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ISOLATION, CHARACTERIZATION AND DETERMINATON OF ANTIBACTERIAL ACTIVITY OF BACTERIAL AND FUNGAL ENDOPHYTES FROM *OCIMUM SANCTUM* AND PHYTOCHEMICAL ANALYSIS

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ABSTRACT

A study was carried out to isolate endophytes from *Ocimum sanctum* (Tulsi) and check their anti-bacterial activitiy against *Escherichia coli, Bacillus megaterium, Pseudomonas aeruginosa, Serratia marsences, Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Salmonella typhi, Salmonella typhi A* and *Salmonella typhi B*. Nine endophytes were isolated including five fungal and four bacterial endophytes and they were found to inhibit the test organisms significantly. Extracellular enzyme production was also assayed and 88.8% of total isolates were found positive for cellulase, 77.7% isolates were positive for pectinase, 55.5% isolates were positive for amylase and 77.7% were positive for tyrosinase. Ethyl acetate extract from fungal isolates were subjected to phytochemical analysis and they showed positive results for presence of alkaloid and flavonoid. These results suggest that endophytes from tulsi are potential to be used as antimicrobial agents and for production of enzymes as well as secondary metabolites.

KEY WORDS: Antibacterial activity, endophytes, enzymes, secondary metabolites, *Ocimum sanctum.*

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1. INTRODUCTION

Endophytes are those microorganisms that inhabit interior of plants especially leaves, stems, roots shows no apparent harm to host[1]. Different groups of organisms such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants[2]. Endophytes are rich sources of bioactive metabolites, which have important potentials in medicine, agriculture and industries[3,4,5]. The need for new antimicrobial agents, in general, comes from the increasing rate of resistance to existing antibiotics. This problem extends beyond the clinical application of antimicrobial drugs, such as agricultural microorganisms are also known to have acquired resistance to commonly used antimicrobial chemicals. Thus there is a need to isolate and synthesize the antibiotics, therapeutic agents and agrochemicals from endophytes, which are highly effective, low toxic and have minor environmental impact. Medicinal plants provide a special environment for endophytes. *Ocimum sanctum* plants are considered as sacred plants in India and known for its antimicrobial, immunomodulatory, anti-stress, anti-inflammatory, antipyretic, anti-asthmatic, hypoglycemic, hypotensive and analgesic activities[6,7,8]. In view of this, *O. sanctum* was selected for isolation of endophytes and their functional characterization. Total nine endophytes were isolated and screened for antimicrobial and enzymatic properties. Also phytochemical analysis by use of ethyl acetate extract from fungal isolates was done.

2. MATERIALS AND METHODS

➢ ***Isolation of endophytes from Ocimum sanctum[9]:***

Isolation of endophytic fungi from *Ocimum sanctum* was carried out using the protocol described by Strobel *et al* with slight modifications. The leaves and stem were washed under running tap water for 5-10 minutes, and the leaves and stem were cut into small pieces. Surface sterilization was carried out with 1.0% sodium hypochlorite (NaOCl) (v/v) for 2 minute followed by treatment with 90% ethanol for 30 seconds. After this the plant materials were further cleaned by passing through two sets of sterile distilled water to remove the trace amount of disinfectant used for surface sterilization process. The treated stem portions were peeled off to remove bark and also the terminal portions were cut to reduce the chances of contamination. To some portions of stem a longitudinal cut was also made. For isolation of fungal endophytes sterile leaf and stem sections were placed on plate containing Potato Dextrose Agar (PDA) media with 200 mg/L concentration of streptomycin to suppress bacterial contamination. While for isolation of bacterial endophytes sterile leaf and stem sections were placed on plate containing Nutrient Agar (NA) medium supplemented with 200 mg/L concentration of bevistin to suppress fungal contamination. The NA plates were then incubated at 37°C till bacterial growth was observed from sample. The PDA plates were wrapped with parafilm and then incubated at 28 - 30 °C till fungal mycelia starts growing from the sample. The isolates obtained were transferred on agar slants having NA and PDA media for preservation of bacterial and fungal isolates respectively and were stored at 4 °C for further studies.

➢ ***Test organisms***

The test organisms *Escherichia coli* (MTCC No. 4315), *Pseudomonas aeruginosa* (MTCC No. 7454), *Staphylococcus aureus* (MTCC No. 3160), *Bacillus subtilis* (MTCC No. 441), *Bacillus megatarium* (MTCC No. 2444), *Mycobacterium luteus* (MTCC No. 2987), *Serratia marsences* (MTCC No. 8569), *Salmonella typhii* (MTCC No. 3231), *Salmonella paratyphii A* (MTCC No. 735), *Salmonella paratyphii B* (MTCC No. 733), were activated in Muller Hinton broth upto a turbidity matching 0.5 Mc Farland standard for antibacterial activity.

➢ ***Antibacterial activity of bacterial Endophytes against the test organisms:***[10]

Fermentation

The bacterial test isolate was inoculated in 20 mL nutrient broth in 100 mL flask. The flask was then incubated at 37 °C in rotatory shaker at 120 rpm for 10 days.

Extract preparation

The fermented broth was then treated to separate the biomass from broth. The broth was then centrifuged at 4000 rpm on orbital shaker for 15 minutes, and then subjected to extraction with ethyl acetate by solvent extraction procedure. Equal volume of ethyl acetate was added to the filterate and mixed well by vigorous shaking for 10 minutes. Tubes were allowed to settle for 5 minutes till two clear immiscible layers are formed. The upper layer containing the extracted compounds was separated and collected in another tube. This filterate extract was evaporated to dryness in hot air oven. The extract residue was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C to be used as stock solution for antimicrobial assay.

Antibacterial assay

Sterile cotton was dipped in activated culture and the test organism was spread over the nutrient agar plate by swabbing. 20 µL of concentrated extract was impregnated in an individual paper disc and the solvent was allowed to evaporate. After sometime the prepared disc was placed over the inoculated plates and then incubated in refrigerator for 1 h. for proper diffusion of the extract. After this the plates were incubated in incubator at 37 °C for 24 h.

➢ ***Antibacterial activity of Fungal Endophytes against the test organisms:***[11]

Sterile cotton was dipped in activated culture and the test organism was spread over the nutrient agar plate by swabbing. 9mm of actively growing fungal culture disc from PDA were cut using a sterile cork borer and placed over the plate seeded with test organism. The plates are sealed with parafilm and then kept in refrigerator at 4 °C for 2 h for diffusion of antimicrobial compounds. Then the plates are incubated at room temperature for 24 h. After incubation the zone of inhibiton was investigated.

➢ ***Qualitative detection of enzyme for bacterial and fungal Endophytes:***[12,13]

For cellulase, pectinase, lipase, and amylase activity the test bacterial isolates were spot inoculated, while 5mm mycelia plugs of fungal isolates were inoculated at the centre of carboxy methyl cellulose agar plate, pectin enriched agar plate, starch agar plate and tributyrin agar plate respectively. After incubation for cellulase activity determination the plates were flooded with congo red for 15 minutes, then destained with sodium chloride salt solution for 10 minutes. For pectinase and amylase activity the plates were flooded with iodine solution.

➢ ***Qualitative phytochemical analysis:***[14,15]

Fermentation and extraction**:** Two or three pieces of the grown culture was cut from the culture plate and inoculated in 250 mL of Erlenmeyer flask containing 100 mL of potato dextrose broth for 21 days at room temperature under stationary conditions with intermittent shaking. The broth culture was filtered to separate the mycelia and filtrate. To the filtrate equal volume of ethyl acetate was added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelium was grinded properly in a pestle and mortar using ethyl acetate as solvent and then it was filtered using cheese cloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in hot air oven. The extract residue was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C for further analysis. This extract was checked for the presence of secondary metabolites such as Alkaloids, Flavonoids, Cadiac glycosides, Tannins.

➢ ***Phytochemical test:***[16,17]

1) Alkaloids

The fungal crude extract was evaporated to dryness in a boiling water bath. The residue filterate was dissolved in 2N HCl. The mixture was filtered and the to the filterate equal amount of Dragondroffs reagent was added. The orange precipitates indicated the presence of alkaloids.

2) Flavonoids

In a test tube containing 0.5 mL of crude extract, 5-10 drops of diluted HCl and small piece of zinc dust was added and solution was boiled for few minutes. In the presence of flavonoids, reddish pink or dirty brown color was produced.

3) Cardiac glycosides

The crude fungal extract was treated with 1mL $FeCl_3$ reagent (mixture of 1 volume of 5% $FeCl_3$ solution and 99 volumes of glacial acetic acid). To this solution add few drops of concentrated H_2SO_4. Appearance of greenish blue color within a few minutes indicated the presence of cardiac glycosides.

4) Terpenoids

In a test tube containing 5 mL of fungal crude extract, add 2 mL chloroform, 3 mL of concentrated H_2SO_4 and allow it to form a layer. A reddish brown precipitate at interface indicated presence of terpenoids.

3. RESULTS AND DISCUSSION

➢ ***Isolated fungal Endophytes*** ***Isolated bacterial Endophytes***

Total nine Endophytes were isolated including five fungal endophytes (F BTL 1, F GTS 2, F GTL3, FGTL4, F GTS 5) and four bacterial endophytes (GTL 1, BTS 2, GTL 3, BTS 4).

➢ ***Antibacterial activity of bacterial endophytes against the test organisms***

The antibacterial activity of isolated bacterial endophytes was tested against representatives of Gram negative and Gram positive bacteria by disc diffusion method and zone of inhibition were investigated.

Table 1
Antibacterial activity of bacterial endophytes against test organisms

Test microorganism	Zone of inhibition the by test organisms (mm)			
	GTL 1	BTS 2	GTL 3	BTS 4
Escherichia coli	-	-	-	-
Salmonella typhi	-	2	3	2
Salmonella paratyphi A	-	3	-	-
Salmonella paratyphi B	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Bacillus subtilis	4	3	2	2
Bacillus megaterium	3	-	4	-
Staphylococcus aureus	2	5	8	-
Micrococcus leuteus	-	2	3	-
Serratia marcescens	-	-	-	-

Abbreviations: G-green, T-tulsi, L-leaves, B-black, S-stem

Results of antibacterial activity of isolated Endophytic bacteria against test organisms

Figure-1
Petri plates showing zone of inhibition by bacterial Endophytes

Abbreviations
S. t. = Salmonella typhi, B. m.=Bacillus megaterium,
S. a.=Staphylococcus aureus, B. s.=Bacillus subtilis, c = control

Bacterial endophytes inhibited six out of ten test microorganisms. Among these test organisms *Salmonella typhi* which is a typhoid causing bacteria was significantly inhibited by three bacterial isolates (BTS 2, GTL 3, BTS 4), while two bacterial isolates (BTS 2, GTL 3) significantly inhibited *Micrococcus luteus* which is an opportunistic pathogen. These bacteria can be further investigated and they might be the future antibiotic producing microorganisms.

- ***Antibacterial activity of Fungal Endophytes against the test organisms***

The antimicrobial activity of isolated endophytes was tested against ten test organism by using disc diffusion method.

Table 2
Antibacterial activity of Fungal Endophytes

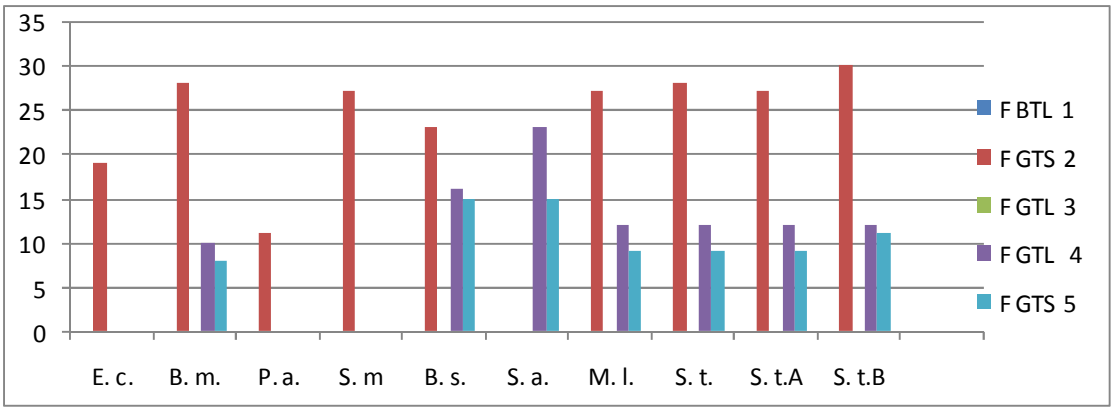
Test microorganism	Zone of inhibition the by test organisms (mm)				
	F BTL 1	F GTS 2	F GTL3	FGTL4	F GTS 5
Escherichia coli	-	-	-	-	-
Salmonella typhi	-	28	-	12	9
Salmonella paratyphi A	-	27	-	12	9
Salmonella paratyphi B	-	30	-	12	11
Pseudomonas aeruginosa	-	11	-	-	-
Bacillus subtilis	-	23	-	16	15
Bacillus megaterium	-	28	-	10	8
Staphylococcus aureus	-	-	-	23	15
Micrococcus leuteus	-	27	-	12	9
Serratia marcescens	-	27	-	-	-

Abbreviations: F- fungal isolate, B-black , T-tulsi, L-leaves, S-stem, G-green

Results of antibacterial activity of isolated Endophytic fungi against test organisms

Figure 2
Petri plates showing zone of inhibition by fungal Endophytes

Graph 1
Graphical representation of zone of inhibition (mm) by test organisms



Three fungal isolates (F GTS 2, F GTL4, F GTS 5) significantly inhibited *Salmonella typhi, Salmonella typhi A*, *Salmonella typhi B* which are representative of typhoid causing bacteria. One fungal isolate (F GTS 2**)** significantly inhibited *Pseudomonas aeruginosa,* a common bacterium that causes disease in animals, including humans. It is the most common cause of infections of burn injuries and of the outer ear. Three fungal isolates (F GTS 2, F GTL4, FGTS 5) significantly inhibited *Micrococcus luteus*. These results indicate that the isolated fungal endophytes posses antimicrobial activity against pathogenic microorganisms hence they need to be further investigated for commercial application.

- ***Enzyme assay for bacterial and fungal Endophytes***

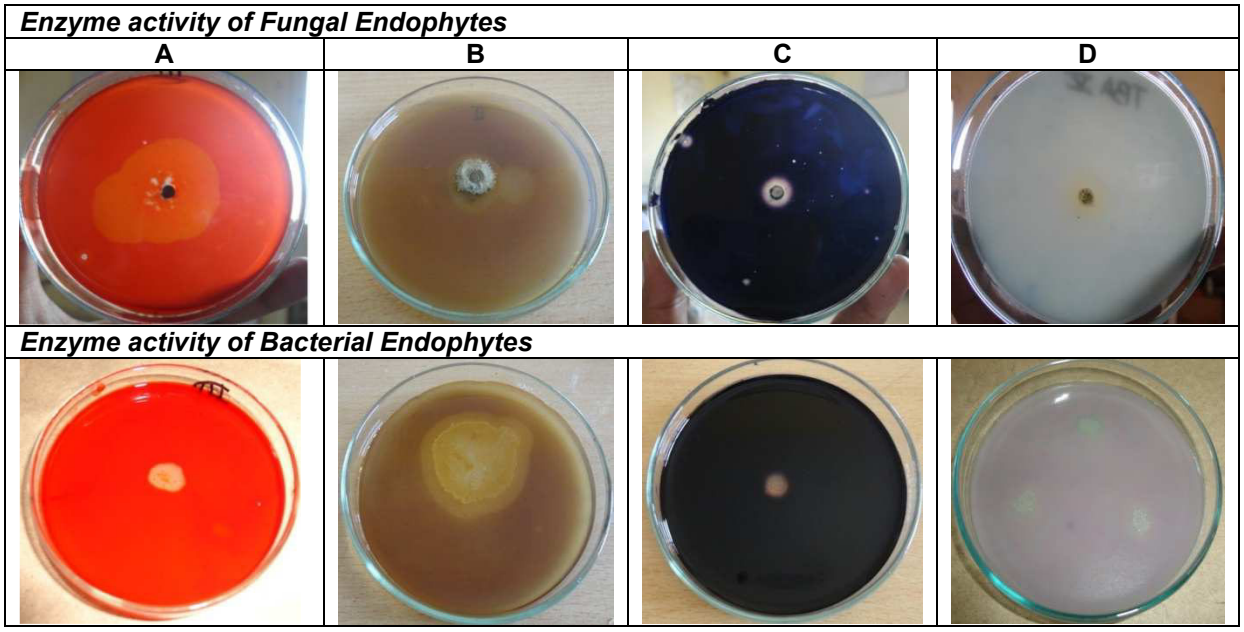
Table 3
Results of qualitative detection of enzyme activity

Test Enzyme	GTL 1	BTS 2	GTL 3	BTS 4	F BTL1	F GTS2	F GTL3	F GTL4	F GTS 5
Cellulase activity	+	+	+	+	+	-	+	+	+
Pectinase activity	+	+	+	+	-	+	-	+	+
Amylase activity	+	+	+	+	+	-	-	-	-
Lipase activity	-	+	+	+	+	+	+	-	+

All the nine endophytes isolated were tested for enzyme assay and 88.8% of total isolates were found positive for cellulase, 77.7% isolates were positive for pectinase, 55.5% isolates were positive for

amylase and 77.7% were positive for tyrosinase. All these enzymes have wide range of industrial applications. Therefore the isolates can be further exploited for large scale production of these enzymes.

Figure 3
Enzyme activity of isolated endophytes



Representative petri plates showing
A; Clear zone indicating degradation of cellulose by cellulase enzyme
B; Clear zone indicating degradation of pectin by pectinase enzyme
C; Clear zone indicating degradation of starch by amylase enzyme
D; Clear zone indicating degradation of tributyrin by lipase enzyme

- ***Qualitative phytochemical analysis***

In this study phytochemical analysis revealed endophytes to be good source of secondary metabolites. The fungal isolates F GTL3 and F GTL4 were found to be positive for alkaloid test, while the fungal isolate F BTL1 and F GTL4 gave positive results for flavonoid test. None of the isolates showed presence of Terpenoids and Cardiac glycosides. However quantitative analysis is to be further carried to ascertain their biological activity.

Table 3
Phytochemical analysis

Secondary metabolite	F BTL 1	F GTS 2	F GTL3	F GTL4	F GTS 5
Alkaloids	-	-	+	+	-
Terpenoids	-	-	-	-	-
Cadiac glycosides	-	-	-	-	-
Flavonoids	+	-	-	+	-

4. CONCLUSION AND FUTURE PERSPECTIVE

The present study revealed the endophytes to begood source of antimicrobial agents, enzymes and secondary metabolites. However there is a need of further in depth studies of these isolated

endophytes. Further growing them on large scale, modifying culture conditions and supplying some stimulants might help in getting better production of particular bioactive compound and enzyme.

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6. REFERENCES

1. Hai-Yan Li, Da-Qiao Wei, Mi Shen, Zuo-Ping Zhou. Endophytes and their role in phytoremediation. Fungal Diversity 54:11–18 (2012).
2. Raghukumar C Marine fungal biotechnology: an ecological perspective. Fungal Diversity 31:5–19 (2008).
3. Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. Nat Prod Rep; 18: 448-59 (2001).
4. Strobel GA. Endophytes as sources of bioactive products. Microbes and Infection; 5:535-44 (2003).
5. Maria G L, Sridhar K R and Raviraja N S. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India.Journal of Agricultural Technology 2005.
6. Bhargava KP, Singh N. Antistress activity of *Ocimum sanctum* Linn. Ind J Med Res; 73:443-51 (1981).
7. Chattopadhyay RR.Hypoglycemic effect of *O sanctum* leaf extract in normal and streptozotocin d Djameliabetic rats. Indian Journal of Experimental Biology; 31: 891–93 (1993).
8. Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN & Roy C.Screening of Indian plants for biological activity. Indian Journal of Experimental Biology; 6: 232–47 (1968).
9. Strobel G, Yang XS, Sears J, Kramer R, Sidhu RS, Hess WM.Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxuswallachiana*. Microbiol; 142: 435-440 (1996).
10. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol; 45: 493–496 (1996).
11. Zhang Yi , Mu J, Feng Y, Kang Y, Zhang J, Gu PJ, Wang Y, Ma LF and Zhu YH . Broad-Spectrum Antimicrobial Epiphytic and Endophytic fungi from Marine Organisms:Isolation, Bioassay and Taxonomy. Mar. Drug; 7: 97-12 (2009).
12. Hankin L and Anagnostakis SL. The use of solid media for detection of enzyme production by fungi. Mycologia; 67: 597-607 (1975).
13. Amirita A, Sindhu P, Swetha J, Vasanthi NS, Kannan KP. Enumeration of endophytic fungi from medicinal plants and screeening of extracellular enzymes. World J Sci Tech; 2(2): 13-19 (2012).
14. Radji M, Sumiati A, Rachmayani R and Elya B.Isolation of fungal endophytes from *Garcinia mangostana* and their antibacterial activity. African Journal of Biotechnology; 10 (1):103-07 (2011).
15. Haque A, Shawkat Hossain M, Rahman MZ, Rezaur Rahman M, Hossain S, Mosihuzzaman M., Nahar N and Khan SI.Isolation of Bioactive Secondary Metabolites from the Endophytic Fungus of *0cimum basilicum*. J. Pharm. Sci; 4(2): 127-13012 (2005).
16. Maniyar Y, Bhixavatimath P. Evaluation of the hypoglycaemic and hypolipidaemic activities of the aqueous extract of the leaves of Ixora coccinea Linn in Diabetic Rats. J Clin Diagn Res; 5(7): 1381-1384 (2011).
17. Handunnetti SM, Kumara RR, Deraniyagala SA, Ratnasooriya WD. Anti-inflammatory activity of Ixora coccinea methanolic leaf extract. Pharmacogn Res; 1(2): 80-90 (2009).
18. M. O. Malpani, P. R. Rajput. Antimicrobial study of whole extraxt, isolated ingredient, and newly synthesized analogue from leaves extract of cassia absus plant. Int J Pharm Bio Sci A; 4(2): (P) 427 – 430 (2013).