC with thier values of 99.36±4.53 mg GAE/mL cell-free supernatant and 88.21±0.94 mg QE/mL cell-free supernatant (Figure 2), respectively, so the antioxidant activity of CZ-Rh7 is the best (Figure 3). In addition, three endophytic bacterial strains of *C. zedoaria* including: CZ-Rh4 (TPC = 86.02±2.26 mg GAE/mL cell-free supernatant, TFC = 79.72±2.50 mg QE/mL cell-free supernatant), CZ-R5 (TPC = 86.02±3.08 mg GAE/mL cell-free supernatant, TFC = 75.94±1.89 mg QE/mL cell-free supernatant), CZ-L11 (TPC = 67.75±4.76 mg GAE/mL cell-free supernatant, TFC = 59.59±3.03 mg QE/mL cell-free supernatant) also showed good antioxidant activity. Thus, it can be concluded that the antioxidant activity of endophytic bacterial strains isolated from *C. zedoaria* depends on TPC and TFC. Endophytic bacterial strains in *C. zedoaria* possess antioxidant activity against various free radicals through four survey methods, as a result, they have the potential to use active components to prevent and cure a wide range of oxidative stress-related disorders in humans.

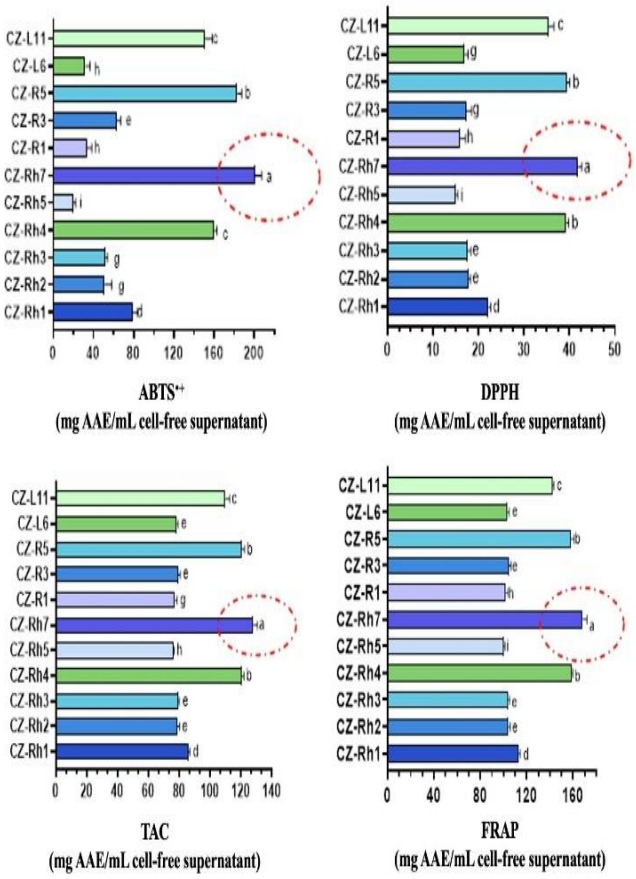


**Figure 3.** Antioxidant content in cell-free supernatant of endophytic bacterial strains

*Results of in vitro anti-inflammatory capacity*

Inflammation is one of the body's natural defense mechanisms, working to combat pathogens, get rid of risky situations, and speed up the healing process. However, inflammation will have several detrimental impacts on the body, including harm to tissues and organs, if it persists for an extended length of time and is not managed. Numerous concerning illnesses, including diabetes, cancer, arthritis, and cardiovascular disease, will result from this. Furthermore, excessive inflammation will also have a major negative impact on the immune system.[44] Natural substances can decrease inflammation, and endophytic bacteria found in medicinal plants are a possible source of them.[45,46] Our research revealed that endophytic bacterial strains isolated from *C. zedoaria* had anti-inflammatory properties *in vitro* by preventing the production of nitric oxide, protecting red blood cells, and preventing denaturation albumin from egg whites and bovine serum. This was also the first time this work has been performed on this species.

The enzyme nitric oxide synthase (NOS), specifically the iNOS enzyme family (found in immune cells and macrophages), produces nitric oxide (an inflammatory mediator). This kind of inflammatory mediator is crucial for controlling the inflammatory reaction brought on by inflammatory substances. The ability of $NO^{\bullet}$ to induce the production of cytokines, prostaglandins, and other substances by endothelial cells is one of its noteworthy processes. This improves the flow of immune cells to inflammatory regions by increasing blood vessel permeability. Notwithstanding the advantages that $NO^{\bullet}$ offers, excessive production of it might have detrimental effects on the body by escalating inflammation.[47] Natural compounds have the capacity to limit the generation of $NO^{\bullet}$, which can help prevent and alleviate inflammation-related disorders. The investigation was undertaken to assess the concentration of chemicals capable of blocking the generation of $NO^{\bullet}$ by endophytic bacterial strains, as shown in (Table 2). Endophytic bacteria isolated from *C. zedoaria* produce compounds that inhibit $NO^{\bullet}$ synthesis at concentrations ranging from 45.39±1.54 to 131.57±2.68 mg AAE/mL cell-free supernatant. The CZ-Rh7 endophytic bacteria produces the greatest quantities of compounds that suppress $NO^{\bullet}$ production.

**Table 2**: Content of compounds with anti-inflammatory *in vitro* activity of cell-free supernatant of endophytic bacterial strains

| No. | Bacterial strains | The content of compounds with anti-inflammatory *in vitro* activity of cell-free supernatant of endophytic bacterial strains is equivalent to the standard substance | | | |
|---|---|---|---|---|---|
| | | $NO^{\bullet}$ (1) | BSA (2) | EWA (3) | RBCs (4) |
| 1 | CZ-Rh1 | 69.29$^{d}$±1.13 | 33.61$^{d}$±0.49 | 20.32$^{c}$±0.33 | 8.99$^{d}$±0.59 |
| 2 | CZ-Rh2 | 54.60$^{e}$±1.13 | 29.01$^{e}$±0.74 | 16.99$^{de}$±0.70 | 4.26$^{e}$±0.43 |
| 3 | CZ-Rh3 | 53.67$^{e}$±2.09 | 29.20$^{e}$±0.62 | 17.15$^{d}$±0.45 | 4.12$^{e}$±0.66 |
| 4 | CZ-Rh4 | 122.93$^{b}$±0.90 | 48.17$^{b}$±0.45 | 31.19$^{a}$±0.30 | 25.77$^{b}$±0.45 |
| 5 | CZ-Rh5 | 45.39$^{f}$±1.54 | 26.91$^{f}$±0.43 | 15.46$^{e}$±0.26 | 1.61$^{f}$±0.28 |
| 6 | CZ-Rh7 | 131.57$^{a}$±2.68 | 50.70$^{a}$±0.66 | 32.70$^{a}$±0.58 | 28.00$^{a}$±0.38 |
| 7 | CZ-R1 | 48.83$^{ef}$±3.07 | 27.87$^{ef}$±0.99 | 16.09$^{de}$±0.70 | 3.20$^{ef}$±0.46 |

| | | | | | |
|---|---|---|---|---|---|
| 8 | CZ-R3 | 53.85$^{e}$±4.13 | 29.20$^{e}$±0.94 | 17.22±0.66 | 4.88$^{e}$±0.18 |
| 9 | CZ-R5 | 123.67$^{b}$±2.17 | 48.36$^{b}$±0.74 | 31.15$^{a}$±0.46 | 25.31$^{b}$±1.14 |
| 10 | CZ-L6 | 51.34$^{ef}$±2.38 | 28.60$^{ef}$±0.53 | 16.49$^{de}$±0.77 | 3.68$^{e}$±0.38 |
| 11 | CZ-L11 | 110.47$^{c}$±3.69 | 44.96$^{c}$±0.88 | 28.55$^{b}$±0.73 | 21.63$^{c}$±1.04 |

Note: (1) is the content of compounds capable of inhibiting the formation of $NO^{\bullet}$ (mg AAE/mL cell-free supernatant); (2) is the content of compounds capable of inhibiting the denaturation of bovine serum albumin (mg DE/mL cell-free supernatant); (3) is the content of compounds capable of inhibiting the denaturation of egg white albumin (mg DE/mL cell-free supernatant); (4) is the content of compounds capable of protecting red blood cells (mg DE/mL cell-free supernatant). Means followed by the same letter or letters in the same column do not vary significantly at the 5% level.

Maintaining red blood cells (RBCs) can help patients avoid illness since they play a vital role in the human body. Active immune cells produce lysosomal components like proteases during the inflammatory response, setting off a series of events connected to systemic lupus erythematosus, glomerulonephritis, and low-form arthritis. Many anti-inflammatory medicines have been proven to inhibit enzyme release from lysosomes while stabilizing their membranes. Because red blood cell membranes and lysosome membranes are similar, anti-inflammatory action is frequently assessed using red blood cell protection.[48] Endophytic bacterial strains in *C. zedoaria* are capable of producing compounds that protect red blood cells with concentrations ranging from 1.61±0.28 to 28.00±0.38 mg DE/mL cell-free supernatant (Table 2).

Protein denaturation occurs when the spatial structure of proteins changes, causing them to function abnormally. Denaturation converts certain proteins into endogenous antigens, which means that the immune system considers the body's own proteins to be alien. This will activate immunological response chains, and the body will produce antibodies that target the denatured protein, resulting in tissue damage and organ weakening. As a result, autoimmune disorders such as arthritis, glomerulonephritis, and lupus erythematosus will develop often and have a harmful impact on the body.[49] Therefore, our study used heat to simulate autoantigen induction due to protein denaturation (for BSA as well as EWA). The content of compounds capable of inhibiting the denaturation of BSA, EWA is presented in (Table 2). Endophytic bacterial strains inhibited heat-induced protein denaturation of BSA and EWA, with values ranging from 15.46±0.26 to 50.70±0.66 mg DE/mL cell-free supernatant. The bacterial strain CZ-Rh7 generates compounds that prevent denaturation of BSA and EWA, which is considerably different ($p<0.05$) from other endophytic bacterial strains. According to the research findings, the capacity to suppress denaturation of BSA and EWA is dependent on TPC and TFC assessed above (section **3.2** and **3.3**). Numerous investigations on the anti-inflammatory properties of extracts from medicinal herbs have shown that the kind and amount of secondary metabolite in the extracts determine their anti-inflammatory action.[50,51] Thus, our work also demonstrates that TPC and TFC are necessary for the inflammation-reducing of endophytic bacterial strains obtained from *C. zedoaria*.

*Identification of some endophytic bacterial strains*

Endophytic bacterial strains CZ-Rh7, CZ-R5, CZ-Rh4 and CZ-L11 have the ability to produce polyphenols, flavonoids, antioxidants and anti-inflammation *in vitro* more effectively than the remaining bacterial strains, so they were chosen for extraction DNA and *16S rRNA* gene sequencing. Based on the morphological characteristics of bacterial colonies and cells (Table 1, Figure 4) combined with the results of *16S rRNA* gene sequencing, the bacterial strain CZ-R5 was determined to belong to the genus *Enterobacter*, CZ- L11, CZ-Rh7, CZ-Rh4 belong to the genus *Bacillus* (Figure 5).

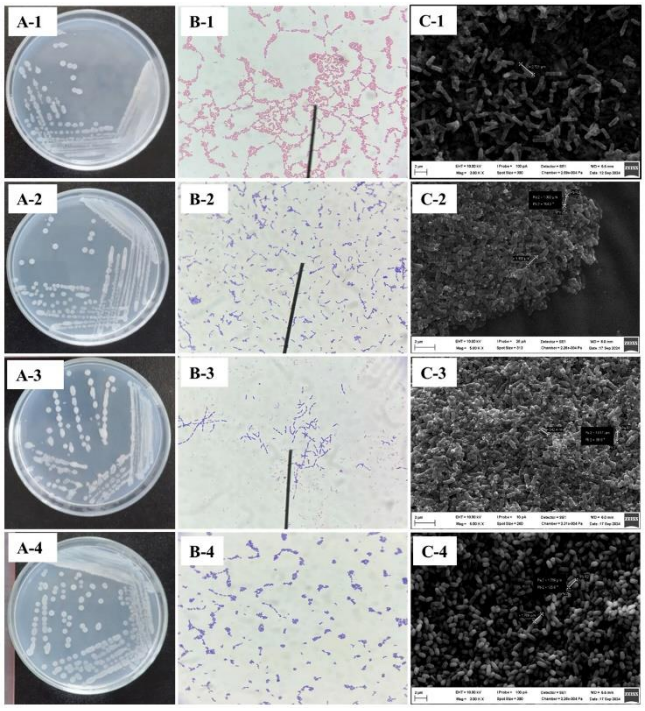


**Figure 4**. Colony and cell morphology of some endophytic bacterial strains in *C. zedoaria* Notes: (A) Colony morphology of bacteria in a 10 cm petri dish, (B) Cell morphology of bacteria with magnification of 400 times, (C) Size of bacteria observed by microscope scanning electron micrograph. 1, 2, 3, 4 lanes are bacterial strains CZ-R5, CZ-Rh4, CZ-Rh7 and CZ-L11, respectively.

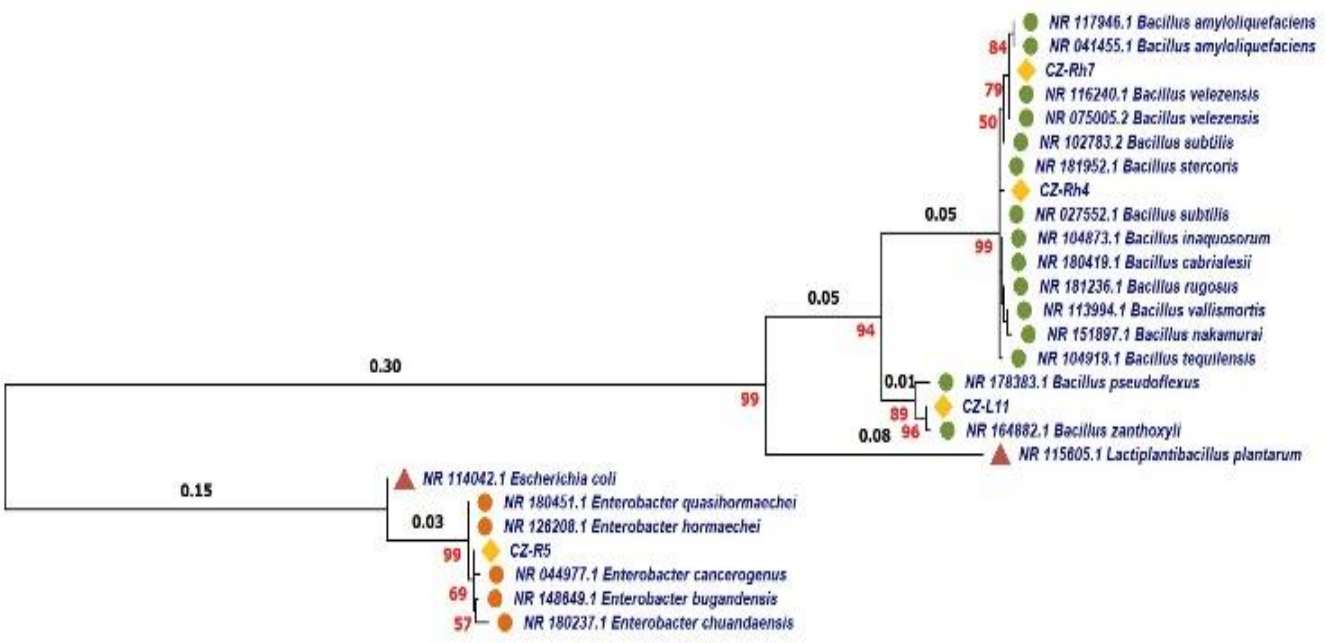


**Figure 5**: Phylogenetic tree of endophytic bacterial strains CZ-Rh7, CZ-Rh4, CZ-L11 and CZ-R5

One previous research also showed that endophytic bacterial strains belonging to the genera *Enterobacter* and *Bacillus* possess many valuable biological activities such as: producing polyphenols, flavonoids, antioxidants and antibacterial properties.[52] Another study found that endophytic bacteria in medicinal plants create pharmaceutically essential compounds such antimicrobials, antioxidants, industrial enzymes, antidiabetic medicines, and anticancer agents.[53] Our study has contributed to providing more scientific basis for the medicinal potential of endophytic bacterial strains. Specially, information on bacterial strains CZ-Rh7, CZ-R5, CZ-Rh4 and CZ-L11 has been published by this study on the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI) (Table 3). With the support of gene editing technology such as CRISPR-Cas9 and biosynthesis, we can improve bacteria to produce more specific pharmaceuticals. There are still a lot of obstacles to overcome, though, like comprehending the biosynthetic mechanism, guaranteeing biosafety, and streamlining the commercial manufacturing process. Endophytic bacteria are a valuable platform in the biotechnology and pharmaceutical sectors, and their full potential may be realized in the future with the aid of biotechnology, artificial intelligence, and materials science.

**Table 3**: Information on bacterial strains CZ-Rh7, CZ-R5, CZ-Rh4 and CZ-L11 on NCBI

| Samples | GenBank |
|---|---|
| *Bacillus* sp. CZ-Rh7 | PQ533188 |
| *Enterobacter* sp. CZ-R5 | PQ533184 |
| *Bacillus* sp. CZ-Rh4 | PQ525292 |
| *Bacillus* sp. CZ-L11 | PQ533185 |

Based on the study's results, endophytic bacterial strains found in *C. zedoaria* present excellent opportunities to replace the source of secondary metabolite exploitation with plant-based antioxidant and anti-inflammatory activities, thereby lowering environmental stress and improving the manufacturing of food, cosmetics, and medications. Numerous studies have specifically shown that endophytic bacteria are capable of producing antibiotics, enzymes that break down toxins, and immunostimulant chemicals, which may find use in the treatment of inflammation, cancer, and infections.

## Conclusion

Eleven endophytic bacterial strains isolated from *C. zedoaria* all had the ability to produce polypohenol, flavonoid, antioxidant and anti-inflammatory. The total polyphenol and flavonoid content of endophytic bacterial strains determined their capacity to serve as antioxidants and anti-inflammatory agents. Bacterial strains CZ-Rh7, CZ-Rh4 and CZ-L11 belonging to the genus *Bacillus* and CZ-R5 belonging to the genus *Enterobacter* had the ability to produce polypohenol, flavonoid, antioxidant and anti-inflammatory *in vitro* relatively stronger and more stable than the remaining bacterial strains. The aforementioned findings demonstrated that *C. zedoaria* includes endophytic bacteria that are cultivable and isolated *in vitro*. Following *in vitro* culture, the cell-free supernatant demonstrated biological activity, suggesting that these endophytic bacteria can produce active chemicals with anti-inflammatory and antioxidant characteristics. This work expands on the potential use of endophytic bacteria in *C. zedoaria* as an antioxidant and anti-inflammatory agent, as well as their ability to be grown outside of the host plant to produce secondary metabolites.

## Conflict of interest

The author reports no conflicts of interest in this work.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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