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BIOTECHNOLOGICAL APPLICATION OF *TALAROMYCES RADICUS* ASSOCIATED WITH *CUCUMIS DIPSACEUS* EHRENB. EX SPACH

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Abstract

Endophytes associated with the plants are rich source of secondary metabolites which represent a potential source of novel natural products for medicine, agricultural and industrial uses, such as antibiotics, anticancer agents, biological control agents and other bioactive compounds. The aim of the present work was to investigate the biotechnological potential of various extracts of *Talaromyces radicus* an endophyte from *Cucumis dipsaceus*. *Talaromyces radicus* was identified using colony morphology and 18S rDNA sequencing technique. Well grown endophytic fungus on potato dextrose broth was extracted sequentially with various solvents and the extracts were subjected to study their biological properties. Accordingly, primary and secondary phytochemicals, enzymes, organic acids and antimicrobial activity were evaluated. *Talaromyces radicus* showed the potential to produce phytochemicals like Flavonoids, Alkaloids, Phenols, Saponins and Tannins, Organic acids and Extracellular enzymes like Amylase, Cellulase, Protease and Lipase. Methanolic extract exerts potent antimicrobial activity against test pathogens followed by ethylacetate.

***Key words*:** *Endophytes, Cucumis dipsaceus*, *Talaromyces radicus, Antimicrobial activity.*

Introduction

Increase in population and thereby the diseases worldwide made it necessary to search new potential sources of natural bioactive compounds for different pharmaceutical, agricultural and industrial applications. These bioactive agents should be renewable, green-friendly and easily accessible (Liu *et al*., 2001). Searching for new drugs led to discovery of many natural and potent sources of novel bioactive molecules. Naturally derived products are chemical substances obtained from living organisms. Plants, animals, marine macro-organisms (sponge, corals and algae) and microorganisms (bacteria, actinomycetes, and fungi) act as excellent producers of natural products. Each natural product are adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes. Endophytes inhabit such a biotope (Schulz *et al*., 2002).

Endophytes are microorganisms (mostly fungi and bacteria) that exist in plants for all or part of their life cycle without causing any evident harm to their host. The importance of endophytes are well known for over a long period as potential sources of pharmaceutical leads, because many of endophytic fungi were reported to produce novel bioactive metabolites such as antimicrobial, anticancer and antiviral agents. Importance of endophytes is increased after the discovery of taxol producing fungi and which shifted natural products research to endophytic fungi (Strobel, 2003).

Cucumis dipsaceus Ehrenb. ex Spach belongs to the family Cucurbitaceae. It is an ethanomedicinal plant of South Africa which helps to treat gonorrhoea, urinary retention and skin infections caused by fungus (Belayneh and Bussa, 2014). Because of its medicinal properties, they were subjected to endophyte studies. *Talaromyces radicus* was isolated from *C.dipsaceus* and studied for their biological activities.

Materials and methods

Isolation and identification of endophytic fungi (Hallmann *et al*., 2006)

Endophytes were isolated from *C.dipsaceus* using standard methods and identified using morphological,

microscopic and molecular techniques.

Fermentation and extraction of secondary metabolites (Haque *et al*., 2005)

Well grown fresh mycelia were separated from the culture plates and transferred in to 1000 mL Erlenmeyer flask containing 300 mL of Potato dextrose broth. The flask was incubated at 28 °C for 15 days with periodical shaking at 150 rpm. After incubation, the culture along with broth was filtered to remove mycelium. The obtained filtrate was resuspended into various solvents such as methanol, chloroform, ethyl acetate, dichloromethane, acetone, benzene and petroleum ether. The organic phase collected from each extraction process was evaporated to dryness and the dried extracts were re dissolved in same solvents and stored at cold condition for further use.

Preliminary qualitative screening of phytochemicals

Various solvents extracts of *T. radicus* were checked for the presence of Alkaloid, Phenols, Flavonoids, Saponins, Steriods and Tannins by using standard procedures (Devi *et al*., 2012).

Alkaloids

The solvent extracts were dried using boiling water bath and the residue was dissolved in 2 N HCl and the filtrate was obtained. The filtrate was divided into 3 equal portions. Each portion was treated with a few drops of respective reagents like Mayers reagent, Dragondroffs reagent and Wagners reagent. The results were represented as creamish precipitate, orange precipitate and brown precipitate respectively, which indicated the presence of alkaloids.

Flavonoids

To 0.5 mL of solvent extracts, few drops of diluted HCL and a pinch of magnesium were added and boiled for few minutes. Appearance of reddish pink colour indicates the presence of flavonoids.

Phenols

The solvent extracts were dissolved in 5 mL of distilled water and few drops of neutral 5% ferric chloride solution were added. In the presence of phenols, dark green color was produced.

Tannins

Few drops of alcoholic $FeCl_3$ was added to solvent extracts, the extracts appear as bluish black colour. The colour disappears by adding diluted H_2SO_4. Formation of yellowish brown precipitate indicates the presence of tannins.

Saponins

The solvent extracts were lyophilized. The lyophilized extract was vigorously shaken with distilled water and allowed to stand for 10 min. Presence of saponin was indicated by the formation of stable froth.

Steroids

The solvent extracts were treated with 1 ml of chloroform solution followed by acetic anhydride. After few minutes, few drops of concentrated H_2SO_4 were added. Appearance of blue green ring indicated the presence of steroids.

Screening for enzymes (Amirita *et al*., 2012; Patil *et al*., 2015)

Plate based assay were conducted to investigate the production of extracellular enzyme by the endophytic fungi. Respective medium was prepared for each enzyme test and mycelial plug were inoculated and incubated for 3-5 days at 37?C. The diameter of the clear zone indicates the amount of enzyme production.

Amylase

For qualitative estimation of Amylase activity, Glucose Yeast Extract Peptone Agar (GYP) medium (glucose-1g, yeast extract -0.1g, peptone- 0.5g, agar -16g, distilled water-1L) with 0.2% soluble starch at pH 6.0 was used. Mycelia of *T.radicus* was inoculated and incubated for 24 to 48 hours. After incubation, 1% iodine in 2% potassium iodide was flooded in the plate. A clear zone around the colony indicates the presence of amylase activity. In quantitative method, *T.radicus* were grown on Czapeks dox broth, mycelium and the filtrate were separated. 1 ml of enzyme broth (filtrate) and 0.5 ml of 1% starch was incubated for 30 minutes at 37°C. After 30 minutes, the reaction was stopped by using Dinitrosalicylic acid as color reagent. The mixture was diluted with 5ml of distilled water. The reddish brown color was observed and measured spectrometrically at 540 nm. Amount of amylase produced were read off from a standard curve by recording the absorbance of different concentration of maltose (standard).

Cellulase

Glucose Yeast Extract Peptone Agar medium with 0.5% Carboxy-methylcellulose was used. After 5 days of mycelia growth, the plates were flooded with 0.2% aqueous Congo red solution followed by destaining with 1M NaCl for 15minutes. Turning of medium to red colour indicated cellulase activity. For quantitative estimation, *T.radicus* was grown in Czapek's Dox broth for 4 days. 10ml of culture filtrate was taken from the flasks after 24, 48, 72 and 96 hours. To the 1ml of culture filtrate, 1ml

of 0.5M citrate acetate buffer (pH 5) and 2.5 ml of 1% carboxymethylcellulose (CMC) was added and incubated for 1 hr. at 37°C. This reaction mixture served as sample for determining the presence of glucose by DNS (Dinitrosalicylic acid) method. One unit of cellulose was defined as 1.0mg of glucose released from 1% CMC after 1 hour at 37°C and pH 5.

Protease

For qualitative estimation, Glucose Yeast Extract Peptone Agar medium supplemented with 0.4% gelatin (pH 6.0) was used. 8% of gelatin solution was prepared using water and sterilized separately. 5ml of gelatin solution was added to very 100ml of medium. Mycelia of *T.radicus* was inoculated and incubated. A clear zone around the colony was formed due to the degradation of gelatin. Saturated aqueous ammonium sulphate solution was flooded in the plate, which forms the precipitate makes the agar opaque and improved the clear zone around the fungal colony. For Quantitative assay, degradation of casein was used to measure protease activity, to the 1ml of culture filtrate, 1ml of 1% (w/v) casein (pH-7.5) was added and incubated for 1hour at 45°C. 3ml of 0.5M Trichloroacetic acid (TCA) was added to stop the reaction and centrifuge at 5000 rpm for 30 minutes and absorbance of reaction mixture was measured at 275nm. Liberation of 1µg of tyrosine standard indicates one unit of protease enzyme activity.

Lipase (Lanka and Trinkle, 2017)

Peptone Agar medium (peptone 10g, NaCl 5g, CaCl 2H O-0.1g, agar- 16g, distilled water-1L; pH 6.0) was used for qualitative estimation of lipase activity. Tween 20 was separately sterilized and 1% was added to the medium. Mycelia of *T. radicus* were inoculated. After the incubation period, precipitate was formed around the colony which indicates the presence of lipase activity. For quantitative estimation of lipase activity, olive oil was used as a substrate, about 5ml of olive oil was added to 20ml of 0.1M phosphate buffer and incubated at 37ºC for 10 min. 1ml of culture filtrate was added to oil mixture and incubated at 40 ºC for 30 min at 120 rpm. This reaction was stopped by the addition of 15 ml of acetone-ethanol (1:1). The reaction mixture was titrated against 0.05 N NaOH along with few drops of phenolphthalein indicator. During titration free fatty acids were released. One unit of lipase activity was defined as the amount of enzyme which produces 1µmol of fatty acids per minute under assay conditions.

Laccase (Sandhu and Arora, 1984)

Qualitative assay was done using Glucose Yeast extract peptone agar (GYP) (glucose-1g, yeast extract 0.1g, peptone 0.5g, agar 16g, distilled water 1000 mL and pH 6) medium supplemented with 0.005% of 1-napthol and mycelia was inoculated and kept for incubation. On oxidation of 1-napthol, the medium changes from clear to blue which indicates the presence of Laccase activity. For qualitative determination, guaiacol was used as the substrate. The reaction mixture consist of 4.80 ml of sodium phosphate buffer with pH 6.0 (100 mM), 0.1 ml of guaiacol (10 mM) and 0.1 ml of culture filtrate. They were incubated for 60°C for 30 min and spectrophotometer reading was measured at 470nm. One activity unit (U) was defined as the amount of enzyme oxidized 1µmol guaiacol per minute.

Screening of organic acids (Khan and Gupta, 2015)

Qualitative acid production assay

Mycelia of *T.radicus* was inoculated on Czapek-Dox broth medium containing (g/l): Sodium nitrate 2.0, Dipotassium hydrogen phosphate 1.0, Magnesium sulphate 0.5, Potasssium chloride 0.5, Ferrous sulphate 0.01, sucrose 30, bromocresol purple 0.04 and incubated for five days. Formation of yellow coloration around the colony indicates the presence of acid production. Acid unitage (AU) value of the colonies were determined as follows,

$$AU = \frac{\text{Diameter of the yellow zone}}{\text{Diameter of the colony}}$$

Quantitative acid production assay

10ml of culture filtrate was taken and titrated against 0.1N NaOH (standard alkaline solution) using phenolphthalein as indicator and strength of acid production was calculated in terms of molarity (M).

Antimicrobial assay (Garcia *et al*., 2012)

Antimicrobial activity of Ethyl acetate and methanolic extract of *T.radicus* was determined against *Stapylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* by agar well diffusion method. The cultures were procured from PSG College of Pharmacy, Coimbatore, Tamilnadu. To evaluate the antibacterial activity, cultures were grown in Nutrient broth for 24h and spread on petridishes containing Nutrient agar medium. The culture extracts were prepared at different concentrations like 20µg, 40 µg, 60 µg, 80 µg and 100 µg. Chloramphenicol (40 µg) was used as positive control and DMSO was used as negative control. The petri dishes were incubated at 37°C in BOD for 24h. Formation of inhibition halos indicates the antibacterial activity.

Antifungal activity of ethyl acetate extract was checked by agar well diffusion method against

Fig.1: a) Pure culture of *T.radicus* b)Reverse position of pure culture plate c) Microscopic picture of *T.radicus*

Aspergillus flavus and *A.niger*. To evaluate the antifungal activity, cultures were grown in potato dextrose broth and spread on petridishes containing Potato dextrose agar medium. Ethyl acetate and methanolic extracts of culture filtrate was prepared at various concentrations like 20µg, 40 µg, 60 µg, 80 µg and 100 µg . Flucanozole (40 µg) was used as positive control. DMSO was used as negative control. Each test was performed in triplicates.

Results

The endophytic fungus, *Talaromyces radicus* was isolated from the healthy leaves of the *C.dipsaceus* and it was identified based on its colony morphology, microscopic and molecular studies (18S rDNA). *T.radicus* colonies is grayish green in colour with narrow margins, mycelium turns white to yellow; texture velvety; sporulation is present; exudates are clear and yellow droplets are formed; reverse centre dark yellowish brown in colour. Conidiophores biverticillate with subterminal branches (Fig.1).

Molecular analysis of the fungal isolate based on ITS 18S rDNA revealed that the consensus sequences

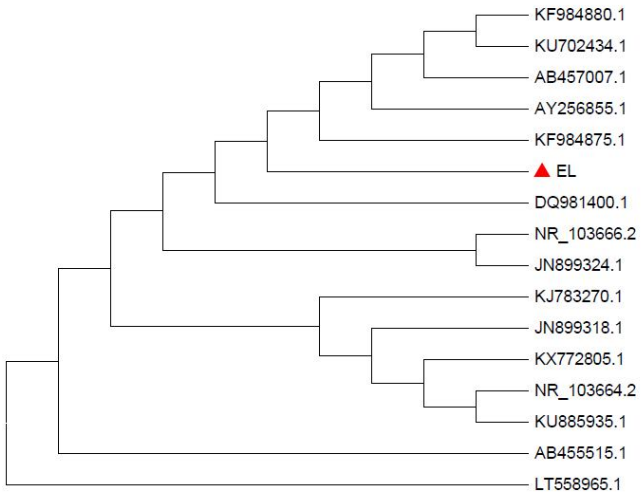


Fig.2: Phylogenetic tree view

Table 1: Sequence alignment view

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB457007.1	Talaromyces radicus genes for, strain: FKI-3765-2	1096	1096	98%	0.0	100%
KF984875.1	Talaromyces radicus strain DTO181D5	1077	1077	96%	0.0	100%
NR_103666.2	Talaromyces radicus CBS 100489	1075	1075	99%	0.0	99%
JN899324.1	Talaromyces radicus strain CBS 100489	1075	1075	99%	0.0	99%
KF984880.1	Talaromyces radicus strain DTO181D4	1070	1070	96%	0.0	99%
KU702434.1	Talaromyces sp. isolate TLT73	1057	1057	95%	0.0	99%
AY256855.1	Talaromyces radicus isolate FRR 3761	1048	1048	93%	0.0	100%
AB455515.1	Penicillium sp. FKI-3389	1037	1037	100%	0.0	98%
DQ981400.1	Talaromyces radicus strain AZ-5	1037	1037	95%	0.0	99%
NR_103664.2	Talaromyces islandicus CBS 338.48	1033	1033	99%	0.0	98%
JN899318.1	Talaromyces islandicus strain CBS 338.48	1033	1033	99%	0.0	98%
KX772805.1	Talaromyces islandicus strain ANF36	1029	1029	99%	0.0	98%
LT558965.1	Talaromyces sp. strain DI16-143	1029	1029	99%	0.0	98%
KU885935.1	Talaromyces islandicus strain EN-501	1024	1024	100%	0.0	97%
KJ783270.1	Talaromyces islandicus strain CICC 4034	1024	1024	99%	0.0	97%

Table 2: Phytochemical screening of various extracts of *T. radicus*

Phytochemicals	Chloroform	Dichloromethane	Acetone	Ethylacetate	Methanol
Alkaloids	++	+	++	+++	+++
Phenols	++	+	++	+++	+++
Flavonoids	++	+	++	+++	+++
Tannins	++	++	++	++	+
Steroids	++	++	++	+++	++
Saponins	++	++	++	++	+++

+++- Highly present; ++- Mild; +- Slightly present

showed a 100% match with *T.radicus* (Table 1 and Fig. 2).

Biochemical analysis

Various solvent extracts of *T.radicus* was screened for the presence of phytochemicals which showed that the ethyl acetate and methanolic extract contains alkaloids, phenols, flavonoids, tannins, steroids and saponins in high amounts (Table-2).

Screening of *T.radicus* for extracellular enzyme production

T.radicus has the ability to produce the extracellular enzymes (Fig.3) like Amylase, Cellulase, Lipase and protease with the exemption of laccase. The incubation period influenced enzyme production and varied from 3 to 7 days. The zones formed around the colonies were measured (Table 3). Quantitative enzyme activity was measured using liquid fermentation method, as Unit/ml (Table 4).

Screening of organic acids

T. radicus exhibited acid production on modified mineral salt medium (Fig. 4), which is visible as yellow halo produced around the colonies. Acid unitage value of *T. radicus* was recorded as 4.37±0.1 and for quantitative estimation, acid production value was observed as 0.47±0.2M.

Table 3: Qualitative enzyme activity of *T. radicus*

Enzymes	Diameter of enzyme activity (cm)
Amylase	#2.37 ± 0.15
Cellulase	2.87± 0.15
Lipase	1.57±0.18
protease	2.67 ± 0.19
laccase	0

#- Mean ±S.E

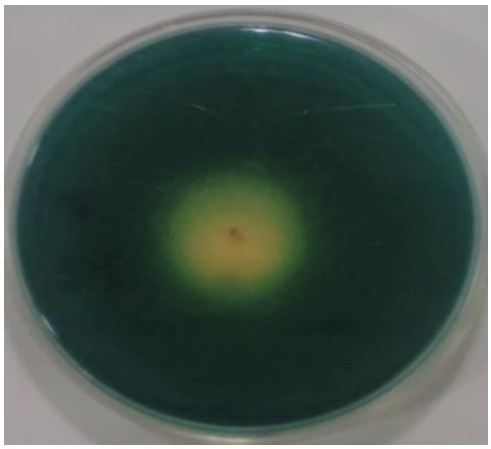


Fig. 4: Production of organic acid by *T. radicus* on modified mineral salt medium

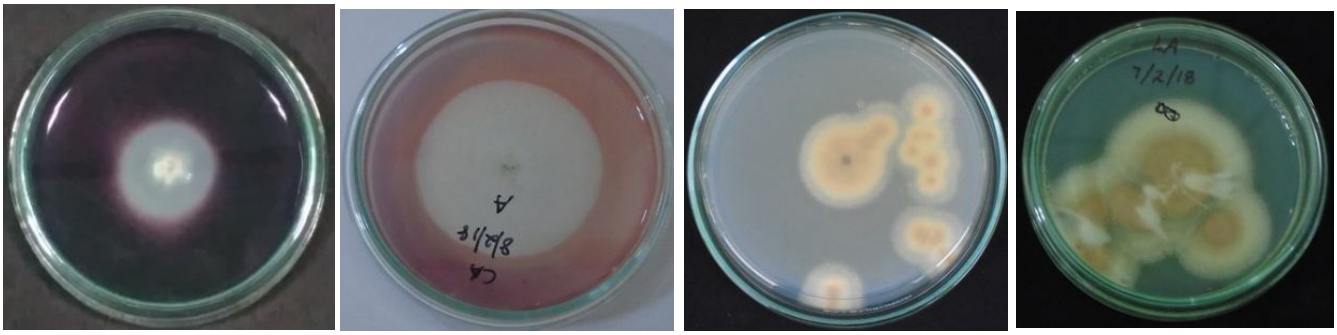
Table 4: Quantitative enzyme activity of *T. radicus*

Enzymes	Unit of activity (ml^{-1})
Amylase	#7.59±0.47
Cellulase	6.37±0.35
Lipase	4.63±0.18
protease	10.13±0.24
laccase	0

#- Mean ±S.E

Antimicrobial activity

Among the different solvent extracts, ethyl acetate and methanolic extract showed high phytochemical contents. Therefore these extracts were screened for their antimicrobial potential (Fig. 5). The methanolic



Amylase **Cellulase** **Protease** **Lipase**

Fig 3: Extarcellular enzyme activity in different solid media

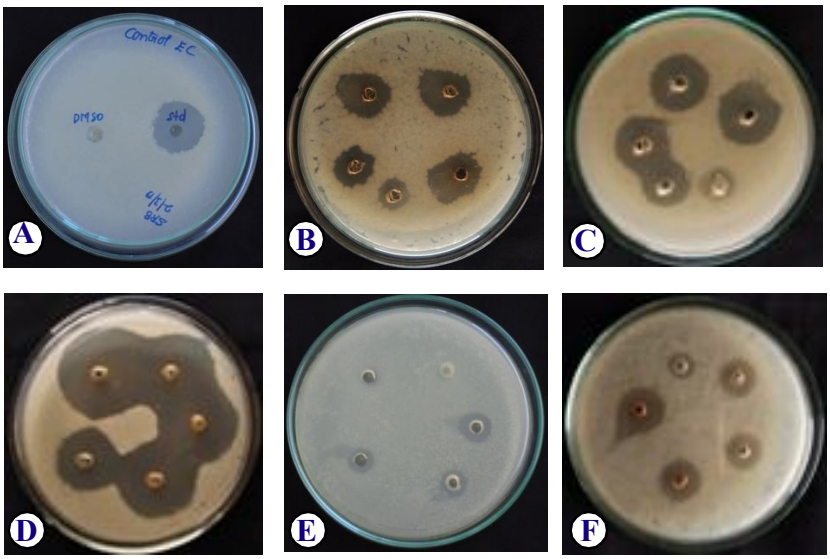
Table 5: Antimicrobial activity of *T.radicus*

Talaromyces radicus	Concentration	Zone of inhibition (mm)					
		Bacterial pathogens				Fungal pathogens	
		S. aureus	*B. subtilis*	*E. coli*	*K.pneumoniae*	*A. niger*	*A.flavus*
Methanolic extract (µg/ml)	20	4.67± 0.6	4.67±0.3	5.33±0.3	-	17.0±0.5	6.0±0.5
	40	7.0± 0.5	8.0±0.5	8.0±0.5	-	21.0±0.5	9.0±0.5
	60	11.0±0.5	11.6±1.2	11.0±0.5	3.67±0.3	26.0± 0.5	10.67±0.3
	80	15.0± 0.5	11.6± 0.8	15.3± 0.3	6.0± 0.5	29.0±0.5	13.0±0.5
	100	17.0±0.5	15.3±0.3	18.0±0.5	6.3±0.3	31.0± 0.5	16.0± 0.5
Ethyl acetate extract (µg/ml)	20	-	-	5.0± 0.5	-	-	-
	40	-	-	7.0±0.5	-	-	-
	60	-	-	7.67±0.6	-	-	-
	80	-	5.0±0.5	10.0± 0.5	-	-	-
	100	-	6.0± 0.5	11.0±0.5	-	-	-
Chloramphenicol (µg/ml)	40	21.0 ± 2.08	17.3±0.8	23.0±0.5	11.0±0.5	-	-
Flucanozole (µg/ml)	40	-	-	-	-	8.0±0.5	5.33± 0.3
DMSO (µl)	40	-	-	-	-	-	-

extract of *T.radicus* showed significant antibacterial activity against *E.coli*, *S. aureus*, *B. subtilis* and *K. pneumoniae*. *E. coli* (18mm) and *S. aureus* (17mm) showed highest antibacterial activity at 100µg/ml followed by *B.subtilis* (15mm).

In ethyl acetate extract, *E.coli* (11mm) showed highest antibacterial activity followed by *B.subtilis* (6mm). *T.radicus* does not show any significant effect on *S. aureus* and *K. pneumonia*. Compared to ethyl acetate extract, methanolic extract showed high potential towards the test pathogens but, both extracts were less significant when compared to the standard (Chloramphenicol) (Table 5).

Methanolic extract of *T.radicus* showed high potential against *A. flavus* and *A.niger* (Fig. 6). Inhibition of test pathogens increased with increase in concentration. But ethyl acetate extract does not show any activity against the test pathogens. Methanolic extracts showed significant antifungal activity when compared to standard (Flucanozole).

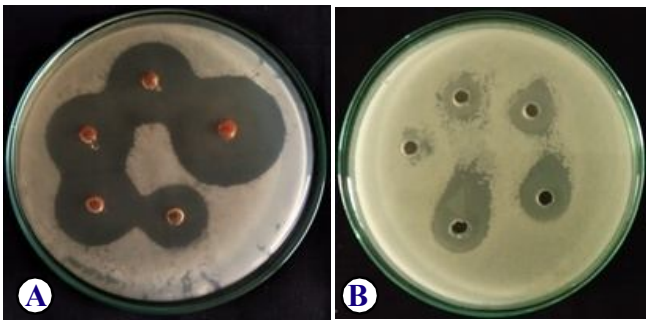


A- Inhibition of pathogen by standard; B-D Methanolic extract against *S.aureus*, *B.subtilis* and *E.coli* ; E and F, Ethyl acetate extract against *B.subtilis* and *E.coli*

Fig.5: Antimicrobial activity of *T.radicus* against bacterial pathogens

Discussion

Endophytes are the group of microorganisms which produce greater number of biologically derived molecules, with various biological activities. Our rationale for studying endophytic microbes is, there are many unexplored area of biochemical diversity from endophytes which leads to the production of new medicinals (Stroble, 2003). *Cucumis dipsaceus* vegetative parts help to treat gonorrhoea, urinary retention and skin infections caused by fungus (Belayneh and Bussa, 2014). The endophytic fungus isolated from the plant *C.dipsaceus* has



A and B-Methanolic extract against *A.flavus* and *A.niger*

Fig.6: Antimicrobial activity of *T.radicus* against fungal pathogens

been recognized as *Talaromyces radicus* based on microscopic and molecular studies. There are previous reports of *T. radicus* isolated as endophytic fungus from *Catharanthus roseus* (Eo *et al*., 2014; Palem *et al*., 2015).

In the present study, biotechnological application of *T. radicus* isolated from *C.dipsaceus* was studied. The presence of phytochemicals is an indicator that they can be exploited as precursors in the development and advancement of synthetic drugs (Jack and Okorosaye-orubite, 2008). Many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic, anticancer activities have been successfully discovered from *Talaromyces* sp.. These bioactive compounds could be classified as alkaloids, peptides, esters, polyketides, quinines, terpenoids, steroids, lignans, phenols and lactones (Zhai *et al*., 2016). Similarly the phytochemical analysis was carried out in all extracts of *T. radicus* confirms the presence of alkaloids, flavonoids, phenols, saponins, tannin and steroid.

The knowledge of enzyme production by endophytic fungi may provide in sights into their possible biotechnological applications and also provide an idea about their life cycles within the plant tissues (Lumyong *et al*., 2002). Enzymes which are not detected on plate based assays were quantified using broth cultural conditions. The enzyme production was detected in solid medium by the discharge of enzyme from the mycelium and its activity in the medium after their production. When the strain does not show its enzymatic activity, it is because that they lack enzyme production or it may be produced but not released from the mycelium or it may be produced and released but inhibited by the medium. In our studies, *T.radicus* did not show any activity for laccase enzymes in agar plate assays and also showed lesser activity for lipase enzymes. This was similar to the results of Bhagobaty and Joshi, (2012) exhibiting the non amylolytic and non laccolytic activity in endophytic fungi from medicinal plants. *Talaromyces emersonii* exhibited prominent amylase activity followed by cellulase activity (Sunitha *et al*., 2013). *Talaromyces purpureogenus* does not have the ability to produce protease. In contrast, *Talaromyces* sp. produces protease at the pH of 9 (Thirunavukkarasu *et al*., 2017).

Some fungi are well recognized to produce high amounts of various useful organic acids for their natural capability (Liaud *et al*., 2014). Fungal natural production of organic acids is thought to have many key roles in nature depending on the species of fungi producing them. Preliminary screening of organic acid production in *T.radicus* was carried out for the first time, their potential of organic acid production in solid state fermentation was high and this is similar to the results of 35 endophytic fungal strains isolated from various mangrove plants of Sao Paulo state, Brazil showed high potential to produce organic acids (Dezam *et al*., 2017).

Endophytic fungi are now recognized as a potential source of anti-microbial secondary metabolites that could be used for various medicinal purposes. Antimicrobial activity of *T.radicus* showed significant effect on different Gram positive and Gram negative bacteria and on different fungi. These endophytes can reduce the growth of the harmful bacteria or fungi by different mode of action. Our results correlated with the findings of Sette *et al*., (2006); Miao *et al*., (2012); Pretsch *et al*., (2014) which reported the antimicrobial activity of *Talaromyces* sp., *T. verruculosus* and *T. wortmanni*. *T.radicus* produces antimicrobial secondary metabolite which may have noble compounds. This endophyte may be used as source of therapeutic agents in pharmaceutical industries.

Conclusion

The endophytes are very useful to the industries by the production of enzymes and metabolites and this can also be used as biocontrol agents. To the best of our knowledge this is the first report of endophytic fungal extracellular enzyme and phytochemicals production from *T.radicus*. In the present investigation, *T.radicus* was iaolated from *Cucumis dipsaceus*. This fungal endophyte has some primary, secondary metabolites and extracellular enzymatic activities to biodegrade different polysaccharides and gelatin. In addition, this fungal strain was found to have antimicrobial activity against various coded test organisms. For biotechnological and ecological application of fungal endophytes various molecular and biochemical studies are required to investigate the role of them in plants.

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