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Comparison of antibacterial activities of the endophytic fungi isolated from different parts of *Heliotropium indicum* using different growth media

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Abstract: Endophytic fungi from medicinal plants are important natural sources of bioactive secondary metabolites for pharmaceutical industries to explore new drugs. The objective of this research was to isolate endophytic fungi from flowers, roots and leaves of *Heliotropium indicum* using two different growth media, Potato Dextrose Agar (PDA) and Maltose Extract Agar (MEA). The antibacterial activity of isolated endophytic fungi was determined against two Gram-positive (*S. aureus* and *B. cereus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. Agar disc diffusion assay was used to check the antibacterial activity at the concentrations of 400 µg/disc, 200 µg/disc, 100 µg/disc, 50 µg/disc, 25 µg/disc and 12.5 µg/disc for the isolated endophytic fungi from flowers, roots and leaves of *H. indicum*. More endophytic fungi were isolated from flowers, roots and leaves of *H. indicum* in MEA media than PDA media. According to the macroscopic morphology studies total of 16 endophytic fungi were isolated from flowers, roots and leaves of *H. indicum*. Among those 11 endophytic fungi were common to both media. Compared to other endophytic fungi isolated from flowers of *H. indicum*, YS-EF-F05 showed the highest antibacterial activity (16.7±1.5 mm– 20.3±0.6 mm) at 400 µg/disc against both Gram-positive and Gram-negative bacteria. Among the endophytic fungi isolated from roots of *H. indicum*, YS-EF-R08 showed the highest antibacterial activity (17.3±2.1 mm –21.0±0.8 mm) against Gram-positive and Gram-negative bacteria at 400 µg/disc. Compared to all endophytic fungi isolated from flowers, roots and leaves of *H. indicum*, YS-EF-L15 showed the highest antibacterial activity (18.3±0.6 mm- 21.0± 1.0 mm) against both Gram-positive and Gram-negative bacteria. The lowest MIC value (25 µg/disc) was shown by endophytic fungi YS-EF-F(01, 03, 05), YS-EF-R(07, 08, 09) and YS-EF-L(11, 15, 16) isolated from three parts of *H. indicum.* Microscopic examination revealed that most of isolated endophytic fungi belong to the genus *Aspergillus.*
keywords: Antibacterial, *Heliotropium indicum*, Disc diffusion, *B. cereus*, *E.coli*, *P. aeruginosa*, *S. aureus*

I. INTRODUCTION

Investigation and isolation of plant based antimicrobial compounds are important in antimicrobial drug development for treating different pathogenic infections. Plants are in contact with different microorganisms including bacteria, fungi, yeasts and viruses. Some microorganisms in plant tissues protect plants from harmful pathogens and environmental factors (Selvi *et al*.,2014;Tolulope *et al*., 2015). Antibacterial endophytes present in medicinal plants can inhibit the growth of microorganisms or kill them (Sandhu *et al*., 2014).

Endophytic fungi reside in healthy inter cellular or intra cellular tissues of plants without showing apparent symptoms or infection. Endophytes are present in different parts of plant tissues to protect their host cells from pathogen infections. An endophyte can be in different forms as a yeast, bacterium or fungus (Arun *et al.,* 2015). Both yeast and bacterial endophytes promote the growth of plants by increasing the total nitrogen content of plants. There are different endophytic bacteria such as *P. agglomerans*, *Methylobacterium*, *Burkholderia*, *Azospirillum*, *Herbaspirillum*, and *Rhizobium*. Some endophytic bacteria produce growth enhancing chemicals such as indole acetic acid or cytokinins (Khan *et al*., 2012).

Generally, endophytes can be found in roots, seeds, flowers, bark or leaves of plants. Endophytic microbes are essential to protect its host cells to inhibit the external biotic effects and abiotic stress (Zhao *et al*., 2010). Endophytes support the propagation of healthy plants without having mutations and infection of pathogenic diseases, and protect plants from herbivores ((Zhao *et al*., 2010). Endophytic fungi contain rich sources of bioactive natural compounds. Over the past two decades scientists have extensively studied antimicrobial, insecticidal, cytotoxic and anti-cancer bioactive compounds presence in endophytic fungi ((Zhao *et al*., 2010). For example, secondary metabolites such as taxol, pestaloside, lavostatin, vanomycin, pesticin and torreyanic acids and enzymes such as xylanase and asparginase had been isolated from endophytes (Sandhu *et*

al., 2014;Bhardwaj *et al*., 2014). Some endophytic fungi have shown antipyretic, anticancer, anti-inflammatory, antimalarial, analgesic and antibacterial activities (Min *et al*., 2016). Endophytes can act as antibacterial agents by inhibiting the synthesis of cell walls, protein, nucleic acid and essential metabolites required for growing of harmful microorganisms (Liwa *et al*., 2015).

Heliotropium indicum ("Eth Sonda" in Singhalese) is an important medicinal plant (Figure1) belongs to Boraginaceae family of plants kingdom. This plant is very common in African and Asian countries. The plant is branched and height is up to 60 cm with pale violet color flowers. *H. indicum* is mainly distributed in tropical warm temperate countries and sub-tropical countries in the world (Abdullah *et al*., 2013). Different parts of this plant had been used in African and Asian countries for traditional medical treatments such as treat for warts, tumors, inflammation, asthma, cough, menstruation disorder, diuretics, rashes, urticaria, pimples on the face and heal the wounds (Abdullah *et al*., 2013).

Figure 1:*Heliotropium indicum* plant

It has been reported that *H. indicum* has many important medicinal values such as gastroprotective (Roy *et al*., 2015), wound healing properties (Roy *et al*., 2015), antimicrobial (Osungunna *et al*., 2011), antitumor (Kugelman *et al*., 1976),analgesic (Boye *et al*., 2012) andanti- inflammatory (Kyei *et al*., 2016) activities. However, the antibacterial activity of endophytic fungi isolated from *H. indicum* has not been reported. Researchers have done some studies on antimicrobial properties of *H. indicum* plant and it has been reported that methanol extract of the leaf of *H. indicum* plant has shown promising antimicrobial activity against five Gram positive and Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiellaspp*, *Proteus mirabilis*, *Staphylococcus aureus)* at 6.25, 12.5, 25,50, 100 and 200 mg/ml. *S. aureus* and *Klebsiella spp*. were inhibited at 50, 100 and 200 mg/ml with minimum inhibitory concentration (MIC) of 3 mg/ml. Plant has showed MIC of 10 mg/ml against *P. aeruginosa* and *P. mirabilis*. For the *E. coli*, MIC value was 20 mg/ml (Osungunna *et al*., 2011).

II. MATERIALS AND METHODS

Collection of Plant materials

Fresh healthy parts of *H. indicum* were collected from Anuradapura district in Sri Lanka. Plant was authenticated at Bandaranayake Memorial Ayurvedic Research Institute, Nawinna, Maharagama. Flowers, roots and leaves of *H. indicum* were used to isolate antibacterial endophytic fungi from *H. indicum*.

Chemicals used for isolating endophytic fungi from *Heliotropium indicum*

Methanol, ethyl acetate, ethanol were used as extraction solvents. Sodium chloride, sulfuric acid, hydrated barium chloride, cotton phenol blue, acetone and ciprofloxacin were used as other essential chemicals.

PDA and MEA media preparation

Potatoes dextrose agar (PDA) and maltose extract agar (MEA) powder (39.0 g each)were dissolved in distilled water (1000 ml) separately and autoclaved in 15 lbs pressure (121 °C for 20 minutes). When temperature was below 40 °C, ciprofloxacin antibiotic solution (15 ml of standard 2.5 mg/ml ciprofloxacin solution) was added to the media. Then the prepared sterilized media was poured into sterilized petri dishes (15 ml) under aseptic condition. When media was solidified these plates were wrapped in aluminum foils and stored in refrigerator at 4 °C.

Isolation of endophytic fungi from different parts of *Heliotropium indicum*

Endophytic fungi isolation was carried out according to the method described by Petrini (Petrini *et al*., 1986). Surface sterilization process of the method was slightly modified. The flowers, leaves and roots of *H. indicum* plant were washed 10 minutes in distilled water, then surface sterilized by immersing for 30 seconds in 70% (v/v) ethanol and 2-3 minutes in sodium hypochlorite (2.5 % (v/v) followed by rinsing for 30 seconds from 70% (v/v) ethanol and washing three times with sterilized distilled water. The excess moisture was blotted using a sterile filter paper. The surface sterilized plant parts were cut into 0.5 cm^2 segments and kept on PDA and MEA media with the ciprofloxacin antibiotic to suppress bacterial growth. Incubation for endophytic fungus was conducted at 28 °C for 10 to 14 days. Isolated endophytic fungi were sub-cultured into five plates of PDA and MEA media per each endophytic fungi without adding ciprofloxacin antibiotic to get pure endophytic fungi. Incubation was done at 28 °C for 14-20 days until matured colonies of endophytic fungi were formed.

Preparation of crude extracts of isolated endophytic fungi

The isolated fungi mycelia and the agar media cultured on five petri dishes were cut into small pieces under aseptic conditions. Pieces were mixed with ethyl acetate solution (100 ml) and sonicated for 30 minutes at 37 °C. Using cotton wool, extracts were filtered into an Erlenmeyer flask and extraction was repeated twice using the same procedure. Three extracts were combined and concentrated by rotary evaporation below the 37 °C until it gets completely dry. Same procedure was followed for all the other endophytic fungi isolated from different parts of *H. indicum* plant.

Screening for the antibacterial activity of the ethyl acetate extracts of endophytes

Completely dried crude extracts of endophytic fungi were dissolved in methanol to prepare 20 mg/ml stock solution. The antibacterial activity of isolated endophytic fungi was carried out using the disc diffusion method (Afzan *et al*., 2005). Two Gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were used to determine the antibacterial activity of the isolated endophytic fungi from parts of *H. indicum* plant. Sterilized paper discs (Whatman No 1 filter papers, 6 mm in diameter) were loaded with 400 µg/disc, 200 µg/disc, 100µg/disc, 50 µg/disc, 25 µg/disc, 12.5 µg/disc of the crude extract. Negative control was carried out for each test organism using 10 µl/disc of methanol solution, and positive control was carried out using 100 µg/disc of ciprofloxacin antibiotic solution. Plates were sealed using glad wrap and incubated at 37 °C for 24 hours. After the incubation period the inhibition zone was measured to the nearest milliliter.

Identification of isolated endophytes from flowers, roots and leaves of *Heliotropium indicum*

Each isolated endophytic fungus was identified using macroscopic and microscopic studies.

Macroscopic studies of endophytic fungi

Macroscopic study was done after growing endophytic fungi on MEA and PDA media. Color, shape and colonies appearance were observed by looking front and back views of isolated endophytic fungi from flowers, roots and leaves of *H. indicum.*

Microscopic studies of endophytic fungi

Preliminary identification of endophytic fungi was carried out using the light microscope at the Department of Plant Science, University of Colombo. Their spores, mycelium and appearance of fungus sporangium were observed.

III. RESULTS AND DISCUSSION

Endophytic fungi isolated in MEA and PDA media

Three different endophytic fungi were isolated in PDA media and five endophytic fungi were isolated in MEA media from flowers of *H. indicum* plant. Four endophytic fungi were isolated in PDA media and five endophytic fungi were isolated in MEA media from roots of *H. indicum*. From leaves of *H. indicum*, four endophytic fungi were isolated in PDA media and six endophytic fungi were isolated in MEA media.

Cultured endophytic fungi in MEA media took 12-14 days to completely cover the surface of media in petri dishes and cultured endophytic fungi in PDA media took more than 18 days to completely cover the surface of media. Therefore growth rate of endophytic fungi in MEA media was higher compared to PDA media. Types of nutrients contain in MEA and PDA media are different, hence it has caused to separate different endophytic fungi in MEA and PDA media.

According to the morphological studies total of 11 and 16 endophytic fungi were isolated from PDA and MEA media, respectively from the parts of *H. indicum* plant. According to the macroscopic studies, all the endophytic fungi isolated from different parts of *H. indicum* plant in PDA media have been also isolated in

MEA media. However, endophytes cultured in MEA media showed five more additional endophytic fungi for parts of *H. indicum* plant compared to endophytic fungi isolated in PDA media.

Antibacterial activity of endophytes isolated from flowers of *Heliotropium indicum*

Antibacterial activity of ethyl acetate crude extracts of flowers of *H. indicum* was determined by measuring average inhibition zone of all endophytic fungi. Endophytic fungi YS-EF-F01, YS-EF-F03, YS-EF-F05 showed promising antibacterial activity (Table 1) against both Gram-positive (*S. aureus* and *B. cereus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) at the concentration range from 25 µg/disc to 400 µg/disc.

The endophytic fungi YS-EF-F05 showed the highest antibacterial activity against *S. aureus* (20.3±0.6 mm)at 400 µg/disc compared to all other endophytic fungi isolated from flowers of *H. indicum.* YS-EF-F02 showed poor antibacterial activity against *B. cereus* and *P. aeruginosa* at 400 µg/disc compared to the other endophytic fungi isolated from flowers of *H. indicum.* However, YS-EF-F02 did not show antibacterial activity against *S. aureus* and *E. coli* at any concentration.

YS-EF-F04 did not show antibacterial activity against both Gram-positive and Gram-negative bacteria at any concentration. Compared to overall antibacterial activity of endophytic fungi isolated from flowers of *H. indicum*.

Antibacterial activity of endophytes isolated from roots of *Heliotropium indicum*

According to the summerized antibacterial activity (Table 2) of the crude extract of roots of *H. indicum*, theendophytic fungi YS-EF-R06 showed poor antibacterial activity against both Gram-positive and Gram-negative bacteria at 400 µg/disc. YS-EF-R06 did not show antibacterial activity against *E. coli* at 400-100 µg/disc.

YS-EF-R07, YS-EF-R08 and YS-EF-R09 showed promising antibacterial activity against both Gram-positive and Gram-negative bacteria at 400 µg/disc. Among these YS-EF-R08 showed the highest value of average inhibition zone against *S. aureus* (21.0 ±0.8 mm) at 400 µg/disc. YS-EF-R07 showed strong antibacterial activity against *B. cereus* compared to the other endophytic fungi isolated from roots of *H. indicum*. YS-EF-R09 showed the highest inhibition zone value (17.8±0.8 mm) against *P. aeruginosa* at 400 µg/disc compared to other endophytic fungi isolated from roots of *H. indicum.*

YS-EF-R10 did not show antibacterial activity against Gram-positive and Gram-negative bacteria at any concentration. According to the observation *P. aeruginosa* has moderate antibiotic resistance for endophytic fungi isolated from roots of *H. indicum.*

Antibacterial activity of endophytes isolated from leaves of *Heliotropium indicum*

According to the summarized antibacterial activity (Table 3) of the crude extract from leaves of *H. indicum*, endophytic fungi YS-EF-L11, YS-EF-L15 and YS-EF-L16 showed very prominent antibacterial activity compared to YS-EF-L12 and YS-EF-L13. Endophytic fungi YS-EF-L11 showed antibacterial activity against all pathogenic bacteria tested at the concentration range from 25 µg/disc to 400 µg/disc.

Endophytic fungi YS-EF-L12 showed the weakest antibacterial activity against only for *B. cereus* and *P. aeruginosa* compared to all other endophytic fungi isolated from leaves of *H. indicum.* Endophytic fungi YS-EF-L12 did not show antibacterial activity against *S. aureus* and *E. coli* at any concentration of the crude extract.

Endophytic fungi YS-EF-L13 showed moderate antibacterial activity against *S. aureus*, *B. cereus* and *E. coli* with the average inhibition zone range from 15.0 ±2.0 mm to 19.0±1.0 mm. YS-EF-L13 did not show antibacterial activity against *P. aeruginosa* at any concentration.

Endophytic fungi YS-EF-L14 did not show positive antibacterial results against Gram-positive and Gram-negative bacteria at any concentration.

Table 1: Average zone of inhibition of endophytic fungi isolated from flowers of *Heliotropium indicum*

Endophytes	Average inhibition zone (mm)				
	Concentration	Gram-positive bacteria		Gram-negative bacteria	
	µg/disc	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*

	400	20.0 ± 1.0	19.5±0.5	17.7±1.5	17.7±0.6
YS-EF-F01	200	14.8±1.0	16.8±0.8	15.7±1.2	12.3±1.5
	100	14.1±0.8	13.0±2.0	11.7±1.5	12.5±1.3
	50	11.1±1.0	10.7±1.5	9.7±1.5	7.7±1.2
	25	7.0±1.0	7.5±0.9	7.1±1.0	6.3±0.6
	12.5	-	-	-	
YS-EF-F02	400	-	11.3±1.2	-	9.3±1.5
	200	-	10.3±0.6	-	8.3±1.5
	100	-	9.3±1.5	-	-
	50	-	-	-	-
YS-EF-F03	400	19.0 ±1.0	16.8±1.60	15.7±1.5	18.0±1.0
	200	12.3±1.5	14.7±1.5	13.2±0.8	16.7±1.5
	100	9.7±1.52	8.0 ±1.0	10.7±1.52	15.0±1.0
	50	7.0±1.0	7.3±1.2	8.2±1.0	10.0±1.0
	25	6.7±0.6	7.0±1.0	7.2±0.8	8.0±1.0
YS-EF-F04	400	-	-	-	-
	200	-	-	-	-
	100	-	-	-	-
YS-EF-F05	400	20.3±0.6	20.0 ±1	18.8±0.8	16.7±1.5
	200	15.7±1.5	17.7±1.5	15.3±1.5	13.0 ±1.0
	100	13.0±1.0	13.0±2.0	12.3±0.6	11.2±1.0
	50	10.0±1.0	11.7±1.5	10.0±1.0	8.0±1.0
	25	6.3±0.6.0	7.0±1.0	6.8±1.0	6.7±0.6
+ve control	(100 µg/disc) Ciprofloxacin	29.0±1.0	31.0±2.0	33.0 ±1.50	31.0±1.0
-ve control	(Methanol)	-	-	-	-

Table 2: Average zone of inhibition of endophytic fungi isolated from roots of *Heliotropium indicum*

Endophytes	Average inhibition zone (mm)		
	Concentration	Gram-positive bacteria	Gram-negative bacteria

	µg/disc	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*
YS-EF-R06	400	8.0±1.0	10.0±1.0	-	7.0±1.0
	200	-	7.7±0.6	-	-
	100	-	-	-	-
YS-EF-R07	400	19.0±1.0	20.0±1.5	17.8±1.8	17.0±1.0
	200	16.2±1.0	15.0±0.6	16.0±1.5	12.0±2.1
	100	15.0±1.0	11.3±1.5	12.7±2.5	10.7±1.5
	50	9.0±1.0	10.3±2.1	8.0±1.7	10.0 ±2.0
	25	7.3±0.6	7.3±1.5	6.8±1.0	6.8±0.3
	12.5	-	-	-	-
YS-EF-R08	400	21.0 ±0.8	19.7±1.0	18.0±1.0	17.3±2.1
	200	16.0±1.0	18.7±0.6	15.7±1.7	13.7±1.5
	100	13.3±1.5	14.3±2.1	13.3±1.5	10.8±1.6
	50	10.7±0.6	12.2±1.9	9.7±1.5	7.7±1.5
	25	6.7±0.6	7.0±1.0	7.2±1.6	6.7±0.6
	12.5	-	-	-	-
YS-EF-R09	400	20.0±1.0	18.3±1.5	16.3±1.5	17.8±0.8
	200	13.0±1.0	12.7±1.5	13.3±0.6	15.7±2.1
	100	10.7±1.5	12.2±1.9	11.3±1.5	13.0±3.7
	50	10.7±0.6	7.7±1.5	9.7±1.5	9.3±1.5
	25	7.3±0.6	7.3±1.52	6.2±0.3	9.0±1.0
YS-EF-R10	400	-	-	-	-
	200	-	-	-	-
	100	-	-	-	-
+ve control	(100 µg/disc) Ciprofloxacin	29.0±1.0	31.0±2.0	33.0 ±1.50	31.0±1.0
-ve control	(Methanol)	-	-	-	-

Table 3: Average zone of inhibition of endophytic fungi isolated from leaves of *Heliotropium indicum*

Endophytes	Average inhibition zone (mm)				
	Concentration	Gram-positive bacteria		Gram-negative bacteria	
	µg/disc	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*

	400	19.3±1.5	17.7±1.5	18.7±1.5	17.3±1.2
	200	15.7±1.52	13.7±2.9	15.3±0.6	16.3±2.1
YS-EF-L11	100	12.8±1.6	11.3±1.52	12.0±1.0	11.3±2.1
	50	8.2±0.8	8.0±1.0	9.0±1.0	7.3±1.5
	25	6.3±0.6	7.8±0.3	6.8±0.8	7.3±1.5
	400	-	10.7±1.5	-	11.0±1.0
	200	-	10.3±1.5	-	9.7±1.5
	100	-	7.6±1.0	-	6.3±0.6
YS-EF-L12	50	-	7.0±1.0	-	-
	25	-		-	
	400	19.0±1.0	15.0±2.0	19.0±1.0	-
	200	11.7±0.6	10.3±1.5	7.0±1.0	-
	100	7.7±1.5	7.0±1.0	7.3±1.2	-
	50	6.3±0.6	-	7.0±1.0	-
YS-EF-L13	25	-	-	-	-
	12.5	-	-	-	-
	400	-	-	-	-
	200	-	-	-	-
	100	-	-	-	-
YS-EF-L14	50	-	-	-	-
	400	20.3±0.6	21.0±1.0	19.0±1.0	18.3±0.6
	200	15.7±1.5	16.3±1.5	16.7±2.5	17.7±2.5
	100	12.3±2.1	11.3±2.1	13.3±1.5	13.3±2.8
YS-EF-L15	50	9.3±2.1	7.7±0.6	8.8±0.3	11.0±1.0
	25	7.3±0.6	6.3±0.7	8.0±1.0	8.3±1.5
	400	14.4±1.0	16.1±1.5	18.5±1.3	16.0±1.6
	200	12.0±1.6	11.3±1.5	16.0±1.6	8.0±1.6
	100	8.7±1.8	10.4±1.1	8.2±1.6	6.1±0.4
YS-EF-L16	50	7.0±1.0	8.0±1.0	7.0±0.8	6.0±0.4
	25	6.7±0.6	7.0±1.0	-	-
+ve control	(100 µg/disc) Ciprofloxacin	29.0±1.0	31.0±2.0	33.0±1.50	31.0±1.0
-ve control	(Methanol)	-	-	-	-

IV. CONCLUSION

The present study revealed that the endophytic fungi in intercellular tissues of flowers, roots and leaves of *H. indicum* have strong antibacterial properties. Number of antibacterial endophytic fungi contain in the leaves are higher compared to the roots and flowers of *H. indicum*.

YS-EF-L15 isolated from the leaves showed the highest antibacterial activity (21.0 ±1.0 mm) than the other endophytic fungi isolated from flowers, roots and leaves of *H. indicum*. YS-EF-F01, YS-EF-F05, YS-EF-R07, YS-EF-R08, YS-EF-R09, YS-EF-L11 and YS-EF-L16 also showed strong antibacterial activity with the average inhibition zone range of 14.4±1.0 – 21.0 ±0.8 mm against both Gram-positive and Gram-negative

bacteria used in this research project. According to the microscopic studies most of the isolated endophytic fungi belong to *Aspergillus* genus.

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Abbreviations

PDA: Potato Dextrose Agar;MEA: Maltose Extract Agar

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