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ENDOPHYTES FROM MEDICINAL PLANTS AS BIOCONTROL AGENTS ; A COMPARITIVE STUDY

[1]S ANU KIRUTHIKA, [2]QURATH UL AIN, [3]BAWANSHISHA SYIEMLIEH & [4]N B KARTHIK
[1]Corresponding author: Dr. S ANU KIRUTHIKA, Associate Professor, Department of Microbiology, Indian Academy Degree College - Autonomous, Bengaluru, Karnataka. E.Mail ID: kiruthika.anu@gmail.com
[2,3,&4] M.Sc Students, Department of Microbiology, Indian Academy Degree College – Autonomous, Bengaluru, Karnataka.

ABSTRACT

Aim: To isolate endophytic microorganisms from the leaves of *Ocimum sanctum, Plectranthus amboinicus,* and from the flower of *Hibiscus rosa-sinensis*..To assess and comparatively study the antibacterial effect of secondary metabolites of endophytic bacteria and fungi.
Methods: Surface sterilization of collected samples, pre-treatment of sterilized samples, isolation, and preliminary characterization of these endophytes was carried out via microscopic observations and biochemical characterization. These endophytes were screened for their antibacterial activity.
Results: A total of 3 fungi and 31 bacteria were isolated from these medicinal plant samples. Out of which only three fungal and four bacterial endophytes were chosen based on the agar plate activity, sub-cultured and screened for the production of bioactive compounds. Later the effect of their secondary metabolites was studied against multidrug-resistant bacteria such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Serretia* sp. All the chosen endophytes except one fungi showed promising inhibitory effects against the test organisms.
Conclusion: The zones of inhibition obtained from the fungal metabolites were wider and had better inhibitory effect on all the test organisms even at a lower concentrations where as, the secondary metabolites obtained from the four bacterial endophytes displayed as varying effect on the above mentioned organisms. Hence its confirmed that the fungal metabolites are more efficient in controlling the growth of these test organisms. The increased concentrations of the bacterial metabolites enhances its antibacterial effects.

Keywords: Endophytic microorganisms, *Ocimum sanctum, Plectranthus amboinicus, Hibiscus rosa-sinensis,* secondary metabolites, bioactive compounds, antibacterial activity, biocontrol agents.

1. INTRODUCTION

Like human beings, plants have certain amount of micro-flora consists of bacteria, actinomycetes and fungi present in its system. These micro-organisms residing within the live plant tissues without causing any significant symptoms or disease in the plants are known as endophytic microorganisms. De Bary in 1866 coined the term endophytes. The term 'Endophyte' is derived from Greek words "Endon" meaning "within" and "phyton" meaning "plants". A wide variety of microorganisms live in close proximity to plants (Anjum *et al*., 2015; Geetha *et al.*, 2019). Numerous surveys have been conducted, the majority of which focused on pathogenic interactions between plants and microorganisms associated with them. However, several studies were conducted on the role of microbial diversity in relation to various plant species, it was assumed that only a small percentage of the microbes interacting with the plant are pathogenic in nature. Most microorganisms that inhabit plants play an important role in the plant's health and development, though some are neutral. A single plant species may contain thousands of microbes, which are classified as epiphytes (microbial inhabitants of the rhizosphere and phyllosphere; those near or on plant tissue) or endophytes (microbes residing within plant tissues in leaves, stems, and roots) (Sushanto *et*

al., 2016). Endophytes are promising, underutilized, and useful sources of novel natural products for use in agriculture, medicine, and industry. Endophytes have long been recognized as a source of pharmaceutical bio-active compounds, as many endophytes have been explored to produce novel bio-active metabolites such as anti-bacterial, anti-fungal, anti-viral, anti-tumor, anti-oxidant, anti-inflammatory, immuno-suppressive drugs, and many other compounds. They are well known for producing various classes of natural products and have been reported to exhibit a wide range of biological activity, including alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, and others (Anjum *et al*., 2015). The most active bioactive compounds produced by these endophytes are antibiotics that are low molecular weight natural bioactive compounds which are basically proteins that are active against other microorganisms. Most of the chemotherapeutically important antibacterial or antifungal drugs are either microbial metabolites or their semi synthetic derivatives obtained through bioconversion or biotransformation processes (Kumar *et al*, 2015).

Taking into account the afore-mentioned benefits, the current study was carried out to isolate and evaluate bacterial and fungal endophytes from selected plant parts and to assess their potential for antibacterial activities.

About the sample plants

1. ***Ocimum sanctum*** (Holy Basil) commonly known as Tulsi, belongs to the family Lamiaceae is a widely distributed medicinal plant in India. Tulsi leaves contains bright yellow-colored volatile oil reported to possess antibacterial properties and is known to work as an insecticide. Traditionally its leaves are used as remedies for cold, bronchitis, cough, digestive issues etc., (Anjum *et al*., 2015). Holy Basil plant is considered as a sacred plant in India, which possesses antibacterial, antipyretic, immunomodulatory, anti-inflammatory, anti-stress, anti-asthmatic, hypotensive, hypoglycemic, and analgesic effects (Bhargava *et al*., 1981; Chattopadhyay *et al*., 1993).
2. ***Hibiscus rosa-sinensis***, commonly known as *Hibiscus* belongs to the family of Malvaceae. *Hibiscus* is used as a herbal medicine to treat hypertension, cholesterol production, and cancer progression, in addition to casual consumption. Traditionally the flowers have been used as anti-asthmatic medications. Previous pharmacological studies revealed that *Hibiscus rosa-sinensis* had reproductive, anti-inflammatory, anti-diabetic, analgesic, fibrinolytic, hypolipidemic, antioxidant, antipyretic, immuno-modulatory, anticonvulsant, antidepressant, memory enhancement, cytotoxic, antimicrobial, antiparasitic, dermatological, anti-haemolytic, urinary, hepatoprotective, neuroprotective effects etc., (Ali, 2018). Hence, further studies can help in developing newer drugs using *Hibiscus* flowers.
3. ***Plectranthus amboinicus L.*** *(Coleus amboinicus),* commonly known as Indian borage is a medicinal plant of Lamiaceae family. It has been extensively studied and recognized for its antifungal, antioxidant and antiinflammatory properties. Traditionally this plant has been used to treat high fever, throat infection, nasal congestion, cough, indigestion constipation and other digestive issues (Patrick *et al*., 2019). This plant's leaves have been shown to have in vivo antimalarial activity against *Plasmodium berghei yoelii*. Carvacrol, flavonoid, rosmarinic, caffeic acid, and chlorogenic acid are all found in the leaves of *Coleus amboinicus*. Flavon salvigenin, 6-methoxygenkwanin, quercetin, chrysoeriol, luteolin, apigenin, flavanon eriodyctol, and flavanol taxifolin are among the flavonoid constituents of this plant (Astuti *et al*., 2014).

Need of the study

In this modern era, excessive use of chemotherapeutic drugs and antibiotics - both natural and synthetic, has led to the development of antibiotic resistance in microorganisms specifically in human bacterial pathogens. But these pathogens are still sensitive to certain medicinal plants due to the presence of diversified compounds in it. Various studies prove that compounds present in plants are not only plant products but also a contribution of endophytes present in them. Therefore, these endophytes can be studied and explored for their potential to produce various bio-active compounds which can be practically used as drugs in treatment of various diseases. It also can be used as antipyretic drugs, anti-diabetic drugs, anticancer drugs etc.

2. MATERIALS AND METHODOLOGY

2.1 Sample Collection

Different medicinal plants such as *Coleus amboinicus* (Mexican mint herb), *Ocimum sanctum* (Tulsi), and *Hibiscus* flower were used as samples. Fresh leaves from Mint, Tulsi plant, and hibiscus flower samples were collected using sterile forceps from Bangalore, Karnataka, and subjected to surface sterilization in order to isolate endophytic microorganisms.

2.2 Surface Sterilization

The freshly collected leaves and flower samples were thoroughly washed under running tap water for 15 to 20 minutes in order to remove debris, soil particles, pollens, etc. from their external surfaces. The samples were then washed thrice using sterile distilled water and pat dried to remove excess water. The plant materials were rinsed again using 70% ethanol and methanol separately for 3 minutes each. Finally, the samples were washed using sterile distilled water and dried using sterile blotting papers and tissues (Anjum *et al.,* 2015).

Sterility check: The effectiveness of surface sterilization was confirmed by inoculating 1ml of sterile distilled water (in which the surface sterilized leaves and flower was dipped) on nutrient agar plates and incubating for 24 hours and checking for the presence of microbial growth (Anjum *et al.,* 2015).

2.3 Preparation and Sterilization of Media

The choice of the media used plays a major role in favoring the growth of endophytes. Nutrient agar being a basic medium supports all types of microbial growth, hence it was used for isolation of endophytes from the chosen samples. Other media such as Muller-Hinton agar, Nutrient agar, and potatoes dextrose agar were also used for the growth of isolates. These media were prepared and sterilized at 121ºC under 15lbs using an autoclave. The sterile media was poured into sterile petriplates under aseptic conditions and respected media containing plates were clearly labeled and used to isolate the endophytes from the samples (Garba *et al.,* 2020).

2.4 Pre-treatment of sterilized samples and isolation of endophytes

The surface sterilized plant samples were crushed using sterilized mortar and pestle, and the finely crushed samples were stored in the sterile Eppendorf tubes. Then the prepared samples were inoculated (using spread plate and pour plate techniques) onto Nutrient agar (NA) plates and Potatoes dextrose agar plates (PDA) to isolate Endophytic bacteria and fungi respectively. The plates with respective samples were wrapped using parafilm tape and incubated at 28±2ºC for 24 to 48 hours. After the incubation period growth of various endophytic bacteria and fungi was observed. Morphologically different bacterial and fungal colonies were chosen and based on their agar plate activity (antagonistic effect) the bacteria and fungi were randomly picked and used in further process. The pure cultures of selected isolates were prepared in duplicates and preserved at 4ºC for further use. The fungal and bacterial pure cultures were stained using LPCB staining technique and Gram staining technique respectively.

2.5 Detection of Bioactive Compound produced by the chosen Endophytes

The bacterial and fungal colonies were sub-cultured in the broth medium and the presence of bioactive compounds from the cultured broth medium was detected using the well diffusion method where the wells were created by puncturing the Muller Hinton agar medium using sterile 1ml micropipette tips. Then the plates were labeled and swabbed with different test organisms. The swabbed plates were incubated for 10 minutes. 20µl of overnight grown bacterial broth samples and 20µl of 7 days old fungal broth samples were inoculated into the respectively labelled wells and all the inoculated plates were incubated for 24 - 48 hours at 28ºC. After the incubation period, the plates were observed for the presence of clear zones which indicates the presence of bioactive compounds. A total of 7 isolates containing three fungal colonies and 4 bacterial colonies showed the clear zones indicating the presence of bioactive compounds in the inoculated brothes.

Test organisms used were - *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Serratia* sp.

Inorder to analyse the effect of plant extract on microorganisms, an experiment was performed by air exposing the plant extract contaning plates and results were observed.

2.6 Effect of plant extract on growth of microorganisms using cold extraction method.

In this method, sample plant leaves were surface sterilized and crushed using a sterile mortar and pestle. The finely crushed sample was then filtered using a whatmann filter paper. The filtrate was then plated using pour plate technique in the aliquotes of 1ml - 5ml respectively. A plate containing only nutrient agar media was used as a control to comparitively analyse the effect of plant extract with increasing concentrations on the air exposed microorganism. After 24 hours of incubation, plates were compared to check the effect of plant extract in its increasing concentrations. With the increase in concentration, the plates showed decrease in the microbial diversity in the air exposed plates. This indicates that the plant extract has lesser effect on air microfloral growth.

Figure. 1. Filtration of plant extract

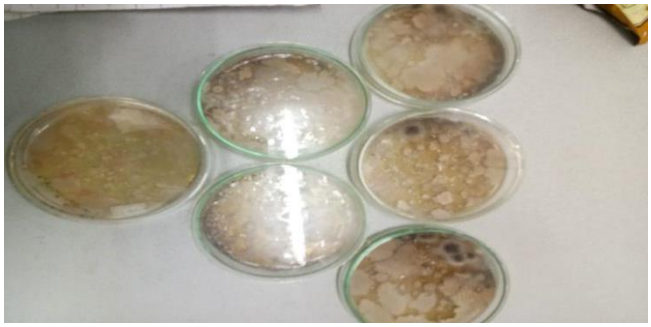

Figure. 1.1 Plates containing Plant extract in different concentrations

Table 1. Effect on Plant extract on Air Micro-flora by Pour Plate technique.

Sl. No.	Volume of NA (ml)	Volume of filtrates plant extract (ml)
(Control)	20.0	0.0
1	19.0	1.0
2	18.0	2.0
3	17.0	3.0
4	16.0	4.0
5	15.0	5.0

3. RESULT AND DISCUSSION

3.1 Isolation of Endophytes

In this project, a total of 3 fungi and 31 bacteria was isolated from three different medicinal plant samples, i.e., from the leaves of *Ocimum sanctum* and *Plectranthus amboinicus* , and from the flower of *Hibiscus rosa-sinensis.* The isolates were named as ISL1 to ISL7 out of which, ISL1 - ISL3 were fungal cultures and ISL4 - ISL7 were bacterial cultures. On LPCB staining of pure fungal cultures, one common fungi was identified and only different fungal colonies were further studied and screened based on their colony morphology. The details of the selected isolates is given below in the tabular form.

Table 2. Total Endophytic Isolates obtained from Three different Medicinal plants. [code ISL stands for isolate]

SL. NO	Medicinal plant name.	Bacteria	Fungi	Selected isolates (Bacteria + Fungi)
1.	***Ocimum sanctum***	9	2	1+2 = 3 isolates
2.	***Plectranthus amboinicus***	10	1	1+1 = 2 isolates
3.	***Hibiscus rosa-sinensis***	12	2	2+2 = 4 isolates

Table 3. Chosen Isolates for further Screening of Bioactive Compounds. [ISL means Isolate]

SL. NO	Medicinal plant name.	Bacteria	Fungi
1.	*Ocimum sanctum*	ISL6	ISL1
2.	*Plectranthus amboinicus*	ISL7	ISL2
3.	*Hibiscus rosa-sinensis*	ISL4, ISL5	ISL3

Figure 2 : Selected medicinal plants
A. *Ocimum sanctum.* B. *Plectranthus amboinicus* C. *Hibiscus rosa-seninsis*

Figure 3: Endophytes isolated from the medicinal plant parts
(a), (b), (e), and (g) are streak plates of Mexican mint, Hibiscus flower and Tulsi leaves samples.(c), (d), and (h) are spread plated samples of Mexicant mint, Hibiscus and Tulsi leaves respectively.

3.2 Screening for Antimicrobial Activity.

The pure cultures of endophytic bacteria and fungi were tested for its antimicrobial activity against the multi-drug resistant human pathogens such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Serretia* sp., by well diffusion method.

Out of three fungal isolates, two isolate, ISL1 and ISL3 showed inhibitory action against all four test organisms. The bacterial isolates ISL4- ISL7 had inhibitory effects on *Serratia* sp. and *Klebsiella* sp with different zones of inhibition. Whereas the ISL1 and ISL3 showed the zone of inhibition of 28mm wide against *Pseudomonas aeruginosa*, and had zone of about 15mm against *S. aureus.* The bacterial isolates ISL4, ISL5 and ISL6 demonstrated a very small zone of inhibition against *P. aeruginosa.* Out of four bacterial isolates only isolate ISL4 and ISL5 were able to inhibit *S. aureus* and showed a very narrow zone of inhibition. This suggests that the increase in the concentration of these bioactive compounds have the ability to significantly inhibit the growth of MRSA.

These results suggest that the fungal extracts can be used to significantly treat the diseases caused by the above mentioned pathogens than the bacterial extracts. Therefore, these isolates can be studied further and used to produce therapeutically important drugs.

Table 4. Effect of the Secondary Metabolites produced by Endophytic Microbes on Human Pathogens. [ISL stands for isolate]

Microbial codes	***S. aureus***	***K. pneumoniae***	***P. aeruginosa***	***Serratia sp.***
ISL2	–	–	–	–
ISL3	+	+	+	+
ISL4	+	+	+	+
ISL5	+	+	+	+
ISL6	–	+	+	+
ISL7	–	+	–	+

Figure. 3 Antibacterial Effect of Isolates on Human Pathogens

Triplets for each sample were maintained, and the zone of inhibition was measured for each sample. The total data is given in the tabular column

Table 3.1. Measured Zones of Inhibition [ISL represents Isolates].
[Zone of inhibition measured in millimeter (mm)]

Isolates	***S. aureus*** (Radius in mm)			***K. pneumoniae*** (Radius in mm)			***P. aeruginosa*** (Radius in mm)			***Serratia sp.*** (Radius in mm)		
ISL 1	16	18	20	18	17	20	24	20	25	26	23	26
ISL 3	15	17	18	22	20	18	26	23	24	25	23	26
ISL 4	5	9	12	24	22	25	10	7	12	13	12	15
ISL 5	14	10	15	15	17	16	7	6	9	10	8	9
ISL 6	0	0	4	12	10	13	6	4	5	7	5	4
ISL 7	0	0	2	7	5	8	0	0	0	5	3	6

All the zones of inhibition were measured from each plates in triplet sets against all the test organisms and the data is given above in the tabular form. The statistical analysis was performed when in the mean and standard deviation for each sample was calculated and the data is given below in the tabular form.

Table. 3.2 Statistical Analysis. (Code SD means Standard deviation)

Isolates	***S. aureus***	***K. pneumoniae***	***P. aeruginosa***	***Serratia sp.***
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
ISL 1	18±1.6	18±1.2	23±2.1	25±1.4
ISL 3	16±2.4	20±1.6	24.3±1.2	24.6±1.2
ISL 4	8±2.8	23±1.2	9±2.0	13.3±1.2
ISL 5	13±2.1	16±0.8	7.3±1.2	9±0.8
ISL 6	1±1.8	11.6±1.2	5±0.8	5.3±1.2
ISL 7	0.6±0.9	6.6±1.2	0	4.6±1.2

3.4Characterization of the Isolates for their Identification

3.4.1 Identification of Fungal Isolates

The pure cultures of fungal isolates were maintained, its colony morphology was observed and lactophenol cotton blue staining was performed. The colony morphology of ISL1 and ISL 3 was same on the MRBA plates and the LPCB staining of this fungal cultures confirmed that though these two fungi were isolated from two different medicinal plants (i.e., from *Ocimum sanctum* and *Hibiscus rosa-sinensis)* was identified as *Aspergillus flavus*. Where as the other fungi which showed a black background and white mycelium was isolated from *P. amboinicus* and *O. sanctum* hence it was maintained as only one pure culture. On LPCB staining it was confirmed that isolate ISL2 is *Alternaria* sp.

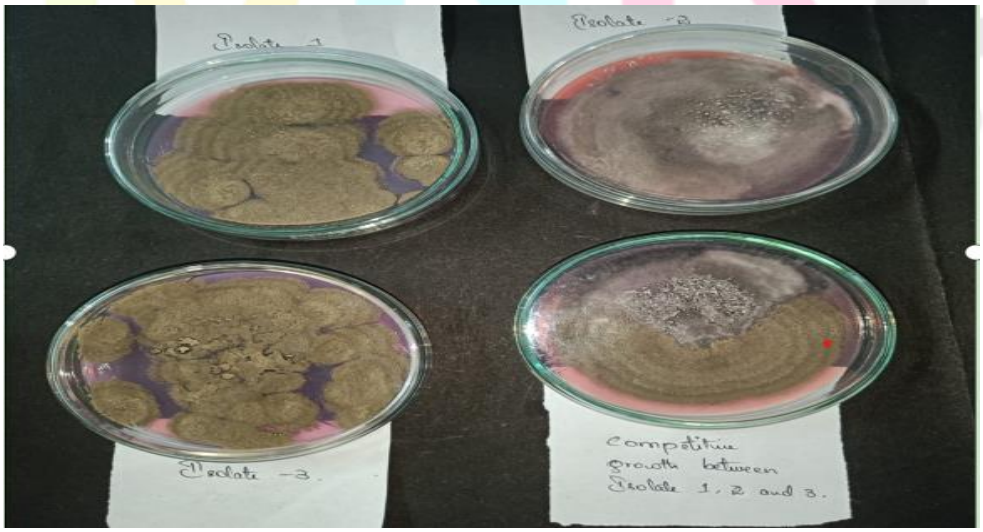

Figure. 5. Pure Cultures of Fungal isolates

A). ISL1, B). ISL2, C). ISL3 and D). Competitive growth between the ISL1 and ISL2

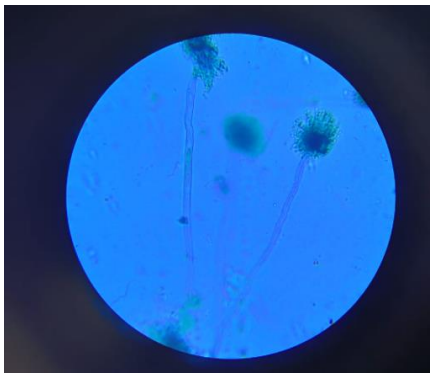

Figure 5.1. Microscopic Observation of ISL1 and ISL3.

ISL1 and ISL3 are found to be same fungi and were identified as *A. flavus.* This isolate displayed a comparitively higher inhibitory action against all the test organisms.

3.4.2 Characterization of Bacterial Isolates

The bacterial isolates ISL4, ISL5, ISL6 and ISL7 were maintained as pure cultures and gram staining was performed. The microscopic observation revealed the shape and gram character of the isolates. The microscopic observations are given below.

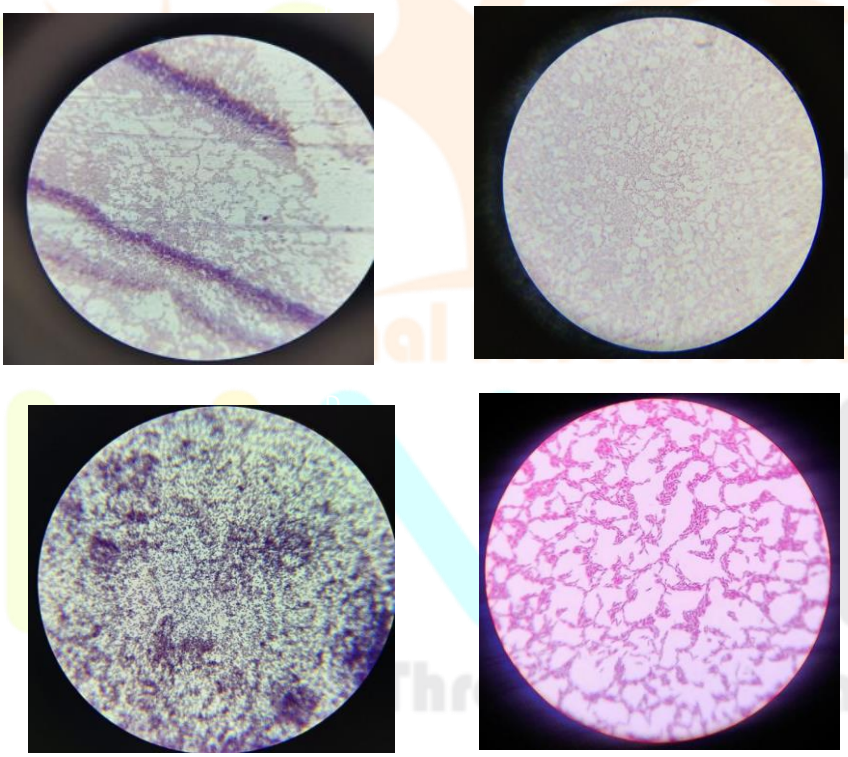

Figure 6. Gram staining [A). ISL 4, B). ISL 5, C). ISL 6 and D). ISL 7.

Table 5. Phenotypic Characterization (Colony Morphology)

Colony Morphology	ISL4	ISL5	ISL6	ISL7
Colour	Creamish	White	Bright yellow - creamish	Creamish
Form	Circular	Irregular	Irregular	Rhizoidal
Margin	Entire	Undulate	Undulate	Undulate
Elevation	Raised	Flat	Flat	Convex
Opacity	Translucent	Translucent	Translucent	Translucent

Table .6. Biochemical Characterization of Bacterial Endophytes.

Biochemical test	ISL 4	ISL 5	ISL 6	ISL 7
Gram staining	+	+	+	–
Shape	cocci	Rods	Rods	Rods
Glucose	+	+	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	+
Motility	+	+	+	+
Citrate ultilization	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Indole	–	–	–	–
Methyl red	–	–	–	–
VP test	–	–	–	–

The bacterial isolates, ISL4, ISL5, ISL6 and ISL7 stained using Gram staining technique and their shapes and gram character were identified. The isolate ISL4 was found to be as gram positive cocci, where as ISL5, ISL6 were gram positive rods and ISL7 was identified as gram negative rods. All four bacterial cutures were found to be motile in nature. These were also identified to be catalase positive, oxidase positive and citrate positive. The ISL4, ISL5, ISL6 and ISL7 isolates showed negative results to IMVIC tests.

4. SUMMARY

In this study, 3 different medicinal plants samples were collected, i.e; the leaves of *Ocicum sanctum* and *Plectranthus amboinicus* and flowers of *Hibiscus rosa-sinensis* from a region of Bangalore, Karnataka. The collected leaves and flower were surface sterilized under 70% ethanol, methanol and distilled water. These plant samples were crushed to isolate the endophytes. A total of 3 fungi and 31 bacteria were isolated from these medicinal plants. The selected isolates were screened for their antimicrobial activity against human pathogens such as *Staphylococcus aureus*, *Klebsiella* sp., *Pseudomonas aeruginosa* and *Serretia* sp., by well diffusion method. Out of 3 fungal isolates, two isolates showed inhibition against all four test organisms. The bacterial isolates ISL4-ISL7 had inhibitory effects on *Serratia* sp. and *Klebsiella* sp. with different zones of inhibition. Whereas the ISL-1 and ISL-3 showed the zone of inhibition of 1.4cm wide against *Pseudomonas aeruginosa*, and had zone of about 15mm against *Staphylococcus aureus*. The bacterial isolates ISL-4, ISL-5 and ISL-6 demonstrated a very small zone of inhibition against *Pseudomonas aeruginosa*. Isolate ISL-4 and ISL-5 were able to inhibit *Staphylococcus aureus* and showed a very narrow zone of inhibition. Characterization of the isolates was done by LPCB staining for fungi and Gram staining and biochemical test for bacteria. On LPCB staining it was confirmed that isolates ISL-1 and ISL-3 (i.e., from *Ocimum sanctum* and *Hibiscus rosa-sinensis*) was identified as *Aspergillus flavus* and isolate ISL-2 is *Alternaria* sp.

5. CONCLUSION

The results obtained in this study indicates the potential of endophytic bacteria and fungi isolated from the chosen medicinal plants as sources of novel natural products with possible applications in medicine, agriculture and the pharmaceutical industry. Furthermore, it is possible that endophytic strains of *Aspergillus* species play a crucial role in the production of beneficial chemical compounds by *Plectranthus amboinicus* and contribute in the medicinal attributes of the plant, as endophytic fungi play important physiological and ecological roles in the life of their hosts. This finding also supported that the increase in the concentration of these bioactive compounds have the ability to significantly inhibit the growth of MRSA. Therefore, these results suggested that the fungal extracts and bacterial extracts can be used to significantly not only to treat the diseases caused by the above-mentioned pathogens but may also be effective against other microbes. With this we conclude that the endophytes can be explored not only to treat the resistant microbes but can also be used as biocontrol agents (the endophytes can be used as PGPR or even as Biofertilizers). Further research and latest developments in research associated with endophytic microorganisms can draw the attention of the research community toward this emerging field and possible exploitation of the available sources for their therapeutic uses in various fields, such as the agriculture, medical, pharmaceutical and food.

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