



## IN VITRO STUDY OF ENDOPHYTIC BACTERIA *BACILLUS CEREUS* FROM TULSI LEAF AGAINST *ESCHERICHIA COLI* IN KAPHAJA PRATHISHYAYA

Geetha Nayak S<sup>1\*</sup>, Ajantha<sup>2</sup>, Shashirekha K S<sup>3</sup>, Anuradha B<sup>1</sup>

<sup>1</sup>Final year PG Scholar, Department of Roga Nidana & Vikruti Vignana, Sri Dharmasthala Manjunatheshwara College of Ayurveda and Hospital, Hassan, India

<sup>2</sup>Professor, Department of Roga Nidana & Vikruti Vignana, Sri Dharmasthala Manjunatheshwara College of Ayurveda and Hospital, Hassan, India

<sup>3</sup>Microbiologist, Department of Roga Nidana & Vikruti Vignana, Sri Dharmasthala Manjunatheshwara College of Ayurveda and Hospital, Hassan, India

\*Corresponding Author Email: nayakgeetz@gmail.com

DOI: 10.7897/2277-4572.083138

Received on: 03/05/19 Revised on: 01/06/19 Accepted on: 13/06/19

### ABSTRACT

Kaphaja Pratishyaya is a frequently observed respiratory disease shows typical Ghana-Shwetha-Snigdha-Srava (thick-whitish-mucoid discharge) characteristic features of Nasa Srava (nasal discharge). Among the microbial flora of these nasal secretion's organisms like *Staphylococcus aureus*, *Escherichia coli* etc can be observed. Tulsi (*Ocimum sanctum*) an herb, is known since Vedic period recognized for its medicinal and therapeutic use. It is referred to as Pratishyayaghna, Kaphakrith and Krimighna. Endophyte is bacterium or fungus that lives within a plant without causing apparent disease for at-least part of its life cycle these are present in all varieties of plants. The present study aims to evaluate sensitivity of secondary metabolites extracted from identified endophytic bacteria in Tulsi leaf (*Ocimum sanctum*) against *Escherichia coli* present in nasal culture of Kaphaja Pratishyaya. Patients of *Kaphaja Pratishyaya* were subjected for nasal swab culture and those with positive results for *Escherichia coli* were further used. The endophytic bacteria *Bacillus cereus* present in the Tulsi leaf was extracted and isolated. Further sensitivity test was performed by Agar well diffusion method and zone of inhibition was measured. The secondary metabolites extracted from *Bacillus cereus* exhibited krimghna (anti-microbial) action against *Escherichia coli*. Comparison of mean values of zone of inhibition at different concentrations of secondary metabolites extracted from *Bacillus cereus* showed higher mean value (21.27) at 62µg/ml concentration with slight difference in the mean value of other concentrations. Secondary metabolites have an affinity for bio-membranes present in the organisms as a consequence they show antimicrobial and cyto-toxic activities.

**KEY WORDS:** Kaphaja Pratishyaya, Endophytic bacteria, Tulsi (*Ocimum sanctum*)

### INTRODUCTION

The word Pratishyaya is understood as "Prati" denoting Abhimukha and Shyaya denoting Gamanam. Pratishyaya is a condition manifested due to the movement of Kapha, Pitta and Raktha from the root of the Nasa (nose) and lodges in Shiras (head) which is facilitated by vitiated by Vata Dosha<sup>1</sup>. Pratishyaya is generally classified into 5 types viz, Vataja, Pittaja, Kaphaja, Sannipataja<sup>2</sup> and Rakthaja<sup>3</sup>. There numerous etiological factors mentioned in the causation of Pratishyaya mentioned by different Acharyas. They include Vegasandharana (suppression of natural urges), Raja-Dhuma Sevana (exposure to dust and smoke), Atibashya (excessive talking), Ajeerna (indigestion), Rutuvaishmya (seasonal variations)<sup>4</sup>, Sheeta Sevana (consumption of cold items), Prajagara (night waking), Avashyaya (fog), Atiswapna (excessive sleeping), jalakreeda (water sports)<sup>5</sup>, shita ambu (consumption of cold water), Shiroabhitapa (trauma to head)<sup>6</sup>. These etiological factors result in the symptoms like Ghana-Shwetha-Snigdha-Srava (thick-whitish-mucoid discharge), Shirogaurava (heaviness of head), Mukhagaurava (haviness over face), Kandu (itching in nose and throat), Shwasa (difficulty in breathing), Kasa (cough) and Aruchi (loss of taste)<sup>7</sup>.

Tulsi (*Ocimum sanctum*) is a well-known medicine commonly used in respiratory diseases. Tulsi (*Ocimum sanctum*) is

mentioned under swasahara varga<sup>8</sup>, Surasadi, Shirovirechana<sup>9</sup>, Kaphagna, and Surasadi<sup>10</sup>.

Microorganisms that inhabit inside the healthy plant tissues are called as Endophytes. An endophyte can be a bacterium, fungi or an actinomycete. They prevent the pathogenic organisms from colonizing the host plant tissue<sup>11</sup>. Natural therapeutic compounds derived from the endophytic bacteria show anti-microbial, anti-oxidant, anti-viral, anti-diabetic, anti-Alzheimer's disease and immunosuppressant<sup>12</sup>.

The current study aims at identification of the endophytic bacteria present in Tulsi (*Ocimum sanctum*) leaf and evaluating its sensitivity against *Escherichia coli* in kaphaja pratishyaya subjects by nasal swab culture.

**AIMS & OBJECTIVE:** To evaluate sensitivity of secondary metabolites extracted from identified endophytic bacteria in Tulsi leaf (*Ocimum sanctum*) against *Escherichia coli* present in nasal culture of Kaphaja Pratishyaya

### MATERIALS AND METHODS

**Source of data:** 30 subjects fulfilling diagnostic criteria were included for the study from OPD and IPD of Sri Dharmasthala Manjunatheshwara College of Ayurveda and Hospital, Hassan.

Study was approved by Institutional ethical committee IEC No. SDM/IEC/55/2017-2018.

**Diagnostic Criteria:** Subjects with Ghana shwetha snigdha nasa srava with or without other following symptoms of Kaphaja pratishyaya: Mukhagauravam, Kandu, Shirogauravam, Swasa, Kasa, Shulkavabhasa and Aruchi.

**Inclusion Criteria:** Subjects between the age group of 18 to 60 years, irrespective of caste, religion, gender fulfilling diagnostic criteria was included in the present study.

**Exclusion Criteria:** Patients with nasal trauma and lesions, Rhinitis medicamentosa, Polypus rhinitis, other systemic illness and Organisms other than *Escherichia coli* was excluded in the present study.

**Research Design:** An observational experimental study

## METHODOLOGY

### Isolation Of *Escherichia coli* By Nasal Swab Culture

using a sterile cotton swab samples of nasal swab were collected from the subjects filling the inclusion and diagnostic criteria. In a circular motion the sterile cotton swab was rubbed against the nasal mucosa. Using streak type of culturing method, the swabs were then transferred over the Mac'Conkey agar and placed in the incubator for incubation at 37°C for 24 hours. Further by colony morphology, gram staining, biochemical and serological test the colonies were observed for the growth of *Escherichia coli*. The samples containing *Escherichia coli* were subjected for the sensitivity test.

### Isolation And Identification Of The Endophytic Bacteria

Tulsi leaves (*Ocimum sanctum*) were collected from Sri Dharmasthala Manjunatheshwara College of Ayurveda, Hassan herbal garden. These collected leaves were subjected for surface sterilization. The healthy and fresh collected leaves were washed under the tap water for 15 minutes to remove the dirt over the leaf. Douse the leaves with sterile distilled water for 60 seconds followed by dilute hydrogen peroxide, ethanol and sodium hypochlorite for 3 minutes each respectively and later with ethanol and sterile distilled water for 1 minute each. After surface sterilization the leaves are kept for dried using sterile filter paper. The surface sterilized leaves were then sectioned vertically and horizontally using a sterile scalpel. The sections of these leaves were then placed over the Muller Hinton Agar plates at equidistant and placed in incubator for incubation at 28±2 °c for about 24 to 48 hours to observe the growth of endophytic bacteria. All the selected organisms were sub-cultured by streak method over the nutrient agar plates and used for further use. The organisms were characterized based on colony morphology and microscopic examination. The isolated endophytic bacterium was sent for further identification of the species based on 16SrRna sequencing. The following procedure was followed: DNA isolation and quantification, PCR amplification of 16SrDNA gene, Sequencing of the PCR amplicon, In silico sequence analysis and bacterial identification, co sequence analysis and bacterial identification.

### Preparation Of Secondary Metabolites

200ml of nutrient broth medium was taken in a conical flask. To this a single pure culture of isolated bacterial endophyte was inoculated and kept for incubation at 28±2 °c. Further it is placed in a rotary shaker at 30°C, for 10 minutes at 120rpm to separate the supernatant fluid and pellets. Upper supernatant fluid was discarded, and the lower sediment pellet was collected in-order to obtain the intracellular antimicrobial compounds. 5 different polar and non-polar solvents such as hexane, chloroform, ethyl acetate, ethanol and methanol were added to this pellet and mixed thoroughly, and this mixture is kept for 2-3hrs. the above mixture was centrifuged for 10 minutes at 120rpm. The supernatant containing antimicrobial compounds was obtained and used further. Different concentrations of these antimicrobial compounds were prepared.

### Sensitivity Test

Sterilize the working area using 70% of ethyl alcohol. Inoculate a loop of *Escherichia coli* by lawn culture method over the Muller Hinton agar plate. 4 wells equidistant wells are prepared by using cork borer. To these four wells, 4 different concentrations of obtained secondary metabolites extracted from isolated endophytic bacteria are added. Test was conducted on 4 different concentrations of secondary metabolites from sub-cultured endophytic bacteria (500µg/ml, 250µg/ml, 125µg/ml and 62µg/ml) separately. Later, incubate the petri-plates at 37°C for 24 hours. After incubation period, the plates are observed for the zone of inhibition. The zone of inhibition is measured with a help of 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

From the present study the following observations and results were obtained to accomplish the objective of the study. The isolated endophytic bacteria *Bacillus cereus* was creamy white in color, entire in shape with smooth surface, raised elevation and entire edge, glistening in luster, translucent in opacity and buttery in consistency. Results of gram staining showed pink colour staining suggestive of gram-negative bacterium. Catalase test results showed to be positive, but coagulase test and motility test showed negative results.

In vitro antibacterial activity of Secondary metabolites extracted from *Bacillus cereus* endophytic bacteria was evaluated by agar well diffusion method. Antibacterial activity of secondary metabolites extracted from *Bacillus cereus* against 30 samples of *Escherichia coli* was observed in different concentration like 500µg/ml, 250µg/ml, 125µg/ml and 62µg/ml. The zone of inhibition ranged 0-30mm. The minimum zone of inhibition was 16mm and maximum zone of inhibition was 30mm (Table 1)

**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	3	10.0	3	10.0	1	3.3	1	3.3
	16	1	3.3	3	10.0	5	16.7	8	26.7
	18	6	20.0	1	3.3	3	10.0	6	20.0
	20	9	30.0	4	13.3	8	26.7	6	20.0
	22	4	13.3	6	20.0	5	16.7	5	16.7
	24	3	10.0	5	16.7	5	16.7	3	10.0
	26	1	6.7	4	13.3	1	6.7	1	3.3
	28	1	6.7	4	13.3	1	3.3	1	3.3
	30	3	10.0	3	10.0	1	3.3	8	26.7
	Total		30	100.0	30	100.0	30	100.0	30

F- Frequency, µg/ml – Mu gram/ milliliter

Further the sensitivity test of Secondary metabolites extracted from *Bacillus cereus* against *Escherichia coli* was carried out to assess the zone of inhibition. The sensitivity test results are tabulated in table 2.

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

CONCENTRATIONS	500µg/ml			250µg/ml			125µg/ml			62µg/ml		
	S	M	R	S	M	R	S	M	R	S	M	R
NO: OF SAMPLES	20	6	4	19	5	6	14	10	6	15	14	1

Here S – sensitive, M – Moderately sensitive, R – Resistant, µg/ml – Mu gram/ milliliter

Mean values of zone of inhibition at different concentrations of secondary metabolites of *Bacillus cereus* against *Escherichia coli* was calculated. The results of mean value have been tabulated in table 3.

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

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	20.80	20.47	20.13	21.27

N - Total samples, µg/ml – Mu gram/ milliliter

Further the means of different concentration were compared between the different concentrations of secondary metabolites of *Bacillus cereus* against *Escherichia coli*. The results obtained are tabulated in table 4.

**Table 4: Comparing the means within the group *Bacillus cereus* among different concentrations of secondary metabolites extracted from *Bacillus cereus***

Group	Concentration (µg/ml)	N	Mean
<i>Bacillus cereus</i>	500	30	20.80
	250	30	20.47
	125	30	20.13
	62	30	21.27
	Total	120	20.67

µg/ml – Mu gram/ milliliter, N = Total samples



**Figure 1 - surface sterilization**



**Figure 2: Growth of endophytic**



Figure 3: adding polar and non-polar substance

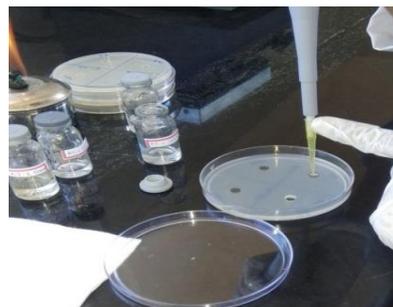


Figure 4: agar well diffusion method

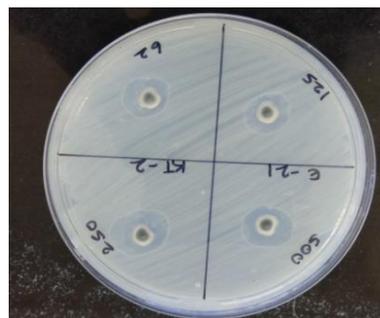


Figure 5 & 6: sensitivity test showing zone of inhibition

## DISCUSSION

Various zone of inhibition was obtained for Secondary metabolites extracted from *Bacillus cereus* against *Escherichia coli* ranging from 16 – 30mm in different concentrations (500 $\mu$ g/ml to 62 $\mu$ g/ml). Maximum zone of inhibition recorded was 30mm and minimum zone of inhibition was 16mm. Thus, from these findings it can be concluded that Secondary metabolites extracted from *Bacillus safensis* is having antibacterial activity against *Escherichia coli*.

Data related to Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* at 500 $\mu$ g/ml showed that zone of inhibition was 30mm in 2 (6.7%) sample, 26mm in 2 (6.7%) samples, 24mm in 3 (10.0%) samples, 22m in 4 (13.3%) samples, 20mm in maximum number of samples 9 (30.0%), 18mm in 6 (20.0%) samples, 16mm in 1(3.3%) sample and minimum zone of inhibition 0mm was observed in 3 (10.0%) sample.

Data related to Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* at 250 $\mu$ g/ml showed that zone of inhibition was 30mm in 2 (6.7%) sample, 26mm in 2 (6.7%) samples, 24mm in 3 (10.0%) samples, 22m in 4 (13.3%) samples, 20mm in maximum number of samples 9 (30.0%), 18mm in 6 (20.0%) samples, 16mm in 1(3.3%) sample and minimum zone of inhibition 0mm was observed in 3 (10.0%) sample.

Data related to Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* at 125 $\mu$ g/ml showed that zone of inhibition was 28mm in 1 (3.3%) sample, 26mm in 2 (6.7%) samples, 24mm in 5 (16.7%) samples, 22m in 5 (16.7%) samples, 20mm in maximum number of samples 8 (26.7%), 18mm in 3 (10.0%) samples, 16mm in 5(16.7%) samples and minimum zone of inhibition 0mm was observed in 1 (3.3%) sample.

Data related to Antibacterial activity of Secondary Metabolites extracted from *Bacillus cereus* at 62 $\mu$ g/ml showed that zone of inhibition was 28mm in 1 (3.3%) sample, 26mm in 3 (10.0%) samples, 24mm in 5 (16.7%) samples, 22m in 6 (20.0%) samples,

20mm in 6 (20.0%) samples, 18mm in maximum number of samples 8 (26.7%) and minimum zone of inhibition 16mm was observed in 1 (3.3%) sample.

In 500 $\mu$ g/ml concentration out of 30 samples of *Escherichia coli* 20 samples are sensitive, 6 samples are moderately sensitive, and 4 samples are resistant. In 250 $\mu$ g/ml concentration out of 30 samples of *Escherichia coli* 19 samples are sensitive, 5 samples are moderately sensitive, and 6 samples are resistant. In 125 $\mu$ g/ml concentration out of 30 samples of *Escherichia coli* 14 samples are sensitive, 10 samples are moderately sensitive, and 6 samples are resistant. In 62 $\mu$ g/ml concentration out of 30 samples of *Escherichia coli* 15 samples are sensitive, 14 samples are moderately sensitive, and 1 sample is resistant.

Mean values of zone of inhibition by Secondary metabolites extracted from *Bacillus cereus* (500  $\mu$ g/ml to 62  $\mu$ g/ml) (table) against *Escherichia coli* are 20.80mm, 20.47mm, 20.13mm and 21.27mm respectively. Thus, Secondary metabolite extracted from *Bacillus cereus* is having antibacterial activity against *Escherichia coli*.

Among different concentrations prepared from the secondary metabolites extracted from *Bacillus cereus*, 62  $\mu$ g/ml shows highest mean value for zone of inhibition compared to other concentrations. This suggests that 62 $\mu$ g/ml shows better antibacterial activity compared to other concentrations. Further, the lowest concentrations of secondary metabolites extracted from *Bacillus* species showed zone of inhibition, exhibiting anti – bacterial activity against *Escherichia coli* suggesting that even lower concentrations are effective against the pathogen *Escherichia coli*. The mechanism behind the antibacterial activity is that living organisms surround themselves with a bio-membrane which is semi-permeable in nature. This helps as a permeation barrier. This barrier prevents in leakage of the cellular metabolites into the surrounding and also the also the uncontrolled influx of external substances. These bio-membranes contain proteins, receptors and transporters which help in mediating communication and exchange of substance with other

cells. Any disturbance or lysis of the bio-membrane will lead to the cell death. Secondary metabolites have an affinity for bio-membranes. As a consequence, they show antimicrobial and cytotoxic activities<sup>13</sup>.

## CONCLUSION

The endophytic bacterium isolated from the tulsi leaf is identified as *Bacillus cereus*. The secondary metabolites extracted from *Bacillus cereus* exhibited krimghna (antibacterial) action against *Escherichia coli* isolated by nasal swab culture in kaphaja prathishyaya. Comparison of mean values of zone of inhibition at different concentrations of secondary metabolites extracted from *Bacillus cereus* showed higher mean value (21.27) at 62µg/ml concentration with slight difference in the mean value of other concentrations.

## REFERENCES

1. Agnivesha, Charaka Samhita with Ayurveda Dipika Commentary, Ed. Acharya Y.T, Chaukhambha Surbharati Prakashan, Varanasi, 2009 (Reprint), Chikitsa Sthana.26/107: p604
2. Agnivesha, Charaka Samhita with Ayurveda Dipika Commentary, Ed. Acharya Y.T, Chaukhambha Surbharati Prakashan, Varanasi, 2009 (Reprint), Chikitsa Sthana.26/105 - 105: p604
3. Susrutha, Susruta samhita with nibandha samgraha and Nyayachandrika commentaries, Ed. Vd. Y.T Acharya, Chaukhambha Orientalia, Varanasi, 2009 (Reprint), Uttara Tantra 24/6: p651
4. Agnivesha, Charaka Samhita with Ayurveda Dipika Commentary, Ed. Acharya Y.T, Chaukhambha Surbharati Prakashan, Varanasi, 2009 (Reprint), Chikitsa Sthana.26/104 - 105: p604
5. Vagbhata, AshtangaHrudaya with Sarvanga Sundara Commentary, Ed. Acharya H.P, Chaukhambha Orientalia, Varanasi, 2005 (Reprint), Uttara Tantra.19/2: p841
6. Susrutha, Susruta samhita with nibandha samgraha and Nyayachandrika commentaries, Ed. Vd. Y.T Acharya, Chaukhambha Orientalia, Varanasi, 2009 (Reprint), Uttara Tantra 23/3: p651
7. Vagbhata, AshtangaHrudaya with Sarvanga Sundara Commentary, Ed. Acharya H.P, Chaukhambha Orientalia, Varanasi, 2005 (Reprint), Uttara Tantra.19/6: p841
8. Agnivesha, Charaka Samhita with Ayurveda Dipika Commentary, Ed. Acharya Y.T, Chaukhambha Surbharati Prakashan, Varanasi, 2009 (Reprint), Sutra Sthana.2/ : p32 (swasahara varga)
9. Susrutha, Susruta samhita with nibandha samgraha and Nyayachandrika commentaries, Ed. Vd. Y.T Acharya, Chaukhambha Orientalia, Varanasi, 2009 (Reprint), Sutra sthana 38/19: p357
10. Vagbhata, AshtangaHrudaya with Sarvanga Sundara Commentary, Ed. Acharya H.P, Chaukhambha Orientalia, Varanasi, 2005 (Reprint), Sutra sthana.15/7: p200
11. Ahmed M, Hussain M, Manoj K. Dhar and Sanjana Kaul, Isolation of microbial endophytes from some ethnomedicinal plants of Jammu and Kashmir Scholars Research Library J. Nat. Prod. Plant Resour 2012, 2 (2):215-220
12. Nawed Anjum, Ramesh Chandra. Endophytic Bacteria: Optimizaton Of Isolation Procedure From Various Medicinal Plants And Their Preliminary Characterization. Asian journal of pharmaceutical and clinical research 2015, volume 8, issue 4.
13. Michael wink. Modes of action of herbal medicines and plant secondary metabolites. Molecular diversity preservation international and multidisciplinary digital publishing institute 2015, 2,251-286; doi: 10.3390/medicines2030251.

### How to cite this article:

Geetha Nayak S et al. In vitro study of endophytic bacteria bacillus cereus from tulsi leaf against *Escherichia coli* in kaphaja prathishyaya. J Pharm Sci Innov. 2019;8(3):112-116. <http://dx.doi.org/10.7897/2277-4572.083138>

Source of support: Nil, Conflict of interest: None Declared

Disclaimer: JPSI is solely owned by Moksha Publishing House - A non-profit publishing house, dedicated to publish quality research, while every effort has been taken to verify the accuracy of the content published in our Journal. JPSI cannot accept any responsibility or liability for the site content and articles published. The views expressed in articles by our contributing authors are not necessarily those of JPSI editor or editorial board members.