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Flavonoid Contents and Antioxidant Activity of an Endophytic Bacteria Associated with Medicinal Plant *Humulus lupulus*

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Abstract

Endophytes are symbiotic microorganisms, most often bacteria or fungi, that reside and multiply within plant tissues without inducing visible signs of disease or negatively affecting the host plant. Endophytic bacteria contribute to plant development by stimulating growth, strengthening defence mechanisms, enhancing tolerance to both abiotic and biotic stresses, and facilitating improved nutrient uptake. Endophytic bacteria serve as an important reservoir of bioactive compounds, notably antioxidant molecules, that play a crucial role in plant defence and human health. Furthermore, there is an escalating scientific focus on the distinctive and relatively unexplored organic compounds derived from diverse endophytic bacteria, owing to their broad functional traits and valuable applications in numerous fields. The heterogeneity of endophyte bacterial isolates across distinct geo-climatic zones underscores the uniqueness of each organism, positioning them as promising reservoirs for the discovery of novel bioactive compounds. Endophytes represent a crucial source of biologically active natural products. This investigation focuses on isolated and characterized endophytic bacteria from the medicinal plant Humulus lupulus. Two endophytic bacteria were isolated. The dried extract of the endophytic bacteria was subjected to phytochemical analysis and evaluation of its antioxidant activity. Total Flavonoid Content (TFC) in the dried extract from the Bacillus licheniformis SKAM1 and Bacillus cereus SKAM2 showed a yield of around 0.48 mg/ml and 0.40 mg/ml, respectively. The antioxidant activity of the dried extract from the Bacillus licheniformis SKAM1 and Bacillus cereus SKAM2 showed antioxidant activity of 80.12% and 76.25%, respectively. To conclude, endophytic bacteria emerge as noteworthy prospects for natural antioxidant applications.

Keywords: Endophytic bacteria, *Humulus lupulus*, antioxidant, total flavonoids content

INTRODUCTION

Plant bacterial endophytes are microorganisms that inhabit plant tissues, either for a portion of their lifespan or for their entire lifespan, without producing any obvious disease symptoms in the host plant [1, 2]. The demonstration of plant endophytic bacteria revealed host-specific characteristics and was influenced by environmental conditions [3]. Endophytic bacteria are thought to engage in a mutualistic relationship with plants [4]. Through their engagements, plants furnish nutrients and habitats for bacteria, while bacterial endophytes, in turn, fulfill essential roles in enhancing plant growth and boosting tolerance to both biotic and abiotic stresses [5]. Countless investigations have concentrated on isolating and inspecting serviceable bacterial endophytes to enhance plant growth or safeguard plants from pathogens. Plant endophytes are attracting scientific attention as a

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source of novel natural products because of their crucial relationship with plants [6]. Plant metabolites provide a rich source of pharmacologically significant compounds that promote drug discovery and account for approximately 80% of the entire range of synthetic pharmaceuticals [7]. However, the classical technique of harvesting secondary metabolites (SM) from medicinal plants encounters several obstacles, such as seasonal variability, mismatches between market needs and availability, erosion of biodiversity, the threatened status of various plant species, and rising costs. It should be noted that plants are not always the exclusive producers of their secondary metabolites (SMs); endophytes – microorganisms residing within the plant's tissues without causing disease – can also produce these metabolites. Examples include vinblastine, vincristine, hypericin, taxol, and camptothecin. Endophytes are associated with nearly every higher plant studied so far [8]. *Humulus lupulus* L. is a climbing, perennial, and dioecious plant that is a member of the Cannabaceae family and the order Rosales [9]. The hop plant's cones enclose many secondary metabolites within lupulin glands, which are primarily utilized in beer production. Hop resins, essential oils, and their derivatives give beer its characteristic bitter flavor and hoppy aroma [10]. Several substances found in hops exhibit multiple biologically potent effects. Among them, 8-Prenylnaringenin is recognized as the most potent phytoestrogen identified to date [11]. The rise of antimicrobial resistance in bacterial pathogens is increasingly posing a serious threat to global public health [12]. Beta acids are distinguished by their potent antimicrobial properties against certain categories of bacteria [13, 14]. Xanthohumol, a prenylated flavonoid, exhibits anticancer properties resistant to certain categories of cancer [15]. So far, research on the hop plant has mainly emphasized areas other than the natural colonization by endophytic and epiphytic bacteria, with only a few exceptions [16–18]. In contrast, it was often regarded that Hops would be resistant to colonization by bacteria due to its metabolites are endowed with antimicrobial activity [16]. The rarity of bacterial diseases further illustrates the considerable immunity of hops to bacterial infections in comparison to those caused by viruses or fungi. However, some bacteria have been isolated from hops, including strains of the genus Streptomyces found in the hop rhizosphere [19]. Pseudomonas fluorescens and Pseudomonas stutzeri isolated from hop cones [20]. This investigation aims to reveal the isolation of endophytes and bacteria from fresh Leaves of the Hops (*Humulus lupulus* L.) plant and their assessment of antioxidant activity.

MATERIALS AND METHODS

Collection of Microorganisms

Recently, our lab isolated endophytic bacteria from *Humulus lupulus*. The endophytic bacteria were characterized through molecular techniques, and their 16S rRNA gene sequence have been deposited in the GenBank database, *Bacillus licheniformis* SKAM1 (Accession No PX363043.1) and *Bacillus cereus* SKAM2 (Accession No PQ305264). The isolated endophytic bacteria were maintained on Luria agar plates and stored at 4°C for later use [21].

Endophytic Bacterial Fermentation

The isolated endophytic bacteria were grown in Luria broth culture medium and incubated for 24 hours at 37°C and 150 rpm using an orbital shaker (Khuner). After fermentation, the culture broth was centrifuged, and bacterial cells were separated and cells were discarded, and the supernatants were dried at room temperature. Dried extracts were used for further studies.

Determination of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was determined using an aluminum chloride-based colorimetric assay, as outlined in previously published protocols [22], with modifications. Quercetin was solubilized in methanol (HPLC grade) to obtain a stock solution of 1mg/ml. The assay executed 5% (w/v) sodium nitrate, 2% (w/v) aluminum chloride, and 1 M sodium hydroxide as reagents. A volume of 100 µl from various quercetin concentrations (ranging from 200 to 1000 µg/ml) was combined with 30 µl of 5% sodium nitrate and incubated for 5 minutes. Afterward, 30 µl of 2% aluminum chloride was added, followed by a 6-minute incubation. Next, 200 µl of 1 M sodium hydroxide was introduced, and the

mixture was diluted with distilled water to a final volume of 1 ml. Absorbance readings were taken at 510 nm using a Thermo Scientific ELISA reader, with distilled water serving as the blank. The total flavonoid content (TFC) in dried extract was quantified by referencing a standard calibration curve plotted from quercetin concentrations against absorbance at 510 nm. All experiments were conducted in duplicate to ensure reproducibility.

Determination of Antioxidant Activity

Antioxidant activity of the dried extract derived from the isolated endophytic bacteria was analyzed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, based on the protocol reported by (23), with minor modifications. Briefly, 900 µl of DPPH solution prepared in methanol was mixed with 100 µl of the dried extract of isolated bacteria. The mixture was incubated at room temperature for 30 minutes. Absorbance was measured at 517 nm. The antioxidant potential of the dried extract was determined by comparing its DPPH scavenging capacity against a standard calibration curve generated using ascorbic acid The percentage inhibition of free radical DPPH was derived using Equation 1, where A_C is the absorbance of the control and A_S is the absorbance of the sample [23].

$$\text{DPPH Scavenging Activity (\%)} = \frac{\text{Absorbance of control } (A_C) - \text{Absorbance of Sample } (A_S)}{\text{Absorbance of control } (A_C)} \times 100 \quad (1)$$

RESULT

Determination of Total Flavonoid Content (TFC)

The total flavonoid content in the bacterial dried extracts was quantified using a standard curve generated from the aluminum chloride colorimetric assay. The Total Flavonoid Content (TFC) in the dried extract from the *Bacillus licheniformis* SKAM1 and *Bacillus cereus* SKAM2 showed a yield of around 0.48 mg/ml and 0.40 mg/ml, respectively (Figure 1).

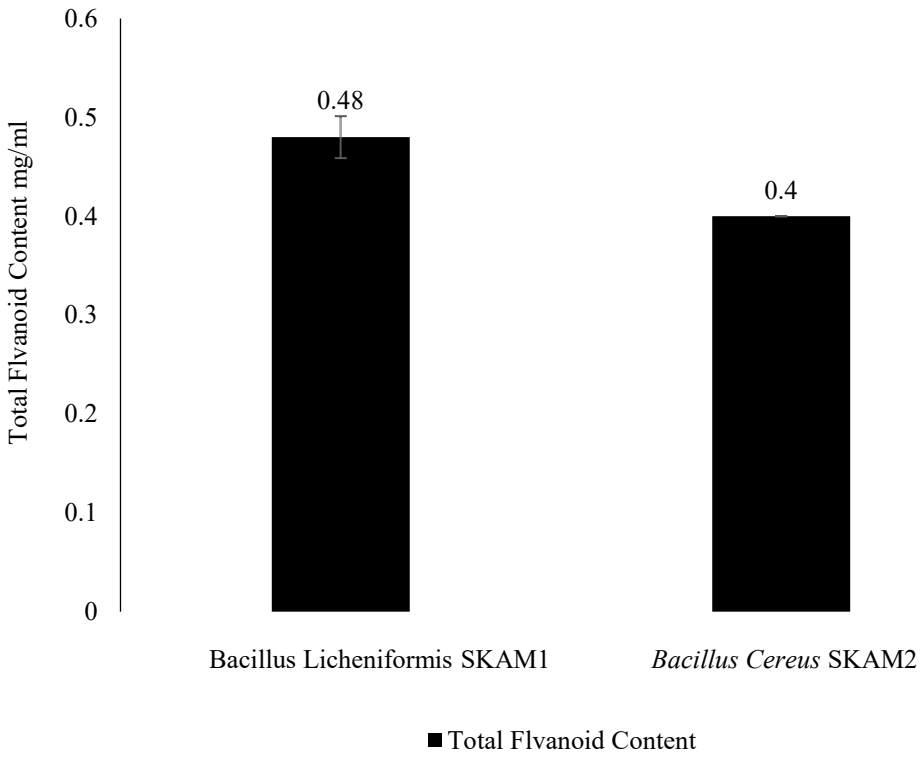

Figure 1. Comparison of total flavonoid content (mg/ml) from a dried extract of *Bacillus licheniformis* SKAM1 and *Bacillus cereus* SKAM2.

Antioxidant Activity

Moreover, the dried extracts of two isolated endophytic bacteria were assessed for their ability to scavenge DPPH free radicals, highlighting the potential antioxidant function of flavonoids. During the assay, DPPH was transformed into its stable, non-radical form (DPPH-H) through the action of hydrogen-donating antioxidants. The antioxidant activity of the dried extract from the *Bacillus licheniformis* SKAM1 and *Bacillus cereus* SKAM2 showed antioxidant activity of 80.12 % and 76.25 %, respectively (Figure 2).

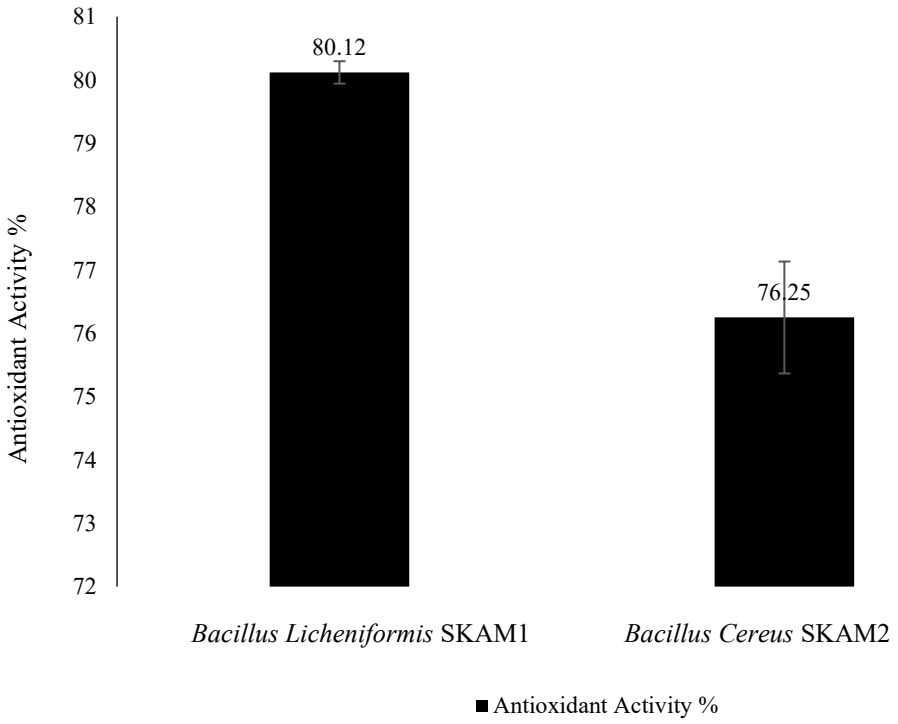

Figure 2. Comparison of antioxidant activity from dried extract of *Bacillus licheniformis* SKAM1 and *Bacillus cereus* SKAM2.

DISCUSSION

The increasing demand for effective antioxidants has inspired scientific investigations into naturally derived bioactive substances for their antioxidant potential. The growing popularity of plant-based products has become more prominent because of their broad range of health-promoting properties such as antioxidant potential [24]. The core objective of this work focuses on assessing the antioxidant potential of endophytic bacteria derived from the medicinal plant *Humulus lupulus*. Antioxidant potential capacity was determined through conducting TFC and DPPH assays. The flavonoid compounds present in these endophytes' bacterial dried extracts contribute to reducing oxidative damage induced by reactive oxygen species. These ROS are key contributors to the onset and a wide range of chronic conditions. These compounds act through several different mechanisms to reduce the impact of reactive oxygen species (ROS) on cellular longevity. This includes scavenging free radicals, regulating antioxidant enzymes, and binding metal ions, like copper and iron, which typically facilitate ROS production. The hydroxyl chemical groups present in these compounds permit them to transfer hydrogen atoms to free radicals or ROS, effectively neutralizing them [25]. The DPPH assay was employed to determine the electron-donating strength of the antioxidants found in the bacterial dried extracts. By transferring electrons, these antioxidants neutralize DPPH radicals, as shown by the color change from purple to yellow. As a result, this assay confirms the presence of potent antioxidants in the bacterial extracts [25, 26]. The results from this research point to the therapeutic potential of the biologically active compounds in the bacterial extract. The current study provided endophyte bacteria

that revealed Total Flavonoid Content (TFC) in the dried extract from the *Bacillus licheniformis* SKAM1 and *Bacillus cereus* SKAM2 showed a yield of around 0.48 mg/ml and 0.40 mg/ml, respectively. The antioxidant activity of the dried extract from the *Bacillus licheniformis* SKAM1 and *Bacillus cereus* SKAM2 showed antioxidant activity of 80.12% and 76.25%. Future research could focus on refining the bioactive compounds and testing their bioactivity. Additionally, studies aimed at purifying and characterizing these compounds may be crucial in validating their potential roles.

CONCLUSIONS

The diverse demographic factors, along with the wide range of associated microscopic organisms, remain to be explored for their potential benefit. This study stands out due to its unique approach, as the isolated endophytic bacteria from the medicinal plant *Humulus lupulus* were analyzed to be a source of flavonoids and antioxidant potential. The occurrence of flavonoids in endophytic bacteria suggests their potential as alternative, economically viable sources of these bioactive compounds. The present investigation delineates prospective avenues for metabolite profiling, emphasizing the integration of Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) methodologies.

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