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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF ENDOPHYTES FROM SELECTED MEDICINAL PLANTS

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Abstract

Bioactive compounds of endophytic fungi exhibit antimicrobial, antioxidant, anticancerous, antiviral and insecticidal properties. They harbor inter or intra-cellular in the plants epidermal layer. They are the most potent microorganisms in establishing interrelationship with plant without harming them or asymptomatically. They have the mechanism which enables them to establish association with the plant and produce similar bioactive compound as plant produce. This research paper focused on the isolation, production, screening and separation of exploitable bioactive compounds from the plants Tulsi and Aloe vera. Total 5 fungi were isolated from segments of the plants. And the organisms taken for the study are both Gram-positive and Gram-negative bacteria i.e. *Staphylococcus aureus*, *Streptococcus pyogens*, *Escherichia coli*, *Pseudomonas aeruginosa* respectively which were isolated from the clinical samples (like wound, blood and urine) and the results of antibacterial screening showed that (BCEF-01*), (BCEF-02*), (BCEF-03*), (BCEF-04*) and (BCEF-05*) inhibited all the test organisms. The inhibitory effect was promising to all the test organisms showing sufficient zone of inhibition with respect to all the fungal extracts.

Introduction:-

Endophytic fungi are ubiquitous fungi that reside inter or intracellular in plant parts for at least a portion of their lives without causing apparent symptoms of infection and represent a large portion of fungal species (Petrini,1991[1]). Endophyte by definition is one which in the tissue beneath the epidermal cell layers and causes no apparent harm to the host. Endophytic fungi can be biotrophic, mutualists, benign commensals, decomposers or latent pathogens. According to Rodriguez *et al.*, (2009) [2], all plants in the natural environment can shelter endophytic fungi, including algae, mosses, ferns, conifers and angiosperms. This fungal group appears to significantly influence the lifestyle of its host. Taxonomically most of the endophytic fungi belong to the phylum Ascomycota and its associated anamorphs, while some species belong to the phyla Basidiomycota and Zygomycota (Huang *et al*., 2001 and 2008[3, 4]). Dreyfuss & Chapela (1994) [5] have estimated that approximately 1.3 million species of endophytic fungi remain to be discovered. This diverse fungal group could impact the ecology, fitness and shape of plant communities, conferring resistance to abiotic (temperature, pH, osmotic pressure) and biotic (bacteria, fungi, nematodes and insects) stresses. Endophytic fungi are an important source of bioactive molecules. These bioactive metabolites have a broad range of biological activities and could be the starting materials for

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pharmaceuticals or lead structures for the development of pharmaceutical or agrochemical products. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Carroll and Carroll, 1978[6]; Azevedo *et al.*, 2000[7]; Strobel, 2003[8]). The substances produced by endophytic fungi originate from different biosynthetic pathways, including isoprenoid, polyketide and amino acid and belong to diverse structural groups, such as terpenoids, steroids, xanthones, quinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasins and enniatins (Schulz *et al.*, 2002[9]). Indeed, these bioactive molecules represent a chemical reservoir for discovering new compounds, such as antibiotic, antioxidant, immunemodulating, anticancer, antiparasitic and antidiabetic compounds, for use in the pharmaceutical and agrochemical industries.

Ocimum species belongs to the family Lamiaceae are very important for their therapeutic potentials. Tulsi leaves contain bright yellow volatile oil reported to possess the antimicrobial properties and also act as insecticide. Tulsi is a fragrant bushy perennial growing up to 1.5 m in height with profusions of white blooms and slightly purple tinted foliage. This herb has been known from as early as the Vedic period and is held sacred by the Hindus and is often planted around temples and used in rosaries. It is native to India, reached Western Europe in 16th century. Tulsi leaves are traditionally used for getting relief from common cold, bronchitis, cough, mild indigestion, diminished appetite and malaise. *Ocimum* (tulsi) are also known for its antimicrobial, immunomodulatory, anti-inflammatory, anti-pyretic, anti-asthmatic, hypoglycemic, hypotensive and analgesic activities. The only side effect reported is constipation.

Aloe vera is a perennial, drought-resisting, succulent plant belonging to the Asphodelaceae family. The name, aloe, is derived from the Arabic "alloeh" or Hebrew "halal" meaning bitter shiny substance. It has a vast traditional role in indigenous system of medicine like ayurveda, siddha, Unani and homoeopathy. *Aloe barbadensis* miller or *Aloe vera*, a semi tropical plant is one of the 250 species of Aloe. There are more than 200 compounds found in *Aloe barbadensis*, about 75 of which have biological activity, *Aloe vera* leaves contain a diverse array of compounds, including anthraquinones (e.g. aloe-emodin), anthrones and their glycosides (e.g. 10-(1, 5‟ anhydroglucosyl)-aloeemodin- 9-anthrone, also known as aloin A and B), chromones, carbohydrates, proteins, glycoproteins, amino acids, organic acids, lipids, sugars, vitamins, minerals. Various studies have revealed that *Aloe vera* leaf possesses many pharmaceutical activities, including antimicrobial, anticancer, antioxidant, antidiabetic, antiulcer, hepatoprotective, immunomodulatory and many more activities. Many of the health benefits associated with *Aloe vera* have been attributed to the polysaccharides contained in the gel of the leaves.

Research on endophytes dates back to over one hundred years. During this period, several aspects of endophyte biology were thoroughly studied, including the diversity, taxonomy, reproduction, host ecology and effects on the host. Because natural selection favors the evolution of beneficial endophytic strains, several endophytes were found to secrete secondary metabolites that protect plants against insect pests, pathogenetic organisms as well as herbivores, thus, endophytes represent a promising source of novel, biologically active metabolites for pharmacological and agricultural applications. Biochemical research revealed that a wide variety of natural products can be obtained from endophytic microbes. Natural products from endophytic fungi were observed to inhibit many pathogenetic organisms including bacteria, fungi, and viruses.

Materials and Methods:-

Sample collection:-

Healthy (showing no visual disease symptom) and mature plants were carefully chosen for sampling. Fresh plant materials (branches, leaves and roots) were collected from ten different sites at Bhoopsandra area, Banglore, India. The plant material was brought to the laboratory in sterile bags and processed within few hours after sampling. Fresh plant materials were used for the isolation work to reduce the chance of contamination.

Glassware, Chemicals and Media:-

The glassware used were made up of borosilicate glass obtained from M/s Borosil India Limited. The chemicals used were of analytical grade obtained from M/s Himedia Laboratories Pvt. Limited & M/s Sigma-Aldrich Pvt. Limited, Mumbai, India. The Media used for the experiments were obtained from M/s Himedia Laboratories Pvt. Limited, Mumbai, India.

Sample Processing:-

For Tulsi:-

Isolation of endophytic fungi from *Ocimum sanctum* was carried out by using the protocol described by strobel *et*

al., (2003) [8] with slight modification. The plant was washed under running tap water for 10 minutes to remove dust and debris. Highly sterile conditions were maintained for the isolation of endophytes. Before surface sterilization, the leaves, branches and roots were cut into small pieces of about 1cm long and sterilized in series with 70% ethanol for 1min, 1% mercuric chloride for 1 min and further cleaned by passing through two sets of sterile distilled water. All of work needs to be performed in the laminar air flow using sterile glassware and mechanical instruments. The sterile samples were placed on plate containing potato dextrose agar (PDA) media with 200mg/L concentration of streptomycin to suppress the bacterial contamination. The parafilm wrapped petridishes were incubated at 25-27°C till the mycelia start growing from the samples. The endophytic fungi was transferred into new agar slant and stored at 4°C for further studies.

For Aloe vera:-
The plant was washed under running tap water for 10 minutes to remove dust and debris. Before surface sterilization plant material was cut into small pieces of about 1cm long and sterilized in a series of 70% ethanol for 1-3 minutes, 4% mercuric chloride for 3-5 minutes, and rinse with 70% ethanol for 2-10 seconds further cleansed by passing through two sets of sterile distilled water. All of work needs to be performed in the laminar air flow using sterile glassware and mechanical instruments. The sterile samples were placed on plate containing potato dextrose agar (PDA) with 200mg/L of streptomycin to suppress the bacterial contamination. The parafilm wrapped petridishes were incubated at 25-27°C till the mycelia start growing from the samples. The endophytic fungi was transferred into new agar slant and stored at 4°C for further studies.

Media preparation:-
Potato Dextrose Agar (PDA) was used for isolation and purification of endophytic fungi. Antibiotic, streptomycin (200mg/L) was added to suppress bacterial growth. The media and antibiotics were purchased from Himedia, India.

Endophyte Fungal identification:-
The identification procedure of endophytic fungi was based on morphology. The five isolated species were described according to their macroscopic features (i.e. the color, shape and growth of cultured colonies) as well as microscopic characteristics (i.e. the structure of hyphae, conidia and conidiophores). The morphology of fungal culture colony or hyphae and the characteristics of the spore were identified by temporary mounts using lacto phenol cotton blue (LPCB) and viewed under the microscope at 40X. Obtained data were then compared with the descriptions of endophytic fungi species from standard identification manuals and matches were recorded. Analysis of the antibacterial activity was carried out on all species identified.

Preliminary antibacterial assay:-
Antibacterial activity of isolated endophytic fungi was tested based on the protocol of Zhang *et al*., (2009) [10] with slight modification. The Petridish containing respective media for growth of bacteria were prepared and the test organism was spreaded on the surface of agar with the help of sterile cotton swab. Nine millimeter diameter of actively growing fungal culture disc from PDA plates were cut using sterile cork borer and placed on the surface of respective agar media seeded with the test bacteria. The plates were sealed with parafilm and kept in refrigerator at 4°C for 6 hours for the complete diffusion of the antimicrobial compounds if any, they were incubated at room temperature for next 12 hours for all bacterial cultures. After incubation the diameter of zone of inhibition was measured in millimeter by using scale.

Secondary metabolite extraction:-
Secondary metabolite extraction was carried out by using the protocol Radji *et al*., (2011) [11]. Positive endophytic fungal isolate was inoculated into 1000 ml conical flask containing 500 ml potato dextrose broth and incubated at room temperature for 21 days under stationary condition 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 min, keep for 5min, till the two clear immiscible layers are formed. The upper layer of ethyl acetate containing extracted compound was separated using separating funnel. The mycelium was grinded properly in pestle and mortar using ethyl acetate as solvent and then it was filtered using cheese cloth. Both mycelia and culture filtrate were pooled together and evaporated to dryness using water bath. The extracted residue was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C to be used as stock solution for antibacterial assay.

Preparation of test organism:-

Endophytic fungal extracts isolated from Tulsi and Aloevera were evaluated for their antibacterial potential against both Gram-positive and Gram-negative bacteria. *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogens*, *Pseudomonas aeruginosa* were isolated from the clinical samples (like wound, blood and urine) obtained from patients attending KIIMs Hospital, K.R market, Bangalore, India and M. S. Ramaiah Memorial Hospital, New Bell road, Bangalore, India. These bacteria were identified on the basis of biochemical tests, by observation of the morphological structure microscopically and colony characters visually. The isolated organisms were stained with Gram's stain and viewed under microscope at 100X. The organisms were isolated in nutrient agar medium and selectively cultured at 37°C for 24 hrs. These bacterial cultures were maintained in nutrient agar slants at 37°C and were stored at 4°C for further study. Each of the bacteria was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

Secondary metabolite antibacterial assay:-

Antibacterial activities of secondary metabolites were tested by agar diffusion method. The bacteria used for antimicrobial assay are the pathogenic *Pseudomonas aeruginosa*, *E.coli*, *Staphylococcus aureus, Streptococcus pyogens* which were isolated from urine, blood and wound samples. Muller Hinton agar was used for the antimicrobial assay. The sterile growth media plate specific for test organism were prepared and inoculated with the test organism. Twenty microliters of crude extract was added on to sterile 6mm paper disc using a micropipette and allowed to dry. Disc containing compounds were placed on the surface of the medium. In another plate 6mm diameter wells were made using a sterile cork borer and 20 microlitres of sample was added to each well. The plates were incubated at 24-48 hours at 37°C. The diameter for zone of inhibition was measured.

Determination of Minimum inhibitory concentration [MIC]:-

Minimum inhibitory concentration was carried out using broth dilution as previously reported by Lennette *et al.*, (1974) [12]. Dilutions of different concentrations of extract and fractions that exhibited sensitivity against the test organisms were prepared using test tubes containing 9 ml of double strength broth. The test tubes were inoculated with (0.2 ml) suspension of the standardized inocula and incubated at 37ºC for 24h. MIC's were recorded as the lowest concentration of extract showing no visible growth of the broth.

TLC Screening:-

About 1 mg of each extract was used for chromatography. A solvent system of dichloromethane: ethyl acetate 7:3 was prepared and placed in a tank and the lid was replaced. The fungal extracts were spotted, each one on separate origin on the plate. Chromatographic separations were carried out using silica gel (E. Merck, type 60), pre-coated silica gel GF254. The plates were placed carefully into the tank and covered with the lid. After development, the plate was removed and the solvent front was marked with pencil and allowed to dry. TLC plates were viewed under UV light at 254 nm for fluorescence spots and at 366nm for fluorescent spots. The metabolites from all the bands obtained were extracted by centrifugation of silica containing band in ethyl acetate at 10,000 rpm for 10 minutes. The supernatant was used for the antibacterial activity against all the clinical pathogens.

Results:-

Medicinal plants have been the main source for drugs over many centuries in many countries, in both developed and developing countries. Traditional medicine products are not officially recognized in many countries, and the European Union presently developing regulatory laws for quality traditional medicines. It is estimated that at least 25% of all modern medicines are derived either directly or indirectly from medicinal plants. Traditional medicines play important role in world health treating millions of people. The medicinal property of herbs is due to the presence of different complex chemical substances as secondary metabolites, which are exclusively accumulated in different parts of the plant. The need for new antimicrobial agent, in general, comes from 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. In the present study, a total of 5 endophytic fungi (Table 1) were isolated from two medicinal plants (tulsi, aloe vera). All the isolated endophytic fungi showed preliminary antibacterial activity against the clinical pathogen. BCEF-01* showed maximum activity against *E.coli* and *Streptococcus pyogens* while BCEF-04* and BCEF-05* showed maximum activity against *Streptococcus pyogens* (Fig.1, Table 2). All the isolated endophytic fungi were cultivated in stationary condition (Fig.2) and the secondary metabolites produced by them are used for the antibacterial activity in different concentrations by agar as well as broth dilution methods. In agar dilution method, the results of antibacterial screening showed that BCEF-01*, BCEF-02*, BCEF-03*, BCEF-04* and

BCEF-05* inhibited all the test organisms. BCEF-01*, showed resistance at concentration of 2.1mg/ml against *P. aeruginosa* while other all test organisms are susceptible at various concentrations of fungal extract. *Staphylococcus aureus* shows maximum zone of inhibition of 15mm at extract concentration 2.6mg/ml (Table 3). For BCEF-02*, all the test organisms were found to be susceptible to the fungal extracts at various concentrations. The maximum zone of inhibition was found to be 17mm for *E. coli* at extract concentration of 2.0 mg/ml (Table 4). For BCEF-03*, *E. coli* was found to be resistant at concentration of 2.1 mg/ml while at rest of the concentrations all the test organisms are susceptible to the fungal extracts (Table 5). For BCEF-04*, *E. coli* is resistant at concentration 1.5mg/ml and 1.6mg/ml and *S. Pyogens* is resistant up to concentration of 1.8mg/ml while *S. aureus* and *P. aeruginosa a*re resistant till the concentration of 2.0mg/ml. Rest of the test organisms are susceptible at various concentrations (Table 6). For BCEF-05*, *Pseudomonas aeruginosa* is resistant at concentration 1.5mg/ml while at other concentrations all test organisms are susceptible to the fungal extract (Table 7). The inhibitory effect was promising to all the test organisms showing sufficient zone of inhibition with respect to all the fungal extracts. The summary for which is given in the Table 8.The secondary metabolites of the endophytic fungi were further determined for Minimum Inhibitory concentration (MIC) against all the clinical pathogens by broth dilution method. The MIC of BCEF-02*, extract for all test organisms was found to be *1.42mg/ml* (Fig.3).

For BCEF-05*, MIC was found to be 1.5mg/ml for *S. aureus* and *S. pyogens*, while for *E. coli* and *P. aeruginosa* the MIC were found to be 1.54mg/ml and 1.56mg/ml respectively (Fig.4). For BCEF-03*, MIC value obtained for *S. aureus, S. pyogens* and *P. aeruginosa* was found to be 2.02mg/ml while for that of *E. coli* MIC value obtained was 2.06mg/ml (Fig.5). For BCEF-01*, MIC value obtained for *E. coli* and *S. pyogens* was found to be 2.04mg/ml while for *S. aureus* and *P. aeruginosa* the MIC values obtained were 2.02mg/ml and 2.06mg/ml respectively (Fig.6). For BCEF-04*, MIC value for *S. aureus* and *S. pyogens* was found to be 2.02mg/ml and for that of *E.coli* and *P. aeruginosa* the MIC value obtained was 2.06mg/ml (Fig.7). Further the secondary metabolites produced by endophytic fungi were subjected to TLC and the bands obtained were used for antibacterial activity (Fig.8).

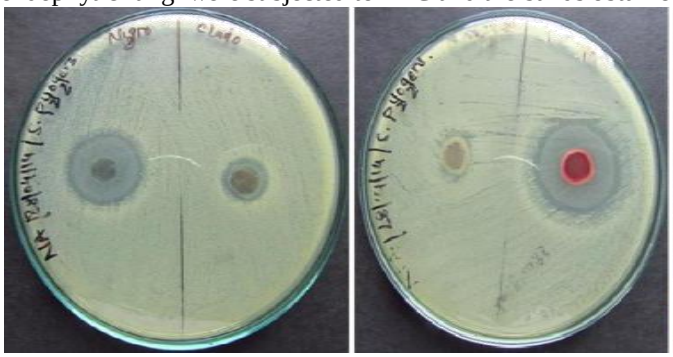

Fig.1:- Preliminary antimicrobial activity

Fig. 2:- Cultivation of endophytic fungi

Table 1:- List of endophytic fungi isolated from different parts of medicinal plants.

S. No.	Code	Plant	Plant part	Identification
1.	BCEF-01*	Tulsi	Root	*Aspergillus* sp.
2.	BCEF-02*	Tulsi	Leaf	*Penicillium* sp.
3.	BCEF-03*	Aloe vera	Root	*Cladosporium* sp.
4.	BCEF-04*	Aloe vera	Leaf	*Nigrospora* sp.
5.	BCEF-05*	Tulsi	Stem	*Gliocladium roseum*

Table 2:- Preliminary antibacterial activity of endophytic fungi.

Name of endophytic fungi	Zone of inhibition (mm)			
	E.coli	*Streptococcus pyogens*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
Penicillium sp.	10	11	10	12
Gliocladium roseum	15	**25**	12	15
Cladosporium sp.	10	20	10	10
Aspergillus sp.	**25**	**25**	15	10
Nigrospora sp.	10	**26**	11	15

Table 3:- Zone of inhibition shown by *Aspergillus* extract (BCEF-01*).

Name of endophytic fungal extract.	Concentration of extract (mg/ml)	Zone of inhibition (mm)			
		Staphylococcus aureus	*E.coli*	*Streptococcus pyogens*	*Pseudomonas aeruginosa*
Aspergillus sp.	2.1	10	9	9	-
	2.2	11	10	10	9
	2.3	12	10.1	10.1	9.5
	2.4	13	10.2	10.2	10
	2.5	14	10.3	10.3	10.1
	2.6	**15**	**10.4**	**10.4**	**10.2**

Table 4:- Zone of inhibition by *Penicillium* extract (BCEF-02*).

Name of endophytic fungal extract.	Concentration of extract (mg/ml)	Zone of inhibition (mm)			
		Staphylococcus aureus	*E.coli*	*Streptococcus pyogens*	*Pseudomonas aeruginosa*
Penicillium sp.	1.5	13	13	12	8
	1.6	13.5	15	13	8
	1.7	14	15.5	13.5	8.5
	1.8	14.5	16	14	9
	1.9	15	16.5	14.5	10
	2.0	**16**	**17**	**15**	**11**

Table 5:- Zone of inhibition by *Cladosporium* extract (BCEF-03*).

Name of endophytic fungal extract.	Concentration of extract (mg/ml)	Zone of inhibition (mm)			
		Staphylococcus aureus	*E.coli*	*Streptococcus pyogens*	*Pseudomonas aeruginosa*
Cladosporium sp.	2.1	8	-	8	8
	2.2	9	9	8.5	8.5
	2.3	9.5	9.5	9	9
	2.4	9.5	10	9.5	9.5
	2.5	10	10.5	10	10
	2.6	**10.5**	**11**	**10.5**	**10.5**

Table 6:- Zone of inhibition shown by *Nigrospora* extract (BCEF-04*).

Name of endophytic fungal extract	Concentration of extract (mg/ml)	Zone of inhibition (mm)		Concentration of extract (mg/ml)	Zone of inhibition (mm)	
		E.coli	*Streptococcus pyogens*		*S. aureus*	*P.aeruginosa*
Nigrospora sp.	1.5	-	-	2.1	9	9
	1.6	-	-	2.2	9.5	9.5
	1.7	10	-	2.3	9.6	9.6
	1.8	10.5	-	2.4	9.7	9.7
	1.9	11	9	2.5	9.8	9.8
	2.0	**11.5**	**10**	2.6	**10**	**10**

Table 7:- Zone of inhibition by *Gliocladium* extract (BCEF-05*).

Name of endophytic fungal extract.	Concentration of extract (mg/ml)	Zone of inhibition (mm)			
		Staphylococcus aureus	*E.coli*	*Streptococcus pyogens*	*Pseudomonas aeruginosa*
Gliocladium sp.	1.5	10	9	9	-
	1.6	10.05	10	10	10
	1.7	11	10.5	10.5	10.5
	1.8	11.15	11	11	11
	1.9	12	12	11.5	11.5
	2.0	**12.5**	**12.5**	**12**	**14**

Table 8:- Summary of antibacterial activity of the fungal extracts.

Endophytic fungi/ code	*S.pyogens*	*S.aureus*	*E.coli*	*P.aeruginosa*
BCEF-01*	++	+++	++	++
BCEF-02*	+++	+++	+++	++
BCEF-03*	++	++	++	++
BCEF-04*	+	+	++	+
BCEF-05*	++	++	++	++

+: the inhibition zone is up to 10mm ++: the inhibition zone is from 11mm to 14mm
+++: the inhibition zone is 15mm and above

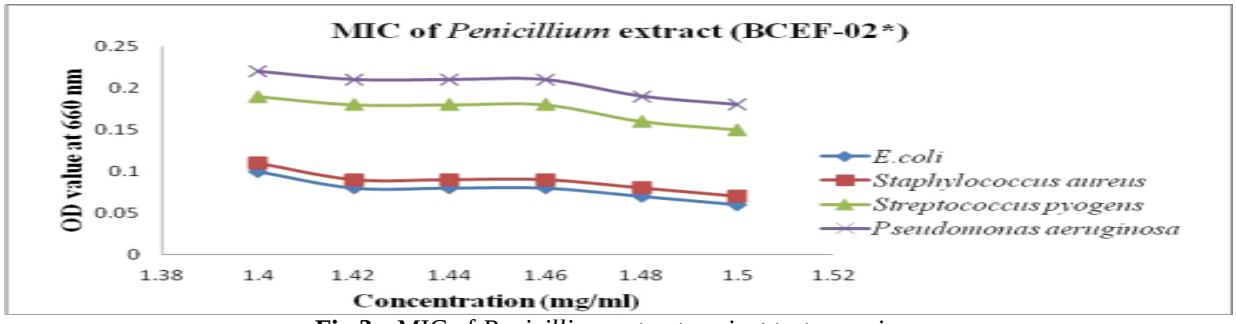

Fig 3:- MIC of *Penicillium* extract against test organisms.

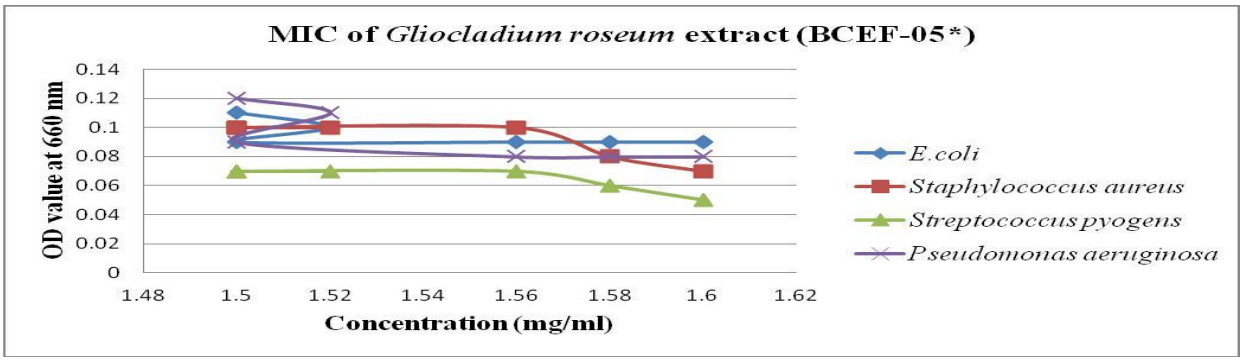

Fig. 4:- MIC of *Gliocladium roseum* extract against test organisms

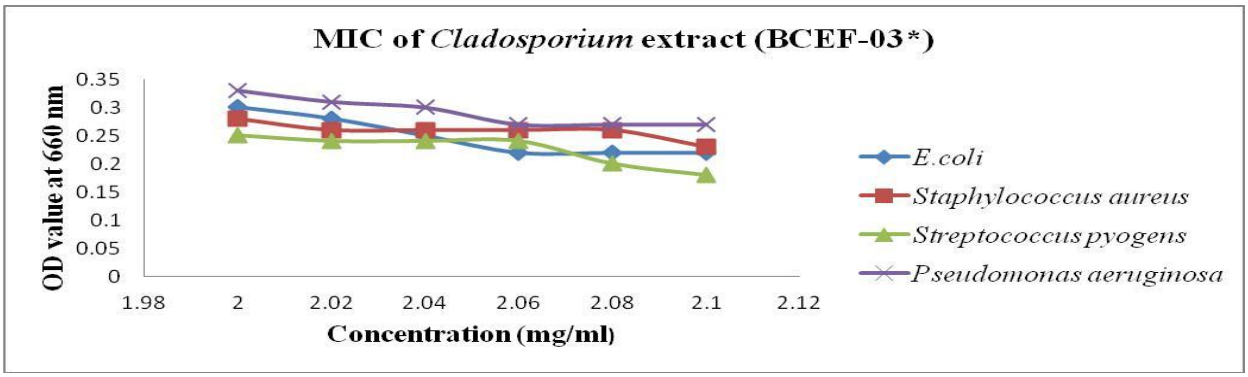

Fig 5:- MIC of *Cladosporium* extract against test organisms.

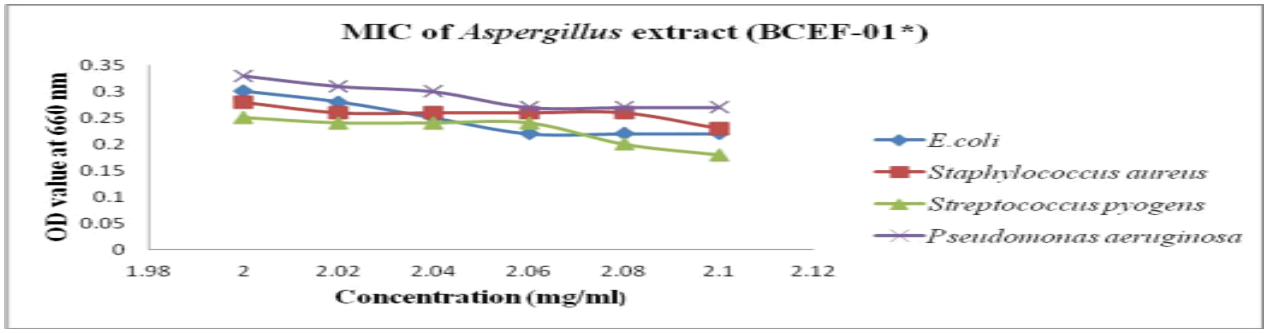

Fig 6:- MIC of *Aspergillus* extract against the test organisms.

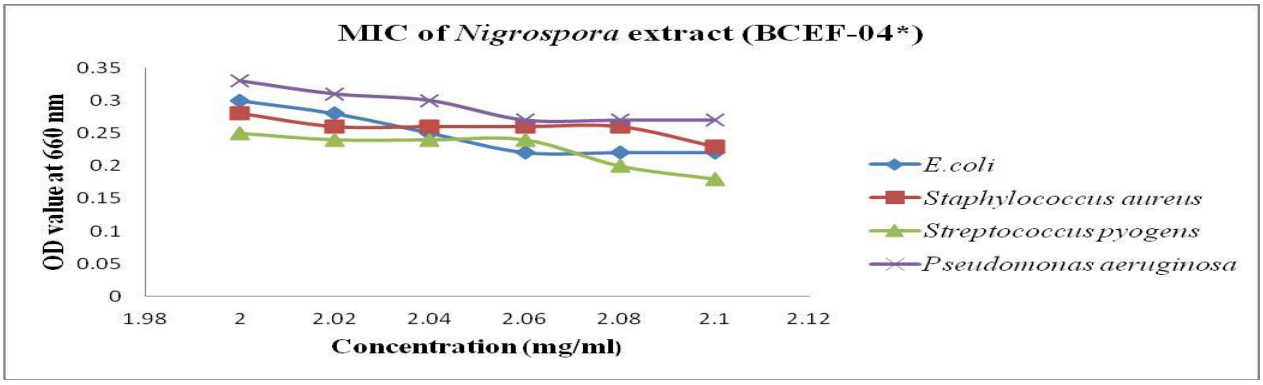

Fig 7:- MIC of *Nigrospora* extract against test organism.

Separation of the metabolites by preparative TLC:-

The fungal extracts were subjected to preparative Thin Layer Chromatography. The spots on the plates were developed by mobile phase (dichloromethane: ethylacetate) in the ratio 7:3 and observed under UV illuminator. Bands obtained were used to check antibacterial activity. And all the fungal extract shows promising antibacterial activity**.**

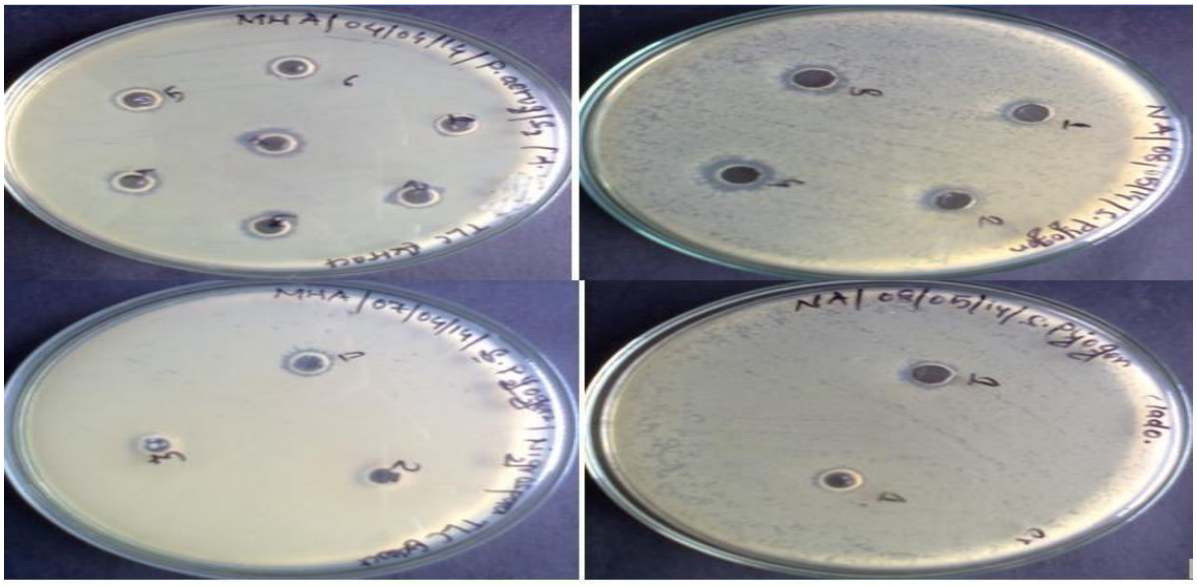

Fig 8:- Antibacterial activity of TLC extracts of different fungal extracts.

Hence the bioactive compounds need to be identified in order to find out which functional group is active against the given pathogens. As for *S. aureus* and *S. pyogens* the novel compound may be used as a potent bioactive chemical

for topical applications against skin diseases and wounds. For identification of the functional group the study recommends the use of HPLC and GCMS. Still the potential for the same promising novel compound needs to be evaluated before it becomes a potential candidate for treatment. Hence, it may be concluded that these endophytic extracts could be used in the treatment of human diseases which have demonstrated potential values.

Discussion:-

Endophytes colonize inside healthy plant tissues to get nutrition and shelter from the host, and in response produce many functional metabolites, which may enhance the host fitness, and have anti-feedant activity and provide resistance against various biotic and abiotic stresses as well. Endophytic fungi have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Bills and Polishook, 1991[13]; Strobel and Daisy, 2003[14]). The results of the present study agree with these earlier findings. The result shows that the fermentation broth of endophytic fungi of *Aspergillus* sp. (BCEF-01*), *Penicillium* sp. (BCEF-02*), *Cladosporium* sp. (BCEF-03*), *Nigrospora* sp. (BCEF-04*) and *Gliocladium roseum* (BCEF-05*) has broad-spectrum of antimicrobial activity against both Gram-positive and Gram-negative pathogenic bacteria like *Staphylococcus aureus, Streptococcus pyogens, Pseudomonas aeruginosa*, and *E.coli.*

Siqueira *et al*., (2011)[15] reported that 16 out of 203 endophytic isolates showed antimicrobial activity with a wider action spectrum inhibiting Gram-positive and Gram-negative bacteria and fungi. Most of the bioactive metabolite compounds from endophytic fungi reported were more effective against Gram-positive bacteria than Gram-negative bacteria and pathogenic fungi (Chareprasert *et al*., 2006[16]). These findings were in agreement with observations by Cos *et al*., (2006)[17] that the Gram-negative bacteria are much more resistant than Gram-positive bacteria. It has been stated that the possible mechanism behind the sensitivity of Gram-positive bacteria may lie in the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space (Duffy and Power, 2001[18]) whereas the Gram-positive bacteria found to be much more susceptible since they have only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971[19]).

Since in microbial-plant relationship endophytes contribute with substances that possess various types of bioactivity (Radu and Kqueen 2000[20]), researchers had been conducting screening for bioactive metabolites and endophytic fungus antimicrobial activity, as was previously revealed (Pela´ez *et al*., 2000[21]; Radu and Kqueen 2000 [20]; Ezra *et al*., 2004[22]). Five different endophytic fungi were isolated from the medicinal plant tulsi and aloe vera and all belong to phyum Ascomycota. These results were correlated with the previous findings of Pavithra *et al*.,(2012)[23]. In the preliminary antibacterial assay the endophytic fungi *Gliocladium roseum and Nigrospora* sp. showed the maximum zone of inhibition of 25mm and 26mm against *Streptococcus pyogens* while *Aspergillus* sp. showed the maximum zone of inhibition of 25mm against *E.coli* and *Streptococcus pyogens.*

The antibacterial activity of 5 extracts of liquid culture of endophytic fungi were evaluated by diffusion agar assay against Gram-positive and Gram-negative bacteria. The activity of the extracts was estimated from growth inhibition (in mm) as follows: <8mm: Inactive; 8–12 mm: weak activity; 13–15 mm: moderate activity; >15 mm: strong activity (Becerra *et al.* 2002[24]). Almost all the endophytic fungi showed the antimicrobial activity against the test bacteria but the endophytic fungi *Penicillium* sp. showed the zone of inhibition of 15mm, 16mm, 17mm and 11mm at the concentration of 2mg/ml against *S.aureus, E.coli, S.pyogens, and P.aeruginosa* respectively. Hence the bioactive compound produced by these fungi can be used for the treatment of disease caused by such organism. Although *Cladosporium* sp. showed less antibacterial activity in this experiment, while in a recent study, Zhang *et al*.,(2011) [25] reported the production of an alkaloid, huperzine A, used in treating the Alzheimer's disease.

The minimal inhibitory concentration (MIC) was defined as the lowest extract concentration resulting in no visible growth after the incubation time. Aligiannis *et al.*, (2001) [26] proposed a classification for plant materials, based at MIC results as: strong inhibitors: MIC up to 0.5 mg/ml; moderate inhibitors: MIC between 0.6 and 1.5 mg/ml and weak inhibitors: MIC above 1.6 mg/ml. In the present study, we have established MIC from 1.42mg/ml to 2.06mg/ml for all the 5 endophytic fungal extracts. *Penicillium* sp. and *Gliocladium roseum* extracts posses moderate MIC.

Besides the antimicrobial and other bioactivities, endophytic fungi *Gliocladium roseum* reported to produce biodiesel (Strobel *et al*., 2008[27]), which may be the future hot cake for endophytic study. Interestingly, the findings of this study provide a strong platform for the isolation and purification of novel natural antimicrobial agents from

endophytic fungi of Tulsi and Aloe vera.

Conclusion and Future Targets:-

Since Stierle *et al.* (1993) [28] discovered a taxol producing endophyte: *Pestalotiopsis microspore*, there have been an increasing interest in bioactive metabolites isolated from endophytes. Many studies proved that endophytes produce novel secondary metabolites as a resistance mechanism to overcome pathogenic invasion (Tan and Zou, 2001[29]). Research on endophytes has been carried out and a great amount of antimicrobial natural products were found. So far, a great number of antimicrobial compounds have been found in a handful of the one million different endophyte species (Petrini, 1991[1]); It is believed that searching for natural products synthesized by endophytes could be a promising way to solve the problem that bacteria are becoming resistant to some commonly used drugs according to WHO and meet the emergency demand of discovering highly effective, low toxicity, and no environmental impacted antibiotics to fight against resistant bacteria species.

The ultimate purpose of endophytes research is to find new antibiotics or pesticides, thus the following aspects should be intensively studied:

1. To find better bioactive antimicrobial substances without any side effect to human, plant and environment.
2. To enhance the antimicrobial activity or decrease the side effects of known metabolites from endophytes by modifying their structures in order to improve the efficacy and specificity to microbes.
3. To optimize conditions of endophyte fermentation that has been found to show bioactivity in order to enhance the yield of active substances synthesized by endophyte.
4. To search for the regulatory gene in synthesis path of antimicrobial compound, and use genetic engineering technology to increase the production of antibacterial substances.

As so many antimicrobial compounds were isolated from endophytes which only occupied a small portion of total endophyte species, it is obviously that there is a great opportunity to find reliable and novel antimicrobial natural products in endophytes, which may be used as clinically effective antibiotics in future.

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