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Isolation, morphological identification and *In Vitro* antibacterial activity of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) leaves

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Abstract
Fresh leaves of *Ocimum sanctum* (Tulsi) was procured and divided into five samples and each sample was again divided into 5 sub samples and separated for isolation of endophytic bacteria. Characterization of bacteria was done according to its morphology by grams staining. The antibacterial effect of endophytic bacteria was studied against three gram positive and gram negative bacteria by disc diffusion method with known antibiotic ciprofloxacin (Ci) as standard. Most of the samples on kings B media depicted irregular shape, flat elevation, undulated, rough, opaque and white in colour. Most of the samples on blood agar showed irregular, raise elevation, undulated, smooth, opaque and all isolates were non haemolytic and non-chromogenic. The growth of all isolates in BHI broth showed turbidity. The microscopic examination revealed that maximum isolates were gram positive and rod shaped. Good antibacterial activity was observed against *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Salmonella* Typhimurium.

Keywords: Endophytic bacteria, *Ocimum sanctum* (Tulsi), leaves, antibacterial activity, ciprofloxacin

1. Introduction
Ocimum Sanctum is a Latin term meaning 'sacred fragrant lipped basil'; native to tropical Asia, this plant is more commonly referred to as tulsi and is now cultivated widely across the world for both religious and medicinal purposes as well as for its essential oil. Tulsi has known to be used for its medicinal qualities for over 1000 years and ancient Ayurvedic texts refer to tulsi as the 'elixir of life'. Tulsi is known to relieve almost all ailments including common colds, digestive problems, breathing problems, stress, blood sugar, heart problems, fever and even ulcers. The oil from the seeds of this herb is now also being used to relieve cancer [1].
Endophytes are microorganisms including bacteria that live in the intercellular spaces of plant without showing any disease symptoms to the host plant [2]. Many studies have emphasized endophytes from medicinal plants and their application in different areas [3]. Recently many known as well as new endophytic bioactive metabolites, possessing a wide variety of biological activities as antibiotic, antiviral, anticancer, anti-inflammatory, antioxidant etc., have been identified [4].
Ocimum sanctum (Tulsi) is a divine tree mainly cultivated in Indian subcontinent, belonging to the botanic family Meliaceae, commonly known as Tulsi [5]. All the parts of *Ocimum sanctum* are commonly used in traditional Indian medicine for household remedy against various human diseases. Different parts of tulsi have been shown to exhibit wide pharmacological activities such as antibacterial [6], immunomodulato [7], anti-inflamatory activity [8], anti-carcinogenic activity [9], antipyretic [10] and anticoagulant [11].
The objective of the present study was to isolate endophytic bacteria from tulsi leaves, their identification and investigate their antibacterial activity against three gram positive bacteria, *Staphylococcus aureus, Streptococcus pyogens* and *Bacillus cereus* and gram negative bacteria *Escherichia coli, Salmonella* Typhimurium and *Klebsiella pneumoniae.*

2. Materials and Methods
2.1 Plant material
Fresh leaves of *Ocimum sanctum* (Tulsi) was collected by selected medicinal plants viz *Ocimum sanctum* (Tulsi). Five samples from each plant were taken and each sample was again divided into 5 sub samples and separated for further isolation of endophytic bacteria. Samples

were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria.

2.2 Sterilization of leaves

The sterlization of leaves and isolation of endophytic bacteria from the leaves was done according to Mahajan *et al*. 2014 [12], with some modifications. Leaves were treated with double distilled water for 2-3 minutes, then surface sterilized with 0.1 per cent sodium hypochlorite for 5 minutes, washed with double distilled water for 2-3 minutes. Later, surface sterilization was done with 0.01 per cent bavistin and was kept in distilled water for 5 minutes. For further sterilization the leaves were exposed to 0.05 per cent streptomycin followed by treatment with double distilled water for 5 minutes. Then the leaves were exposed to 70 per cent ethanol and again were kept in double distilled water for 5 minutes and were excised with autoclaved scalpel and forceps in laminar air flow chamber, than air dried in laminar flow.

2.3 Sterility check

To confirm that the surface of leaves were effectively sterilized 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures were planted onto nutrient agar media and incubated at 37^0C for 24 hrs. Bacterial growths were observed after 24 hrs. Also surface sterilized leaves were rolled on nutrient agar plates and incubated at 37^0C for 24 hrs and checked for possible microbial growth.

2.4 Preparation and sterilization of media

King's B (KB) media, mueller hinton media, blood agar media and BHI broth were prepared by adding agar into the distilled water. Hot plate was used for the proper mixing of media and autoclaved at 121°C for 15-20 minutes at 15 lbs.

2.5 Inoculation of leaves

The media was poured into different autoclaved petri plates and leaves of the plants were embedded in petri plates. These plates were then incubated at 37°C for 24 hrs. Characterization of the bacteria was done according to its morphology and by grams staining. After that a single colony was transferred into BHI broth and incubated at 37°C for 24 hrs.

2.6 Purification of endophytic bacteria

For purification of endophytic bacteria sub culturing was mainly done by streaking a loop full of BHI broth on the fresh pre solidified blood agar plates and then incubated at 37°C for 24 hrs. After incubation the colonies were transferred into BHI broth and then incubated at 37°C for 24 hrs and purity is checked by grams stain and stored at -20^0C in deep freeze for further work.

2.7 Antibacterial activity of endophytic bacteria

2.8 Procurement of known culture: Table 01

2.9 Preparation of inoculums

The above prepared bacterial inoculums were evenly spread on sterile mueller hinton agar plates as described by Bauer *et al.* (1969) [13] and antibacterial effect was studied by the disc diffusion method in these plates. The known antibiotic ciprofloxacin (Ci) was simultaneously used and placed as control for antibiotic sensitivity. The dried discs were immediately used and incubated at 37^0C for 24 hrs.

Table 1: list of culture procured from

S.No.	Bacteria	ATCC Catalogue No.
1.	*Escherichia coli*	25922
2.	*Klebsiella pneumonia*	700603
3.	*Salmonella* Typhimurium	13311
4.	*Bacillus cereus*	11778
5.	*Staphylococcus aureus*	6538
6.	*Streptococcus pyogens*	12386

2.10 Preparation of Antibacterial disc

For determination of antibacterial activity of endophytic bacteria, broths were centrifuged at 4°C at 12000rpm for 30 minutes. Supernatant of each of these broths were taken, sterile discs were soaked in these broths in a sterile test tubes for 24 hrs and dried in laminar flow. After drying the discs were used immediately for disc impregnation in the inoculated plates as described by Kirubaharan *et al*. (1999) [14] with slight modifications. Ciprofloxacin discs were used as control drug to compare the effect of treatment during *in vitro* study.

2.11 Antibacterial test

The prepared bacterial inoculums were evenly spread on a sterile mueller hinton agar plate as per method described by Bauer *et al.* (1969) [13]. The known antibiotic Ciprofloxacin (Ci) was simultaneously placed as a control for antibiotic sensitivity. The dried disc was incubated at 37°C for 24 hrs. Results were recorded as positive (growth) or negative (no growth) and zone of inhibition of growth exerted by these impregnated discs.

3. Results

3.1 Preliminary characterisation of isolated endophytic bacteria

3.1.a Growth of endophytic bacteria in kings B medium

Growth characteristics of endophytic bacteria isolated from tulsi leaves showed that 80 per cent were irregular in shape while 20 per cent circular in shape, 64 per cent were flat elevation on petri plate while 36 per cent raised elevation, margin of the 80 per cent colonies were undulated while 20 per cent entire, the surface of the growth was rough in 80 per cent while 20 per cent smooth and 96 per cent growth were opaque and white in colour. (Table 02)

Table 2: Growth of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) on kings B media

Sl. No.	Isolate No.	Form	Elevation	Margin	Surface	Opacity	Chromogenesis
1	T1a	Irregular	Flat	Undulated	Rough	Opaque	Absent
2	T1b	Irregular	Raised	Undulated	Rough	Opaque	Absent
3	T1c	Irregular	Flat	Undulated	Rough	Opaque	Absent
4	T1d	Circular	Flat	Undulated	Rough	Opaque	Absent
5	T1e	Irregular	Raised	Undulated	Smooth	Opaque	Absent
6	T2a	Irregular	Flat	Undulated	Rough	Opaque	Absent

7	T2b	Irregular	Raised	Undulated	Rough	Opaque	Absent
8	T2c	Circular	Raised	Undulated	Smooth	Opaque	Absent
9	T2d	Irregular	Flat	Undulated	Rough	Opaque	Absent
10	T2e	Irregular	Flat	Entire	Smooth	Opaque	Absent
11	T3a	Irregular	Raised	Undulated	Rough	Glistening	Absent
12	T3b	Irregular	Flat	Undulated	Rough	Opaque	Absent
13	T3c	Circular	Flat	Entire	Rough	Opaque	Absent
14	T3d	Circular	Flat	Undulated	Smooth	Opaque	Absent
15	T3e	Irregular	Flat	Entire	Rough	Opaque	Absent
16	T4a	Irregular	Raised	Undulated	Rough	Opaque	Absent
17	T4b	Irregular	Flat	Undulated	Rough	Opaque	Absent
18	T4c	Irregular	Flat	Undulated	Smooth	Opaque	Absent
19	T4d	Irregular	Flat	Undulated	Rough	Opaque	Absent
20	T4e	Irregular	Flat	Undulated	Rough	Opaque	Absent
21	T5a	Irregular	Raised	Entire	Rough	Opaque	Absent
22	T5b	Irregular	Raised	Undulated	Rough	Opaque	Absent
23	T5c	Irregular	Flat	Entire	Rough	Opaque	Absent
24	T5d	Circular	Raised	Undulated	Rough	Opaque	Absent
25	T5e	Irregular	Flat	Undulated	Rough	Opaque	Absent

3.1.b Growth of endophytic bacteria on 5 per cent sheep blood agar medium:

Colonies of endophytic bacteria grown on kings B agar were transferred to the 5 per cent sheep blood agar plates and incubated at 37^0 C for 24 hrs. The growth of endophytic bacteria from *Ocimum sanctum* (Tulsi) was studied. Growth characteristics of endophytic bacteria isolated from tulsi leaves presented that 86 per cent were irregular in shape while 24 per cent circular in shape, 84 per cent were raised elevation on petri plate while 16 per cent flat elevation, margin of the 84 per cent colonies were undulated while 16 per cent entire, the surface of the growth was smooth in 88 per cent while 12 per cent rough, the growth was opaque in 92 per cent isolates. All the isolates were non haemolytic and non-chromogenic. (Table 03)

Table 3: Growth of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) on 5 per cent sheep blood agar

Sl. No.	Sampl No.	Form	Elevation	Margin	Surface	Opacity	Chromogenesis
1	T1a	Irregular	Raised	Entire	Smooth	Opaque	Absent
2	T1b	Irregular	Raised	Undulated	Smooth	Opaque	Absent
3	T1c	Irregular	Raised	Undulated	Smooth	Opaque	Absent
4	T1d	Circular	Flat	Undulated	Smooth	Glistening	Absent
5	T1e	Irregular	Raised	Undulated	Smooth	Opaque	Absent
6	T2a	Irregular	Raised	Entire	Rough	Opaque	Absent
7	T2b	Irregular	Raised	Undulated	Smooth	Opaque	Absent
8	T2c	Irregular	Raised	Undulated	Smooth	Opaque	Absent
9	T2d	Irregular	Raised	Undulated	Smooth	Opaque	Absent
10	T2e	Irregular	Raised	Undulated	Smooth	Opaque	Absent
11	T3a	Circular	Raised	Undulated	Smooth	Opaque	Absent
12	T3b	Irregular	Raised	Undulated	Smooth	Opaque	Absent
13	T3c	Circular	Flat	Undulated	Smooth	Opaque	Absent
14	T3d	Circular	Raised	Undulated	Smooth	Opaque	Absent
15	T3e	Irregular	Raised	Undulated	Smooth	Opaque	Absent
16	T4a	Irregular	Raised	Undulated	Smooth	Opaque	Absent
17	T4b	Irregular	Raised	Undulated	Smooth	Glistening	Absent
18	T4c	Circular	Raised	Entire	Rough	Opaque	Absent
19	T4d	Irregular	Raised	Undulated	Smooth	Opaque	Absent
20	T4e	Irregular	Flat	Undulated	Smooth	Opaque	Absent
21	T5a	Irregular	Raised	Entire	Smooth	Opaque	Absent
22	T5b	Irregular	Raised	Undulated	Smooth	Opaque	Absent
23	T5c	Irregular	Raised	Undulated	Rough	Opaque	Absent
24	T5d	Circular	Raised	Undulated	Smooth	Opaque	Absent
25	T5e	Irregular	Flat	Undulated	Smooth	Opaque	Absent

3.1.c Growth of endophytic bacteria in BHI broth:

Colonies of endophytic bacteria grown on blood agar were transferred to the sterile BHI broth tubes and incubated at 37^0 C for 24 hrs. The growth of endophytic bacteria from *Ocimum sanctum* (Tulsi). Endophytic bacteria from tulsi leaves shown characteristics as 100 per cent isolates with turbidity, 88 per cent isolates with flocullant growth and 100 per cent isolates with pellicle formation. No sediment formation was seen in 88 per cent isolates and 56 per cent isolate showed ring formation. (Table 04)

Table 4: Growth of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) on BHI broth

Sl. No.	Isolate No.	Turbidity	Flocullant	Pellicle	Sediment	Ring formation
1	T1a	Present	Present	Present	Absent	Present
2	T1b	Present	Present	Present	Absent	Absent
3	T1c	Present	Present	Present	Absent	Absent
4	T1d	Present	Present	Present	Absent	Absent
5	T1e	Present	Absent	Present	Absent	Absent
6	T2a	Present	Present	Present	Absent	Absent
7	T2b	Present	Present	Present	Absent	Present
8	T2c	Present	Absent	Present	Absent	Present
9	T2d	Present	Present	Present	Absent	Present
10	T2e	Present	Present	Present	Present	Absent
11	T3a	Present	Present	Present	Absent	Absent
12	T3b	Present	Present	Present	Absent	Present
13	T3c	Present	Present	Present	Absent	Present
14	T3d	Present	Present	Present	Absent	Absent
15	T3e	Present	Present	Present	Absent	Absent
16	T4a	Present	Present	Present	Absent	Absent
17	T4b	Present	Present	Present	Present	Present
18	T4c	Present	Present	Present	Absent	Absent
19	T4d	Present	Absent	Present	Absent	Present
20	T4e	Present	Present	Present	Absent	Absent
21	T5a	Present	Present	Present	Absent	Present
22	T5b	Present	Present	Present	Absent	Present
23	T5c	Present	Present	Present	Present	Absent
24	T5d	Present	Present	Present	Absent	Present
25	T5e	Present	Present	Present	Absent	Absent

3.2 Microscopic examination

The microscopic examination of endophytic bacteria was done by using Grams stain. The results of grams staining are follows: 80 per cent isolates shown gram positive reaction while 20 per cent were gram negative, 84 per cent endophytic bacteria were rod shape and 16 per cent were cocci, microscopic examination showed that more than one type of endophytic bacteria were present in 72 per cent of isolate. (Table 05)

Table 5: Grams staining of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi)

Sl. No.	Isolate No.	Grams staining	Shape	Types of bacteria
1	T1a	Positive	Bacillus	<1
2	T1b	Positive	Cocci	1
3	T1c	Negative	Bacillus	<1
4	T1d	Positive	Bacillus	<1
5	T1e	Negative	Bacillus	<1
6	T2a	Positive	Bacillus	<1
7	T2b	Positive	Bacillus	<1
8	T2c	Positive	Bacillus	1
9	T2d	Positive	Bacillus	<1
10	T2e	Positive	Cocci	<1
11	T3a	Positive	Cocci	1
12	T3b	Positive	Bacillus	<1
13	T3c	Positive	Bacillus	<1
14	T3d	Negative	Bacillus	<1
15	T3e	Positive	Bacillus	1
16	T4a	Positive	Bacillus	<1
17	T4b	Negative	Bacillus	<1
18	T4c	Positive	Bacillus	<1
19	T4d	Positive	Bacillus	1
20	T4e	Positive	Bacillus	<1
21	T5a	Positive	Cocci	<1
22	T5b	Negative	Bacillus	1
23	T5c	Positive	Bacillus	<1
24	T5d	Positive	Bacillus	<1
25	T5e	Positive	Bacillus	1

3.3 *In vitro* antibacterial activity

The antibacterial activity of endophytic bacteria was evaluated against various gram positive and gram negative pathogenic bacteria namely *Bacillus cereus, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae and Salmonella* Typhimurium. Results were recorded for zone of inhibition around the disc. The inhibitory zone around the disc indicated absence of bacterial growth reported as sensitive and absence of zone reported as resistant.

3.3.a For gram positive bacteria

The *in vitro* antibacterial activities of the endophytic bacteria against different gram positive bacteria are shown in Table 06. The endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) shown antibacterial activity as 72 per cent of isolates inhibited growth of *Staphylococcus aureus*, 76 per cent of isolates inhibited growth of *Streptococcus pyogenes* and 12 per cent isolates inhibited growth of *Bacillus cereus.*

Table 6: *In vitro* antibacterial activity of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) against gram positive bacteria

Sl. No.	Isolate No.	*Staphylococcus aureus*	*Streptococcus pyogens*	*Bacillus cereus*
1	T1a	S	S	R
2	T1b	S	S	R
3	T1c	R	R	S
4	T1d	S	S	R
5	T1e	R	S	R
6	T2a	S	R	R
7	T2b	S	S	R
8	T2c	R	S	R
9	T2d	S	S	R
10	T2e	S	R	R
11	T3a	R	R	R
12	T3b	S	S	R
13	T3c	S	S	R
14	T3d	S	S	R
15	T3e	R	S	S
16	T4a	R	R	R
17	T4b	S	S	R
18	T4c	S	S	R
19	T4d	S	S	R
20	T4e	R	S	S
21	T5a	S	S	R
22	T5b	S	S	R
23	T5c	S	R	R
24	T5d	S	S	R
25	T5e	S	S	R

3.3.b For gram negative bacteria

The *in vitro* antibacterial activities of endophytic bacteria against different gram negative bacteria have been shown in Table 07. The endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) shown antibacterial activity as 84 per cent of isolates inhibited growth of *Escherichia coli*, 80 per cent of isolates inhibited growth of *Salmonella* Typhimurium and 28 per cent isolates inhibited growth of *Klebsiella pneumoniae.*

Table 7: *In vitro* antibacterial activity of endophytic bacteria isolated from *Ocimum sanctum* (Tulsi) against gram negative bacteria

Sl. No.	Isolate No.	*Escherichia coli*	*Salmonella* Typhimurium	*Klebsiella pneumonia*
1	T1a	S	S	S
2	T1b	S	R	R
3	T1c	S	S	R
4	T1d	S	S	R
5	T1e	S	S	R
6	T2a	S	R	R
7	T2b	R	S	R
8	T2c	S	S	S
9	T2d	S	S	R
10	T2e	R	S	R
11	T3a	S	S	R
12	T3b	S	R	R
13	T3c	S	S	S
14	T3d	S	S	R
15	T3e	R	S	R
16	T4a	S	S	R
17	T4b	S	R	R
18	T4c	S	S	S
19	T4d	S	S	R
20	T4e	S	S	R
21	T5a	S	S	R
22	T5b	R	R	R
23	T5c	S	S	S
24	T5d	S	S	S
25	T5e	S	S	S

4. Discussion

Twenty five strains of endophytic bacteria were isolated from leaves of *Ocimum sanctum* (Tulsi). Endophytic bacteria are found in virtually every plant on earth [15]. Different plant parts such as root, stem and nodule [16], leaves, stems and root [17] can also be used for isolation of endophytic bacteria. Costa *et al.* (2012) [18] had isolated culturable endophytic bacteria from common bean (*Phaseolus vulgaris*) leaves.

The preliminary identification of the bacterial isolates was done based on various morphological features of isolated endophytic bacteria. The colony characteristics of endophytic bacteria isolated from tulsi are having irregular in shape while flat elevation on petri plate, margin of the colonies were undulated, the surface of the growth was rough, opaque and white in colour. The microscopic examination of endophytic bacteria has shown that among the endophytic bacteria isolated from tulsi, 84 per cent isolate were gram positive while 20 per cent gram negative, 88 per cent endophytic bacteria were rod shape and 16 per cent were cocci, Microscopic examination showed that there were more than one type of endophytic bacteria present in 72 per cent of isolate. The isolation of endophytic bacteria was in agreement with the findings of Hung and Annapurna (2004) [16], had found equal percentages of gram positive (49 per cent) and gram negative (51 per cent) bacteria. Sobral *et. al*. (2005) [17] and Ebrahimia *et al*. (2010) [19] has also found equal percentage of gram positive and gram negative bacteria. However, Baghat *et al*. (2014) [20] found the 90 per cent of gram positive bacteria.

As summarized in results antibacterial activity of endophytic bacteria was calculated by the presence of zone of inhibition produce by endophytic bacteria against pathogenic bacteria. All the isolates from endophytic bacteria were screened for the antibacterial activity against pathogenic bacteria *Bacillus cereus, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae and Salmonella* Typhimurium.

The overall *in vitro* antibacterial results shown that maximum sensitivity was observed against *Escherichia coli.* Most of the isolates from tulsi had shown antibacterial activity against both gram positive (*Staphylococcus aureus, Streptococcus pyogens*) and gram negative bacteria (*Escherichia coli*, *Salmonella* Typhimurium). Verma *et al.* (2009) [21] observed antibacterial activity of endophytic actinomycetes from *Azadirachta indica* against *Escherichia coli*. Ebrahimia *et al*. (2010)[19] observed antibacterial activity of endophytic bacteria isolated from leaves of *Hypericum scabrum* against *S. aureus.* Jalgaonwala *et al*. (2010) [22] observed antibacterial activity of endophytic bacteria isolated from roots of *Aloe vera* possess strong antibacterial activity against *S. typhi* in dual culture assay. Roy and Banerjee (2010) [23] isolated endophytic bacteria from a medicinal plant *Vinca rosea*. One of the isolated endophytes produced potential antimicrobial activity against *Bacillus cereus, Klebsiella pneumoniae, Escherichiae coli*. Pal *et al.* (2012) [24] reported the antimicrobial activity of the bacterial endophytes of *P. foetida* indicating the inhibitory effect of majority of the isolates against *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. The present study is also very near to all the above authors.

5. Conclusion

Endophytic bacteria were present in leaves of *Ocimum sanctum* (Tulsi), gram positive rod shaped bacteria were present in leaves of *Ocimum sanctum* (Tulsi). Endophytic bacteria from tulsi possess maximum antibacterial activity against *Staphylococcus aureus, Streptococcus pyogens, Escherichia coli, Salmonella* Typimurium *and Klebsiella.*

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