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ORIGINAL ARTICLE **OPEN ACCESS**

In-vitro study of anti-bacterial and time-kill efficacy assay of *Enterobacter roggenkampii* BLS02 from *Barleria lupulina* Lindl.

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ABSTRACT
Antibacterial properties of the medicinal plant (Barleria lupulina) may be attributed to the presence of bioactive compounds. Secondary metabolite found in endophytic bacteria have long been documented. 16S rRNA Sequencing has already been used to identify the endophytic bacterium Enterobacter roggenkampii (BLS02) from B. lupulina. Well and disc agar diffusion techniques were used to test E. roggenkampii from B. lupulina for antibacterial activity. Antibacterial secondary metabolites with the greatest potential against six of the most common human diseases were further chosen to establish the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Tests on Listeria monocytogenes indicated that 20 µg/mL of antibacterial activity was the most effective. There was a range of 50 to 100 µg/mL MIC and MBC for L. monocytogenes and Pseudomonas aeruginosa, respectively. In this study S. typhi, K. pneumoniae, P. aeruginosa, S. aureus, L. monocytogenes, and E. coli were all subjected to a time-killing test after six hours, followed by S. aureus, L. monocytogenes, and E. coli.
***Keywords*:** *Barleria lupulina*, antimicrobial activity, *Enterobacter roggenkampii*, MIC and MBC.

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INTRODUCTION

Quality and productivity of medicinal plants are affected by environmental factors including temperature, lighting, moisture and soil conditions. Soil fauna and their existence are also important factors to consider [1]. It is possible that bacterial endophytes may have a significant influence on the medicinal plant efficacy and safety [2]. When bacterial colonization occurs at important periods of plant development, the seed may already have a stable endophytic bacterial population [3]. These endophytes take use of the plant internal environment to defend itself against substantially altering external circumstances [4]. As the global population grows, drug supplies become scarcer, prices rise, side effects from many allopathic medications increase, and drug resistance to commonly prescribed antibiotics and other infectious disease treatments emerges, natural remedies for a wide range of human ailments are becoming increasingly popular. Natural solutions for a broad spectrum of human problems are getting more and more essential [5]. Around 80% of the world's 4 billion inhabitants, according to the WHO, cannot afford Western pharmaceutical items and must instead depend on traditional treatments made from plant components [6]. This claim is backed up by a comprehensive inventory of medicinal plants, which covers over 20,000 distinct species and provides sufficient evidence to substantiate it [7]. Biochemical substances that may be extracted from green plants and used as chemical feedstock or raw material in a range of scientific study are many and varied. Due to their vast variety of uses, secondary metabolites from plants are important to the pharmaceutical business [8]. In several medical systems across the world, medicinal plants are used for their health-enhancing qualities. For example, *B. cristata* is only one of more than 300 species of Barleria that may be found in the wild, including *B. albostelleata,* and *B. prionitis* in addition to the more common Barleria. There has been a lengthy history of ethnomedical research on these species. According to the complete inventory of medicinal plants, which contains more than 20,000 unique species and has adequate evidence to support the statement, this premise is supported [9]. As a source of chemical feedstock or raw material for a broad variety of scientific inquiries, green plants contain a vast spectrum of biochemical compounds. These plant secondary metabolites (endophytic microorganisms) are essential in the pharmaceutical industry since

they may be used for many different things, including antibiotics. In the present study, the ability of Endophytic bacteria (*Enterobacter roggenkampii*) isolated from medicinal plant (*Barleria lupulina*) promote the antibacterial activity against some pathogenic bacteria. These endophytic bacteria are very significance for upcoming as a work in medicine in the field of pharmaceutical industries and medical field.

MATERIAL AND METHODS

Collection

Young and fresh stems of *B. lupulina* were collected from plants that were growing in the Botanical Garden of the Department of Botany and Microbiology (Figure 1) at Gurukul Kangri (Deemed to be University), located in Haridwar (29°55′18′′N 78°7′39′′E/29.92167°N 78.12750°E), Uttarakhand (29.92167°N 78.12750°E) (India).

Isolation and characterization of endophytic bacteria

Samples of stems were washed many times with tap water after drying at room temperature to eliminate any surface debris. Afterwards, 70% ethanol was used for 3 to 5 minutes to clean the stem samples, followed by 2 minutes of 4% NaOCl cleaning. Finally, the roots were washed 5-6 times with double distilled water for 2-5 minutes to remove any remaining traces of pesticides from the soil. The sterile distilled water was used to serially dilute and distribute on the surface of a suitable medium of nutrients after cutting the stem samples into small pieces. For a period of two to seven days, the plates were incubated at 37°C. NA plates were used to identify and purify colonies of bacteria that varied physiologically in the laboratory.

Identification of endophytic bacteria

The endophytic bacteria were discovered by sequencing the 16S rRNA gene using 27F primers using the Sanger sequencing technology developed by ABI from Macrogen in South Korea [10].

Optimization of Incubation Time

TSB (Tryptic Soy Broth) was used to cultivate endophytic bacteria isolates in order to observe the growth curve's stationary phase. Throughout the course of the experiment, the formation of secondary metabolites was observed at regular intervals.

Test organisms

Standard test bacterial strains including *Pseudomonas aeruginosa* (MTCC 424), *Staphylococcus aureus* (MTCC 7443), *Escherichia coli* (MTCC 118), *Salmonella typhi* (MTCC 733) *Klebsiella pneumonia* (MTCC 432) and *Staphylococcus aureus* (MTCC 7443), were produced from the microbial type culture collection (MTCC), Chandigarh (India). The Luria Bertani (LB) slants were kept at a temperature of 4° C in order to preserve the bacterial strains.

Antibacterial activity

Agar well diffusion method

The antibacterial activities of secondary metabolites produced by *B. lupulina* bacteria were investigated in this study. Agar plates containing Mueller-Hinton medium were plated with swab samples of bacterial cultures (MHA). Using a sterile cork borer, wells with a diameter of 6 mm were punched out. In each well, 80 μg/mL of each of the secondary metabolites produced by the bacteria was dispensed. All of the plates were placed in an incubator that measured bio-oxygen demand (BOD) and were heated to 37° C for a period of 24 hours to determine the zone of inhibition in bacteria [11].

Disc diffusion method

Disc diffusion was also used to investigate whether or not secondary metabolites produced by bacteria have antibacterial capabilities. A lawn was created by distributing 100 μg/mL of each bacterial culture onto Mueller Hinton Agar medium plates, which resulted in a concentration of 1 108 CFU/mL. On a bacterial lawn, discs of Whatman filter paper of 5mm in diameter were coated with 10 μg/mL of the secondary metabolites produced by the bacteria. In order to determine which antibacterial secondary metabolites are the most efficient, an experiment with inhibition zones determined in (mm) [12].

Determination of minimum inhibitory concentration, minimum bactericidal concentration

A stock solution of bacterial secondary metabolites with a concentration of 20 μg/mL was created by following the steps of a two-fold serial dilution technique, and then the solution was diluted in sterile Luria Bertani broth to achieve concentrations ranging from 20 to 0.048 μg/mL. For each test tube, a bacterial inoculum dilution of 100 μg/mL and a bacterial secondary metabolites dilution of 100 μg/mL were used. After the incubation, the minimum inhibitory concentration (MIC) was determined to be the lowest concentration that prevented the growth of bacteria. The investigated bacterial strains were sub-cultured combined with the bacterial secondary metabolites on agar plates using Nutrient Agar Medium (NAM) for the dilution process. This was done in order to evaluate the bacterial secondary metabolites MBC. It was determined that the MBC concentration was the one that put an end to bacterial development [13].

Time-kill efficacy assay

The time kill efficacy test to determine if any bacteria were able to survive in the bacterial secondary metabolites of *B. lupulina* was performed with a little amount of modification. During the course of the experiment, several time intervals at 0, 2, 4, and 6 hours were used. The test microorganisms were each placed in their own jar of sterile Luria Bertani broth, which was then heated to 37°C for a period of 24 hours. In order to test the time-kill effectiveness of bacteria, the standard tube dilution technique was used as the evaluation method. At 37°C, 1 mL of a dilution of 10-3 bacteria was exposed to an equivalent volume of a culture containing 50 μg/mL of *E. roggenkampii* for various amounts of time (0, 2, 4 and 6 h). After transfer, the incubated suspension at a concentration of 100 μg/mL was distributed over the agar plates using the spreader [14].

Statistical analysis

The findings of the experiments were analyzed by computing the mean and standard error of the data obtained from all three repetitions. With the assistance of Microsoft Excel 2016, an analysis of variance (ANOVA) was carried out on the data. It was determined that the results were statistically significant when p was less than ($p<0.05$).

RESULT AND DISCUSSION

Isolation and Identification of Endophytic Bacteria from *B. lupulina*

The nutrient agar medium was used to isolate the endophytic bacteria that were discovered in the stem of the *B. lupulina* plant. It was decided to continue with the original way of life of the colony of choice. It was discovered that the endophytic bacteria were rod-shaped and Gram-negative. The bacterial strain was further characterized by sequencing its 16S rRNA using a PCR-based method. The DNA that was amplified by PCR has a length of 1495 base pairs. The 16S rRNA gene sequence data for BLS02 showed similarities with those of *Enterobacter roggenkampii* (accession number MW024073). There is evidence that bacterial endophytes in plants may suppress populations of plant-harming diseases, insects, and nematodes [15]. Endophytes are known to be responsible for the production of secondary metabolites, thanks to the work of a significant number of researchers [16]. On the other hand, there is no research that has been published on the generation of secondary metabolites by endophytes of *B. lupulina*. On the stem of *B. lupulina*, the endophytic strain BLS02 was found, which was subsequently discovered to be *E. roggenkampii*. It was determined that BLS02 is a member of the genus Enterobacter based on its 16S rRNA sequence, which had a similarity of 98.5% with that of *E. cloace*. This result was in agreement with the previously published results [10].

Antibacterial activity assay of *E. roggenkampii* culture

the agar-well and disc-diffusion methods that were used in the process of evaluating the antibacterial activity of bacterial secondary metabolites Figure 2 and Figure 3 illustrate, respectively, the findings of the agar well diffusion showed that the culture of *E. roggenkampii* was the most effective against *L. monocytogenes*, while the culture of *S. typhii* was the least effective. Disc-diffusion cultures of *E. roggenkampii* are the most successful against *S. aureus* and *P. aeruginosa*; however, these cultures are also less effective against *S. typhii*. The concentration of bacterial secondary metabolites at 20 μg/mL was shown to have a greater potential against *L. monocytogenes* compared to the concentrations of 10 μg/mL and 5 μg/mL. It was discovered that the leaves and stems of the plants had antibacterial action because they had a high concentration of chemical components such as alkaloids, flavonoids, and tannins. It was observed that these compounds have pharmacological as well as physiological effects [17]. The antibacterial and antioxidant qualities of medicinal plants are garnering a large amount of attention from the food and pharmaceutical industries due to the availability of naturally occurring compounds that have the potential to replace man-made antioxidants and antimicrobials [18]. Plant polyphenols have an important part to play in the body's natural defense mechanisms against pathogenic bacteria.

Time-killing assay

Qualitative analysis was shown in Table 1 of the time-killing experiment at various time points. Secondarily, bacteria were more effective at killing *S. typhi* and *K. pneumoniae* than they were at killing other bacteria, such as *P. aeruginosa* and *S. aureus*, as well as *L. monocytogenes* and *E. coli*. Bacterial survival and time-killing test outcomes are both impacted by the concentration of bacterial secondary metabolites and time. As measured by the time kill test, which assesses how long an agent's bacteriostatic effect lasts at constant doses, antimicrobial agents and the mortality of the microbial population are proven to be strongly connected [19].

CONCLUSION

The endophytic bacteria of *B. lupulina* have been discovered according to the sequencing data of the 16S rRNA gene, BLS02 is genetically related to the pathogen *Enterobacter roggenkampii*. Secondary metabolites

generated by Gram-negative, rod-shaped endophytic bacteria (*E. roggenkampii*) exhibit strong antibacterial action against *S. aureus* and *P. aeroginosa*. All six harmful bacteria were subjected to a time-killing experiment, and *S. aureus, P. aeruginosa*, and *K. pneumoniae* decided to remain.

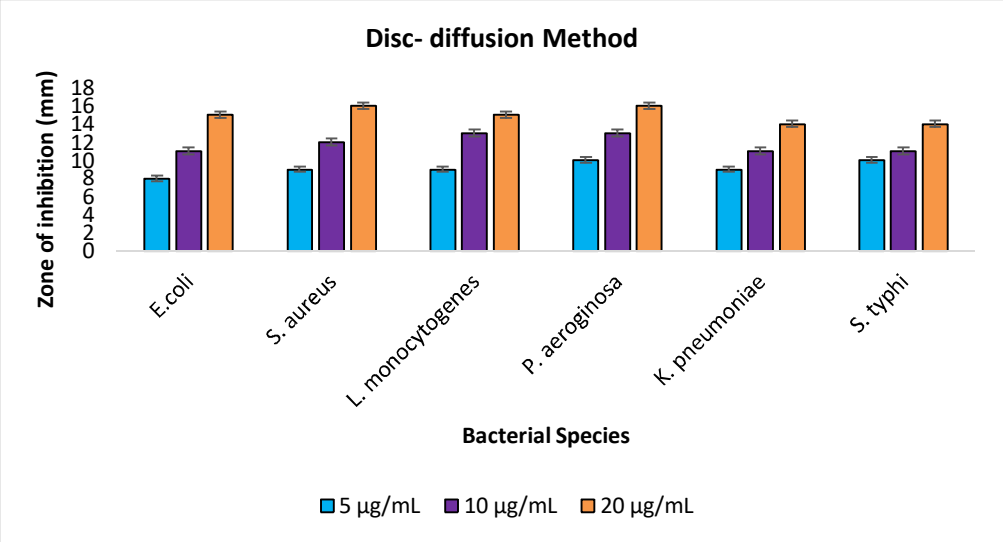

Figure 1: Antimicrobial activity by well-diffusion method.

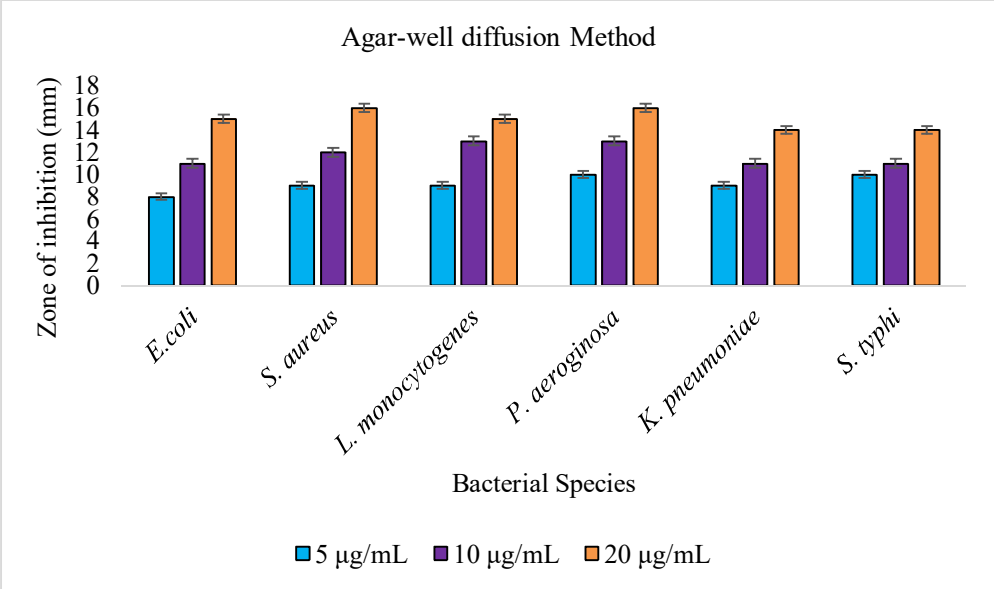

Figure 2: Antimicrobial activity by disc-diffusion method.

Table 1: Survival of bacteria/time-killing assay of Bacterial secondary metabolites

Bacterial secondary metabolites				
Bacterial species	Log CFU/mL			
	0 hr	2 hr	4 hr	6 hr
L. monocytogenes	3.451	3.443	3.321	3.233
P. aeroginosa	4.625	4.312	4.305	4.240
E. coli	3.403	3.160	3.020	2.234
S. typhi	2.502	2.175	2.124	NG
S. aureus	4.034	3.446	3.375	3.245
K. pneumoniae	4.340	3.250	2.674	NG

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ABBREVIATIONS

(ABI) Applied biosystem; (ANOVA) Analysis of variance; (BOD) Biological oxygen demand; (CFU) Colony forming count; (LB) Luria bartani; (MBC) Minimum bactericidal concentration; (MHA) Muller Hinton agar; (MTCC) Microbial type culture collection; (PCR) Polymerase chain reaction; (MIC) minimal inhibitory concentration; (WHO) World health organization.

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