

# Antibacterial Effect of Endophytic Bacteria Isolated From *Tulsi* Leaf against *Escherichia coli* by Nasal Swab Culture

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## ABSTRACT

Endophyte is often a bacterium or fungus that resides within a plant without causing apparent disease and by preventing the pathogenic organisms from colonizing them. *Tulsi* (*Ocimum sanctum*) is said to have endophytes in them. *Tulsi* is a commonly found herb generally used in respiratory tract diseases. The microbial flora of the host with kaphaja pratishyaya (allergic rhinitis) is predominant with *Staphylococcus aureus*, *Escherichia Coli* etc. Identification of the organisms plays an important role because the nasal mucosa when exposed to allergens can create a favourable environment for the growth of certain organisms which are not identified as normal nasal commensals. Hence, the present study is aimed at evaluating the antibacterial effect of endophytic bacteria isolated from *tulsi* leaf against *Escherichia coli* by Nasal swab culture from *Kaphaja Pratishyaya*.

**Key Words:** Endophyte, *Tulsi*, *Kaphaja Pratishyaya*

## INTRODUCTION

Endophyte is an endosymbiont often a bacterium or fungus that lives within a plant for at-least part of its life cycle without causing apparent disease. They prevent the pathogenic organisms from colonizing the host plant where they reside benefiting them. They may be either confined at the point of entry or reside within the cells, the intercellular spaces or the vascular system and can spread throughout the plant. Endophytic bacteria are found in stems, leaves, roots, seeds, fruits, ovules, tubers and legume nodules. Secondary metabolites prepared from the endophytes are utilized in medicine, fuel, agriculture etc. endophytic bacteria produce natural therapeutic compounds that have impending uses in pharmaceutical industry. <sup>[1]</sup>

*Tulsi* (*Ocimum sanctum*) is a commonly found herb in every Indian house

generally used in respiratory tract diseases. It's considered to be drug that sustains healthy condition by removing different diseases. It possesses *Katu*, *Tiktha Rasa* and *Laghu Ruksha Guna*, *Ushna Veerya* and *Katu Vipaka*. <sup>[2]</sup> It is said to exhibit the following *Rogagnata* and *Karma: Swasahara*, <sup>[3]</sup> *Kaphahrit*, *Krimisudana*, *Pratishyayaghna*, *Aruchigna*, *Kasaghna* and *Swasaghna*. <sup>[4]</sup>

Normal microbial flora denotes the population of microorganisms that inhibit the skin and mucous membrane of the normal persons. On the mucous membrane, the resident flora prevents colonisation by pathogens and possible disease through bacterial interference. Disruption of the normal flora can predispose to other pathogenic microbes. Even though most elements of the normal microbial flora inhabiting the human skin, nails, eyes, nose,

oropharynx, genitalia, and gastrointestinal tract are harmless in healthy individuals, these organisms can cause disease in compromised hosts. Studies show that the microbial flora of the host with allergic rhinitis is predominant with *Staphylococcus epidermis*, *Staphylococcus aureus*, *Klebisella pneumoniae*, *Escherichia Coli*. Identification of the organisms is plays a important role because the nasal mucosa when exposed to allergens can create a favourable environment for the growth of certain organisms like *Klebisella pneumoniae*, *Escherichia Coli* which are not identified as normal nasal commensals. These organisms can derange the mucosal immune mechanisms.

*Escherichia Coli* are rod shaped, Gram negative, motile with flagella or non motile and are non sporing bacteria. They usually grow in all laboratory media. They are facultative anaerobe with circular, smooth and raised colonies. They show pink lactose fermenting colonies on MacConkey's. *Escherichia coli* gives positive result methyl red test and indole test. [5] *Escherichia coli* is a commensal of intestine of humans. Also it is most common organism that causes urinary tract infection, pneumonia, meningitis and septicaemia. Certain strains of *Escherichia coli* are observed in the nasal flora of subjects with allergic rhinitis and chronic sinusitis.

*Pratishyaya* is characterized by symptoms like *Naasavrava*, *Ghranauparodha*, *Shirashoolam*, *Shiro Gauravam*, *Jwara*, *Kasa*, *Kaphotklesha*, *Swarabheda*, *Aruchi*, *Klama* and *Indriyanam Asamarthyam*. *Kaphaja Pratishyaya* being one among the types of *Pratishyaya* includes symptoms like *Kasa*, *Aruchi*, *Ghana-Shveta-Srava*, *Kandu*, *Shuklavabhasa*, *Guru Shiro-Mukha*, *Shwasa*, *Vamana*, *Mukha Madhuryata*, *Shoonnakshi*. [6-8] The symptoms of rhinitis resembles with that of the condition of *Pratishyaya* mentioned in the Ayurvedic medical literature among the types of rhinitis, allergic rhinitis is the one which presents the symptoms similar with that of

*Kaphaja Pratishyaya*. Hence the aim of the study was to evaluate the antibacterial effect of endophytic bacteria isolated from tulsi leaf against *Escherichia coli* by Nasal swab culture of *Kaphaja Pratishyaya*.

### Aims & Objective

To assess the antibacterial effect of secondary metabolites extracted from endophytic bacteria in *Tulsi* leaf (*Ocimum sanctum*) against *Escherichia coli* present in nasal swab culture of *Kaphaja pratishyaya*.

## MATERIALS AND METHODS

### Methodology:

- 1) Collection of the nasal mucosal swab from subjects with *kaphaja pratishyaya*
- 2) Identification of the endophytic bacteria from the *Tulsi* leaf (*Ocimum sanctum*)
- 3) Sensitivity test

### Collection of the nasal swab:

The subjects with *kaphaja pratishyaya* were subjected to nasal swabbing by introducing a sterile cotton swab into the nasal cavity and rubbed smoothly in circular motion. The swab was then transferred to MacConkey agar plate by streak culture and further subjected for incubation at 37°C for 24 hours. The colonies were then observed for growth of *Escherichia coli* by colony morphology. Further to distinguish to distinguish gram positive and gram negative organism staining technique was carried out. Biochemical and serological reactions were carried out for further identification.

### Isolation and identification of the endophytic bacteria from the tulsi leaf:

#### Drug collection:

Fresh *Tulsi* leaves was (*Ocimum sanctum*) collected from Sri Dharmasthala Manjunatheshwara College of Ayurveda, Hassan herbal garden.

#### Surface sterilization:

The collected leaf was first cleaned by washing under running tap water for 15 minutes. Rinse the plant material with sterile distilled water for 60 seconds, then

with dilute hydrogen peroxide for 3 minutes followed by ethanol for 3 minutes followed by sodium hypochlorite for 3 minutes and later with ethanol for 1 minute finally with sterile distilled water for 1 minute. Plant material was then dried in between the folds of sterile filter papers.

#### **Isolation of endophytic bacteria:**

The plant material was sectioned into 2-3 inches using sterile scalpel after the surface sterilization technique and were placed on the Muller Hinton agar plates. The plates were then kept for incubation at  $28 \pm 2$  °c for about 24 to 48 hours to promote the growth of endophytic bacteria. Based on the colony morphology and microscopic examination the organisms were characterized. Further the isolated endophytic bacteria were sub-cultured on nutrient agar plates by streak method till further use.

#### **Identification of isolated endophytic bacteria:**

The isolated endophytic bacterium was sent for further identification of the species based on 16SrRna sequencing. The following procedure was followed:

- 1) DNA isolation and quantification
- 2) PCR amplification of 16SrDNA gene
- 3) Sequencing of the PCR amplicon
- 4) In silico sequence analysis and bacterial identification
- 5) co sequence analysis and bacterial identification

#### **Preparation of secondary metabolites from subcultured endophytic bacteria:**

Single pure efficient bacterial endophytes were inoculated into 200ml of nutrient broth medium and kept for incubation in a rotary shaker at 30°C, 120rpm for 10 minutes. The supernatant was discarded and the pellet was collected in order to obtain the intracellular antimicrobial compounds. To this pellet 5 different polar and non-polar solvents such as hexane, chloroform, ethyl acetate, ethanol and methanol were added and mixed

thoroughly and this mixture is kept for 2-3hrs. Later the above mixture was centrifuged. The supernatant containing antimicrobial compounds was obtained. Different concentrations of these compounds were prepared.

#### **Sensitivity test by Agar well diffusion method:**

Clean the work place using 70% of ethyl alcohol. Inoculate a loop of *Escherichia Coli* from 24 hrs culture into Muller hinton agar plate. Make 4 equidistant wells on the plate with cork borer. Add different concentrations of obtained secondary metabolites from subculture of endophytic bacteria. Test will be conducted on 4 different concentrations of secondary metabolites from subcultured endophytic bacteria (500µg/ml, 250µg/ml, 125µg/ml, 62µg/ml) separately. Incubate the petri dishes at 37°C for 24 hours. After incubation period, the zone of inhibition is measured with a ruler.

#### **Assessment criteria:**

The disc diffusion study is assessed by following zones:

1. Sensitive (S) zone - 22-26mm zone of inhibition
2. Intermediate (I) zone - 20-18mm zone of inhibition
3. Resistant (R) zone – below 16mm zone of inhibition

#### **OBSERVATIONS AND RESULTS**

The following observations and result were obtained to accomplish the objective of the present study. *Bacillus cereus* (F837/76) is the endophytic bacteria present in the tulsi leaf. It showed following colony characteristics like creamy white in color, irregular in shape with granular surface, low convex elevation and crenated edge, dull in luster, translucent in opacity and powdery in consistency. The results of grams staining showed pink colour staining suggestive of gram negative bacterium. It showed positive Catalase test but coagulase

test and motility test showed negative results.

**Table 1: Distribution based on Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* at different concentrations**

Secondary metabolite extracted	Zone of inhibition against <i>Escherichia coli</i> in mm	500µg/ml		250µg/ml		125µg/ml		62 µg/ml	
		F	%	F	%	F	%	F	%
<i>Bacillus cereus</i>	0	4	13.3	1	3.3	1	3.3	1	3.3
	16	4	13.3	1	3.3	1	3.3	6	20.0
	18	3	10.0	2	6.7	8	26.7	5	16.7
	20	3	10.0	5	16.7	3	10.0	4	13.3
	22	6	20.0	7	23.3	5	16.7	5	16.7
	24	4	13.3	6	20.0	7	23.3	5	16.7
	26	2	6.7	3	10.0	3	10.0	1	6.7
	28	4	13.3	1	3.3	2	6.7	1	6.7
	30	4	13.3	4	13.3	1	3.3	1	3.3
	Total	30	100.0	30	100.0	30	100.0	30	100.0

**Table 2: Sensitivity test for Secondary metabolites extracted from *Bacillus safensis***

CONCENTRATIONS	500µg/ml			250µg/ml			125µg/ml			62µg/ml		
	S	M	R	S	M	R	S	M	R	S	M	R
NO: OF SAMPLES	19	7	4	21	7	1	18	10	2	18	11	1

Here S – sensitive, M – Moderately sensitive, R – Resistant

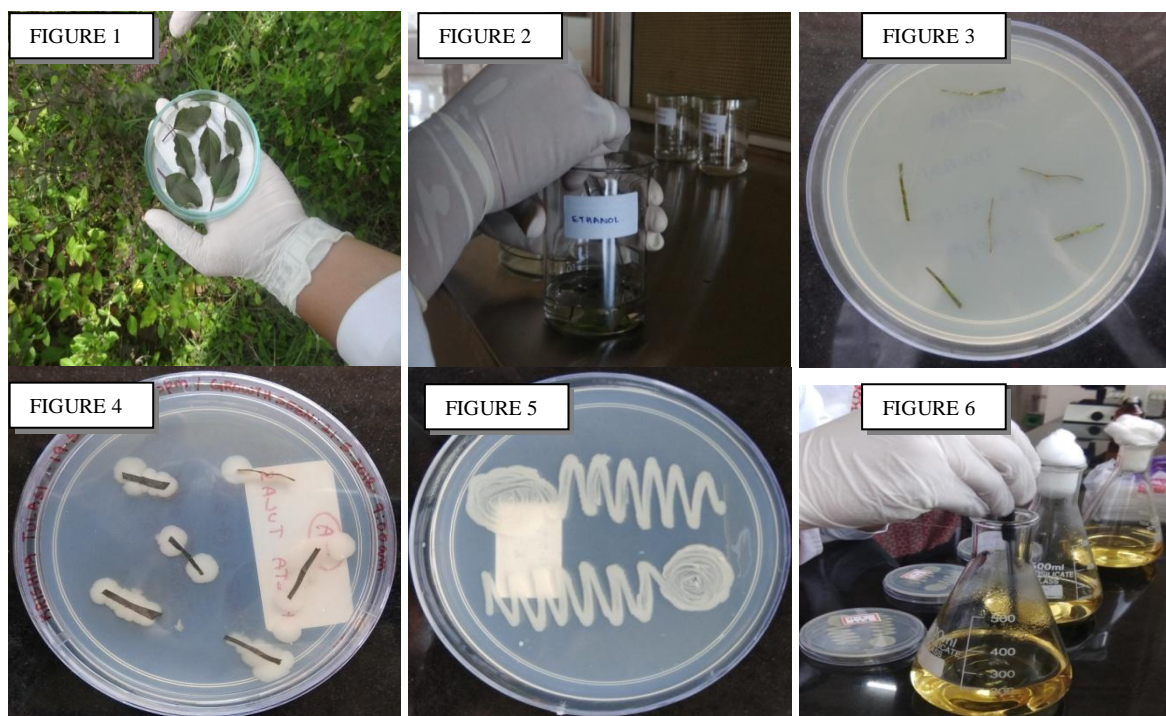
**Table 3: Mean values of zone of inhibition at different concentrations of Secondary metabolites extracted from *Bacillus safensis***

Different concentrations of Secondary metabolites extracted from <i>Bacillus cereus</i>	500µg/ml	250µg/ml	125µg/ml	62µg/ml
N	30	30	30	30
Mean	20.73	22.53	21.07	22.67

**Table 4: Comparing the means within the groups with different concentrations**

Group	Concentration (µg/ml)	N	Mean
<i>Bacillus cereus</i>	500	30	20.73
	250	30	22.53
	125	30	21.07
	62	30	22.67
	Total	120	21.73

Explanation on the above mentioned tables and results are discussed in discussion below.



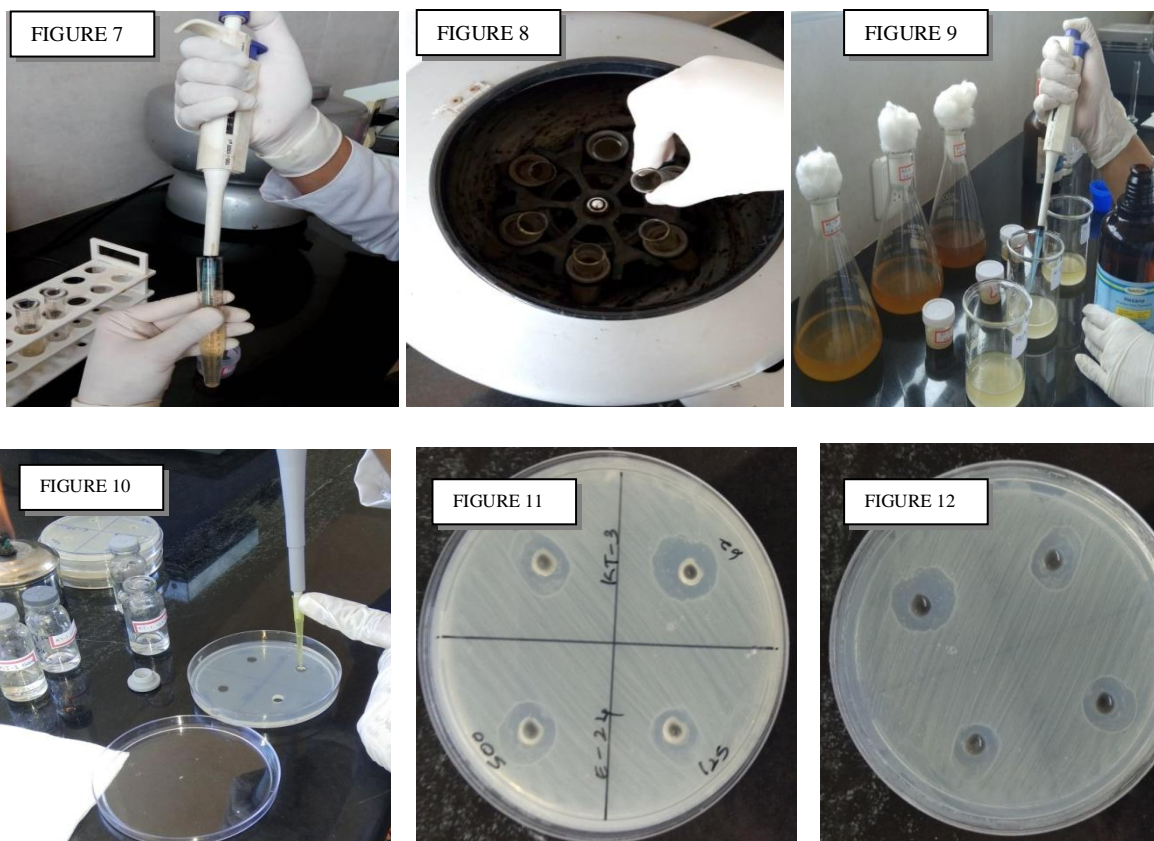


Figure 1: Collection of Tulsi leaf  
 Figure 2: Surface sterilization  
 Figure 3: Placement of the sectioned leaf over the agar medium  
 Figure 4: Growth of Endophytic Bacteria  
 Figure 5: Subculture of Endophytic Bacteria  
 Figure 6: Inoculation of Endophytic Bacteria in nutrient medium  
 Figure 7: Adding of the above solution into centrifuge tubes  
 Figure 8: Placement of centrifuge tubes with above solution in centrifuge  
 Figure 9: Adding polar and non polar solvents  
 Figure 10: Agar Well diffusion method for Sensitivity  
 Figure 11 & 12: zone of inhibition

## DISCUSSION

The isolation procedure of bacterial endophytes is a critical step. Surface sterilization accounts to the first obligatory step in isolation of endophytic bacteria in order to kill the surface microbes. Surface sterilization removes all the exophytic bacteria (bacteria present over the surface of the leaf) and dirt substances and soil debris over the surface of the leaf. Surface sterilization is accomplished by treating the part of the plant with oxidants and sterilizing agent. The sterilizing agents in the present study used are hydrogen peroxide, ethanol and sodium hypochlorite. [9] Hydrogen peroxide, sodium hypochlorite demonstrates broad spectrum efficacy against bacteria, bacterial spores etc with

low cost and also safe in terms of safety issues. [10] Ethanol demonstrates efficacy against the bacteria but it shows very poor sporadic activity thus, ethanol alone will not be enough in surface sterilization of the plant material. [11]

In the present study, antimicrobial activity of Secondary metabolites extracted from the isolated endophytic bacteria namely; *Bacillus cereus* was analysed against *Escherichia coli*. The assay was done at different concentrations of the Secondary metabolites were prepared from the isolated endophytic bacteria, *Bacillus cereus* to understand its activity. Here anti-microbial study is done by Agar well diffusion method in Muehler Hinton agar. Mueller Hinton agar it is a non selective and

non differential medium which means that almost all the organisms plated on this agar will enhance the growth of the same. It is a loose agar which helps for a better diffusion. A better diffusion leads to true zone of inhibition.<sup>[12]</sup>

Secondary metabolites extracted from *Bacillus cereus* had shown various zone of inhibition against *Escherichia coli* ranging from 16 – 30mm against various concentrations (500µg/ml to 62µg/ml). Maximum zone of inhibition recorded was 30mm and minimum zone of inhibition was 16mm. thus Secondary metabolites extracted from *Bacillus safensis* is having antibacterial activity against *Escherichia coli* (table1).

At 500µg/ml concentration, the Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* showed that zone of inhibition was 30mm in 4 (13.3%) sample, 28mm in 2 (6.7%) samples, 26mm in 4 (13.3%) samples, 24m in maximum samples 6 (20.0%), 22mm in 3 (10.0%) samples, 20mm in 3 (10.0%) samples, 18mm in 4 (13.3) samples and minimum zone of inhibition 0mm was observed in 4 (13.3%) sample.

At 250µg/ml concentration, the Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* showed that zone of inhibition was 30mm in 4 (13.3%) sample, 28mm in 1 (3.3%) sample, 26mm in 3 (10.0%) samples, 24m in 6 (20.0%) samples, 22mm in maximum samples 7 (23.3%), 20mm in 5 (16.7%) samples, 18mm in 2 (6.7%) samples, 18mm in 2 (6.7) samples, 16mm in 1 (3.3%) sample and minimum zone of inhibition 0mm was observed in 1 (3.3%) sample.

At 125µg/ml concentration, the Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* showed that zone of inhibition was 28mm in 2 (6.7%) sample, 26mm in 3 (10.0%) samples, 24mm in 7 (23.3%) samples, 22m in 5 (16.7%) samples, 20mm in 3 (10.0%) samples, 18mm in maximum samples 8 (26.7%), 16mm in 1 (3.3%) sample and

minimum zone of inhibition 0mm was observed in 1 (3.3%) sample.

At 62µg/ml concentration, the Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* showed that zone of inhibition was 32mm in 1 (3.3%) sample, 30mm in 1 (3.3%) samples, 28mm in 2 (6.7%) samples, 26m in 5 (16.7%) samples, 24m in 5 (16.7%) samples 22mm in 4 (13.3%) samples, 20mm in 5 (16.7%) samples, 18mm in maximum samples 6 (20.0%) and minimum zone of inhibition 16mm in 1 (3.3%) sample.

Out of 30 samples of *Escherichia coli* at 500µg/ml concentration, 19 samples are sensitive, 7 samples are moderately sensitive and 4 samples are resistant. Out of 30 samples of *Escherichia coli* at 250µg/ml concentration, 21 samples are sensitive, 7 samples are moderately sensitive and 2 samples are resistant. Out of 30 samples of *Escherichia coli* at 125µg/ml concentration 18 samples are sensitive, 10 samples are moderately sensitive and 2 samples are resistant. Out of 30 samples of *Escherichia coli* at 62µg/ml concentration 18 samples are sensitive, 11 samples are moderately sensitive and 1 sample is resistant.

Mean values of zone of inhibition by Secondary metabolites extracted from *Bacillus cereus* (500µg/ml to 62µg/ml) against *Escherichia coli* are 20.73mm, 22.53mm, 21.07mm and 22.67mm respectively. Thus Secondary metabolites extracted from *Bacillus cereus* are having antibacterial activity against *Escherichia coli*.

The Highest mean value for zone of inhibition of secondary metabolites extracted from *Bacillus cereus* among different concentrations was at 62µg/ml when compared to other concentrations. This suggests that 62µg/ml concentration shows better anti-bacterial activity compared to other concentrations with very minimal difference in the mean value of zone of inhibition. All living organisms are surrounded by a semi-permeable bio-membrane which functions as a permeation barrier preventing the leakage of cellular

metabolites into the surrounding but also the uncontrolled influx of external substances. Bio-membranes also contain proteins, receptors and transporters which mediate a communication or exchange of substance with other cells. If the bio-membrane is disturbed or lysed, usually cell death occurs. Many secondary metabolites have an affinity for bio-membranes. As a consequence they show antimicrobial and cyto-toxic activities. <sup>[13]</sup>

## CONCLUSION

The nasal swab culture of kaphaja pratishyaya showed the presence of the gram negative microorganism *Escherichia Coli*. Endophytic bacteria residing in the tulsi leaf was identified as *Bacillus cereus*. Further the secondary metabolites extracted from *Bacillus cereus* exhibited krimghna karma (antibacterial action) against *Escherichia coli* present in nasal swab culture of kaphaja pratishyaya subjects. Comparison on mean values of zone of inhibition showed *Bacillus cereus* showed higher value at the lowest concentration 62µg/ml with minimum difference in the mean value of zone of other concentrations.

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