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T Janaki
Department of Botany,
K. M. Centre for P. G. Studies (Autonomous), Puducherry-8, India.

BK Nayak
Department of Botany,
K. M. Centre for P. G. Studies (Autonomous), Puducherry-8, India.

T Ganesan
Department of Botany,
K. M. Centre for P. G. Studies (Autonomous), Puducherry-8, India.

Correspondence
T Janaki
Department of Botany,
K. M. Centre for P. G. Studies (Autonomous), Puducherry-8, India.

Screening mangrove actinomycetes for anticandida activity

T Janaki, BK Nayak and T Ganesan

Abstract
Candida albicans is the most prevalent pathogenic and causal agent of oral candidiasis in man. Even though many azole based antimycotics are available, still candidiasis persists. The present investigation aims at finding better anticandida compound for controlling the candidiasis. The 25 aerobic mangrove actinomycetes were isolated from the soil collected near the root region of *Avicennia marina* (*Forsk*). *Vierh* - (*Avicenniaceae*), of Ariyankuppam backwater area. Out of 25, 13 actinomycetes (52%) showed anticandida activity. The most active isolate was identified as *Streptomyces cacaoi* subsp. *cacaoi,* Gene bank accession no: KP872910, 100µl culture filtrate shown maximum inhibitory zone measuring as 28mm in well diffusion method. Agar plug method has given inhibitory zone as 32mm. Methanolic partially purified compound fraction of M20 has shown 28mm of inhibitory zone, it was higher than the antibiotic clotrimazole used. The presence of pyrimidine nucleosides - neutral and acidic Polyoxins (230 nm), (270-290 nm) and Heptaene antifungal antibiotics (406-417 nm) are confirmed from the UV-Visible spectral analysis. It was concluded that the pyrimidine and polypeptide nucleoside compounds are responsible for anticandida activity

Keywords: *Avicennia marina*, anticandida activity, *Streptomyces cacaoi* subsp *cacaoi*, UV-Vis spectral analysis.

Introduction
Candida species are opportunistic pathogens (Odds, 1994) [30]. It is a diploid, dimorphic fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans (Ryan & Ray, 2004, Enfert & Hube, 2007) [33, 8] and infection of the nail plate. It is the most common species residing in the oral cavity, in both healthy and diseased, and is the agent of most oral candidal infections (Samaranayake and MacFarlane, 1990; Zegarelli, 1993; Silverman *et al*., 1996) [34, 46, 38]. *C. albicans* is the most significant pathogenic species. Other species pathogenic in humans include *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. dubliniensis*, and *C. lusitaniae*. *Candida* are almost universal in low numbers on healthy adult skin and *C. albicans* is part of the normal flora of the mucous membranes of the respiratory, gastrointestinal and female genital tracts.
Even though many azole based antimycotics are available, still candidiasis persists in the human world. Repeated administration of antifungal drugs produces resistance and continuous use of other antifungal drugs may also produce resistant strains. *C. albicans* has been reported to develop resistance to antimycotic drugs (Cowen *et al*., 2002) [6]. The wide spread antibiotic resistance by microbes initiated the search and find new antibiotics to control the resistant microbes. In nature, antagonistic microbes play a vital role by suppressing the activity and spread of human pathogens. Actinomycetes are the strongest antagonists among microbes. The antibiotic substances elaborated by them display antibacterial, antifungal, anticancer, antiprotozoic and antiviral properties of the ten thousand known antibiotics produced by microbes over a decade ago, about 70% are of actinomycete origin: of them, representatives of the genus *Streptomyces* account for two thirds (Miyadoh, 1993) [29]. Actinomycetes are potent source of antibiotics, besides vitamins and enzymes, and such antagonistic actinomycetes of marine origin are being regularly reported. Few reports that soil is a major source of actinomycetes (Vijayakumar *et al*., 2007; Dhanasekaran *et al*., 2008) [42, 7]. Members of actinomycetes which live in marine environment are poorly understood and only few reports are available pertaining to actinomycetes from mangroves (Sivakumar, 2005; Janaki *et al*., 2014, Janaki, 2016) [39, 16, 18]. Mangrove ecosystem is the most productive ecosystem diversified with variety of microbes (Kathiresan and Bingham, 2001) [19]. The search of new and novel antibiotics and other bioactive microbial metabolites is important for the fight against new emerging pathogens (Good fellow *et al*., 1989, Berdy, 2005, Busti *et al*., 2006) [9, 2, 3]. Isolation of actinomycetes from unique unexplored natural habitats is of interest to avoid re-isolation of strains that produce known bioactive metabolites. Neglected habitats are proving to be a good source of novel actinomycetes and bio active compounds.
The present investigation aims at finding better anticandida compound for controlling the candidiasis, with the help of mangrove actinomycetes selectively isolated from the soil near the (root region) of *Avicennia marina (Forsk). Vierh - Avicenniaceae,* from the Ariyankuppam back water area, Puducherry, India.

Materials and Methods

Collection of soil sample

Soil sample was collected near the mangrove plant, *Avicennia marina (Forsk). Vierh – (Avicenniaceae)* in Ariyankuppam back water area, Pondicherry (Lat 11º46´03'' to 11º53´40'' North and Longi 79º49´45" to 79º48´00" East) and packed in sterile plastic containers and transported immediately to the laboratory. The pH of the fresh soil sample was determined (Reed and cummmings, 1945) [32]. Then the soil sample was air dried for 7-10 days at 40 ºC, Crushed and sieved to remove the shells and debris and stored.

Soil analysis

Physico-chemical nature of soil sample was analysed in soil testing laboratory, Department of Agriculture, Puducherry, India.

Isolation of mangrove actinomycetes

The soil sample was subjected to dry heat (70 ºC for 15 min) (Hayakawa *et al*., 1991) [10] (Janaki *et al*., 2014) [16] pretreatment to enhance the chances of isolating rare and novel actinomycetes. After pretreatment, one gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last two dilutions ($10^{-5}$and 10^{-6}) was inoculated by pour plate method (Zheng *et al*., 2000) [47] using Starch casein agar (Kuster and Williams, 1964) [21] supplemented with Fluconazole 80µg/ml and Nalidixlic acid 75µg/ml. Plates were incubated at 30± ºC for up to 30 days. Plates were periodically examined for actinomycetes colonies. Selected colonies were transferred to Yeast Malt extract agar slants and maintained in the same medium.

Screening of actinomycetes for anticandida activity

Candida albicans (MTCC-183) was procured from Microbial Type Culture Collection (MTCC)-Chandigarh, India and maintained in nutrient broth, pH 7.0. 12-24 hours culture was used for anticandida activity assay.

In vitro screening for anticandida activity

All the 25 isolates were primarily screened for anticandida by agar plug method (Mohanraj *et al*., 2011) [26] using four different media- i.e. - Starch casein agar, potato dextrose agar, nutrient agar and yeast malt extract agar. Selected actinomycetes were subjected for secondary screening by agar well diffusion method (Murrey *et al*., 1995) [28]. Anticandida activity of the isolates was tested and confirmed further by cross streak method (Lemos *et al*., 1985) [22]. The anticandida activity of metabolites was determined based on the diameter of zones of inhibition after 24-30 hrs of incubation.

Morphological characteristics of active actinomycete M20

Media recommended by International Streptomyces Project ISP1-ISP7, ISP9 media, nutrient glucose agar- modified (Waksman, 1957) were used for colony characterization of active culture M20. SEM analysis of cover slip culture of M20 was carried out with scanning electron microscope (SEM) Hitachi, Model: S-3400N at CIF, Pondicherry University, Puducherry.

Physiological and biochemical characteristics of isolate M20

Growth and activity in different pH (6, 7, 7.5, 8, 9, 10, 11, 12), temperatures (25 ºC, 30 ºC, 37 ºC, and 45 ºC), concentrations of sodium chloride (0%, 2%, 4%, 6%, 8%, 10%, 12% and 14%), utilization of 21 different sugars by the isolate M20 was studied in carbon utilization medium (Shirling and Gottlieb, 1966) [36]. Antibiotic sensitivity of isolate M20 was studied using 10-different readymade antibiotic discs (Himedia) by Kirby-Bauer method. Production of extra cellular enzymes chitinase (Hsu and Lockwood, 1975) [11] also tested.

Molecular characterization

Template DNA was prepared by using standard procedure. 1 µl of template DNA was added 20 µl of PCR reaction solution. By using 2 universal primers: 518F/ 800R, performed 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72 ℃ for 60 sec. The purified PCR products of approximately 1,400 bp were sequenced by using 2 universal primers: 518F 5'CCAGCAGCCGCGGTAATACG 3', 800R 5' TACCAGGGTATCTAATCC 3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The isolate M20 was identified and phylogenetic tree was constructed.

Partial purification of methanolic crude extract by column chromatography

Using Chloroform: methanol: acetic acid (8.5:1.5: 0.2 ml) as the mobile phase two colour spots were resolved in both in ascending paper chromatography and TLC. Since methanol extraction produced highest activity, the culture filtrate (1-litre) was extracted in methanol, after drying, produced six gram of dark brown oily residue. The residue was mixed with silica gel and applied to the silica gel column (230–400 mesh), and eluted with chloroform: methanol: acetic acid (85:15:2 ml) to give 2 fractions. The yellow compound fraction was used further investigation. The partially purified compound fraction after drying, re dissolved in 2ml of the respective solvents. 10 mg of the extract was loaded to six mm sterile filter paper discs and used for bio assay against *Candida albicans.*

Ultra Violet-Visible Spectrum analysis

UV-Visible spectral analysis of crude culture filtrate and partially purified methanol compound fraction was carried out by using Hitachi U-2010 Spectrophotometer, Wavelength Range: 200 nm to 800 nm.

Results

Isolation and maintenance of actinomycetes

The wet pH of mangrove soil sample collected from *Avicennia marina* was 7.7. The soil analysis results showed that there were very low available Nitrogen, P_2O_5 and Cu. Micro-nutrients like Zn and Fe were high in their available form, Mn was medium. Totally 25 actinomycetes were isolated from soil sample of *Avicennia marina* by dry heat (70 ºC for 15 min) pretreatment method. Dry heat method yielded bioactive actinomycetes for antimicrobial activity. The isolated actinomycetes were subcultured in yeast malt extract agar-ISP2.

Anticandida activity of mangrove actinomycetes

Table 1: Agar plug method

S. no	Isolate code	Zone of inhibition (mm)
1	M1	15
2	M2	10
3	M3	-
4	M4	8
5	M5	10
6	M6	-
7	M7	-
8	M8	-
9	M9	14
10	M10	16
11	M11	-
12	M12	-
13	M13	-
14	M14	12
15	M15	16
16	M16	18
17	M17	-
18	M18	-
19	M19	14
20	M20	32
21	M21	10
22	M22	-
23	M23	-
24	M24	-
25	M25	12

Table 2: Agar well diffusion method

S.no	Isolate code	Zone of inhibition (mm)
1	M1	10
2	M2	10
3	M4	6
4	M5	16
5	M9	12
6	M10	15
7	M14	12
8	M15	8
9	M16	14
10	M19	12
11	M20	30
12	M21	12
13	M25	18

More than half of the isolates (52%) showed anticandida activity in preliminary screening. From the screening results 13 isolates with strong activity were selected for secondary screening to test their ability to produce the active compounds by liquid medium. Based on the secondary screening results, 5 isolates with strong activity were selected and subjected to cross streak method. Finally, the isolate M20 was selected as the most active for anticandida activity.

A. Agar plug method B. Agar well diffusion method C. Cross streak method

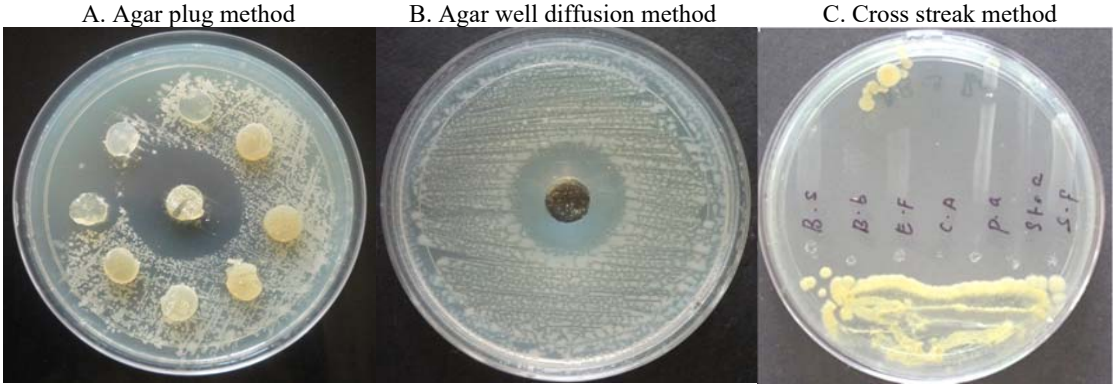


Plate 1: Anticandida activity of mangrove actinomycetes

Plate - A shows the Candida inhibitory activity of isolate M20 measuring 32mm, B shows that the 100µl culture filtrate of isolate M20 inhibits the growth of *C.albicans* and C shows the total absence of *C. albicans.*

Morphological characteristics of the active actinomycete-M20

Colony morphology, colour of aerial and substrate mycelium, pigment production etc., differs in different medium tested. SEM analysis revealed the spore coil structure of the isolate clearly and confirmed the group and family of the isolate M20 and belongs to Streptomycetaceae, genus *Streptomyces*. The isolate M20 has long sporophores, spirals long and open.

A B

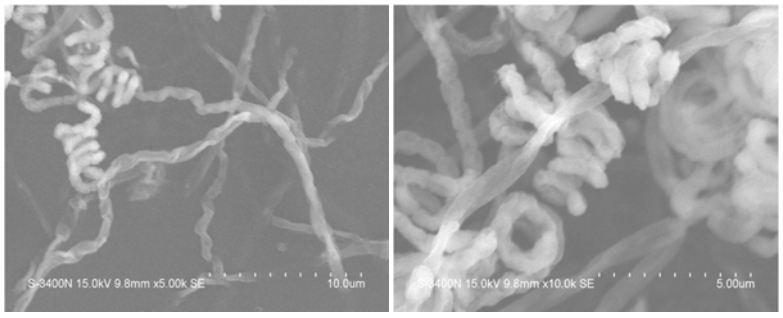


Plate 2: SEM photographs of isolate M20 – A. Well-developed mycellium and attached spore coil, B. Spore coil in close up.

Physiological and biochemical characterization of M20

Observed the growth and activity of M20 in pH 7, 7.5, 8, 9 and 10 with maximum activity in pH 7.5. Maximum growth and activity was observed in 30 ºC. Growth and activity was noticed from 0%-10%, maximum activity observed in 6 & 8% of sodium chloride. Isolate M20 utilized all the sugars except Inosital, Inulin, Dulcitol and Sorbital. The isolate was very sensitive to gentamicin (10mcg) -42mm and less sensitive to carbenicillin (100mcg)-4mm, resistant to ampicillin (10mcg) and amoxicillin (25mcg). The isolate M20 was chitinase positive.

Molecular characterization

The sequence was submitted to Gene Bank with the accession No. KP872910. Phylogenetic analysis of 16S rRNA gene (1400bp) of M20, species of *Streptomyces* was carried out with 18 different reference species of *Streptomyces* available in the Gene Bank database. The isolate M20 branched along with *Streptomyces cacaoi subsp-cacaoi* (NRBC 12748(T)-AB184115 in the analysis. The phylogenetic tree was constructed by neighbour joining analysis. 16sRNA sequencing results revealed that the isolate shared 98.6% similarity with the *Streptomyces cacaoi subsp. cacaoi.* Therefore the isolate M20 was designated as *Streptomyces cacaoi subsp. cacaoi.*

Compound separation

The methanolic partially purified yellow compound fraction (10mg) showed maximum anticandida activity (28mm) than the control clotrimazole (10mg) (12mm). The yellow compound fraction was investigated further by UV-Visible spectrophotometer for antibiotic group analysis

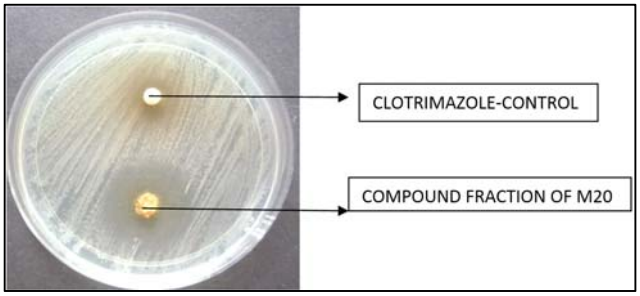
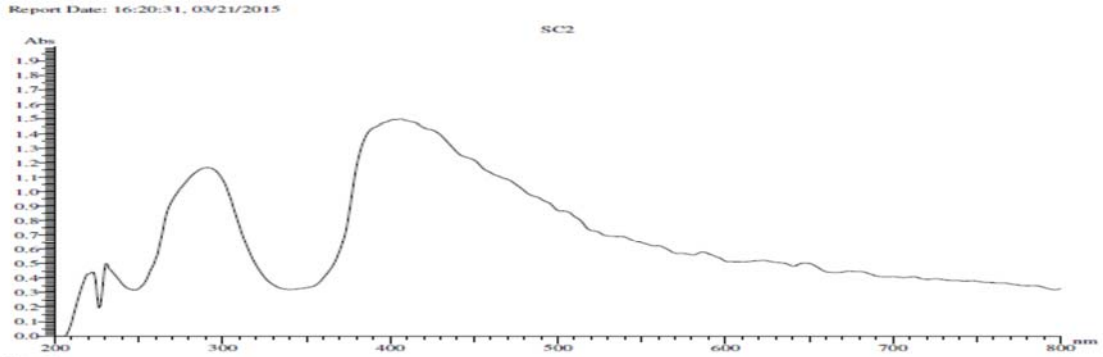


Plate 3: Anticandida activity

Ultra Violet- Visible Spectrum of methanolic crude culture filtrate extract.

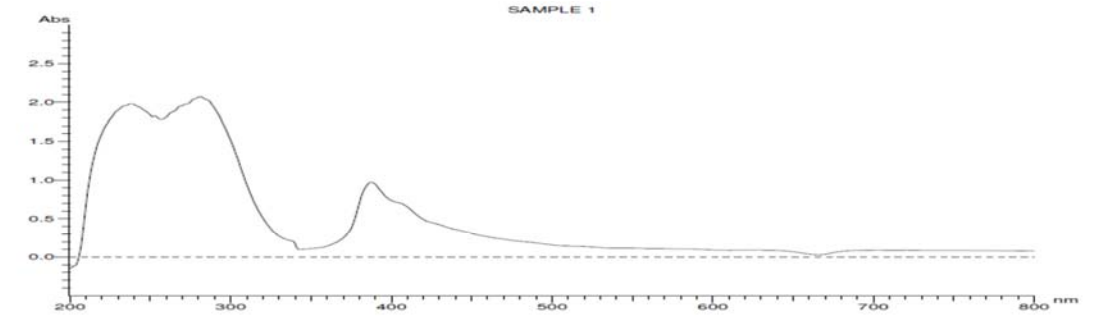
The maximum absorbance of methanolic crude culture filtrate extract (SC2) ($\lambda_{max)}$ at 406.0, 291.0, 230.0, 222.0 nm.



Peaks

Peak #	Apex (nm)	Height (Abs)	Area (Abs*nm)
1	406.0	1.500	321.996
2	291.0	1.169	69.068
3	230.0	0.501	8.194
4	222.0	0.442	5.410

Ultra Violet- Visible Spectrum of partially purified yellow compound fraction



Peaks

Peak #	Start (nm)	Apex (nm)	End (nm)	Height (Abs)	Area (Abs*nm)	Valley (nm)	Valley (Abs)
1	800.0	387.0	342.0	0.976	89.050	342.0	0.099
2	342.0	282.0	257.0	2.071	108.654	257.0	1.780
3	257.0	239.0	200.0	1.979	83.569	200.0	-0.146

UV-Vis spectral analysis of methanolic crude extract of M20 has shown 4 peaks and maximum absorbance at 406 nm followed by 291 nm. UV-Vis spectral analysis of methanolic partially purified yellow compound fraction of M20 has shown 3 peaks and maximum absorbance at 284 nm. The presence of pyrimidine nucleosides - neutral and acidic Polyoxins (230 nm), (270-290 nm) and Heptaene antifungal antibiotics (406-417 nm) are confirmed from the UV-Visible spectral analysis.

Discussion

Clotrimazole is known to suppress candidal adhesion to human buccal epithelial cells, a factor which may aid its therapeutic efficacy (Macura, 1988) [24]. Further, sub-inhibitory concentrations of clotrimazole have been shown to curtail the proteinase production by oral *C. albicans* isolated from HIV-positive and HIV-negative individuals (Wu *et al*., 1996) [45]. In contrast, clotrimazole had no effect on the adherence of *C. albicans* to vaginal epithelial cells, *in vitro*, regardless of whether the drug was used to pre-treat the fungi or the vaginal epithelial cells or was added to the yeast/vaginal cell mixture (Odds and Webster, 1988) [31]. The inappropriate use of the more useful azoles as the first drug of choice may result in eventual emergence of resistant strains and rendering the drug worthless. Drawbacks of all azoles are fungistatic, not fungicidal. This is an important consideration in the treatment of chronic, immune-compromised patients, such as those with AIDS, and in the treatment of infections at critical sites (e.g., candidal meningitis) (Siegman-Igra and Raban, 1992) [37]. Further, none of the azoles is entirely benign, and they are expensive. Hepatotoxicity may be common to all of them (Lesse, 1995) [23], and the potential for endocrine toxicity exists, particularly at higher doses. Antifungal drug resistance has been extensively reviewed (White *et al*., 1998) [44]. The emergence of resistance to the triazoles, is disturbing. Repeated administration of antifungal drugs produces resistance and continuous use of other antifungal drugs may also produce resistant strains. *C. albicans* has been reported to develop resistance to antimycotic drugs (Cowen *et al*., 2002) [6]. Hence, the search for new drugs against Candida infections is a major challenge to current research in Candidiasis. Despite the long list of currently available antibiotics only a limited number of antifungal agents are currently available for the treatment of life-threatening fungal infections. Further, the need for new, safe and more effective antifungals is a major challenge to the pharmaceutical industry today. New sources of antimycotic agent are very much needed, particularly in view of the opportunistic capabilities of *C.albicans.* The present investigation aims at finding better anticandida compound for controlling the candidiasis, with the help of mangrove actinomycetes selectively isolated from the soil near the *Avicennia marina (Forsk). Vierh - Avicenniaceae,* from the Ariyankuppam back water area, Puducherry, India.

Totally 25 actinomycetes were isolated from soil sample by dry heat (70 ºC for 15 min) pretreatment method. Dry heat method yielded bioactive actinomycetes for antimicrobial activity (Baskaran *et al.,* 2011, Janaki *et al*., 2014) [1, 16]. Anticandida activity of Streptomycete group from marine and mangrove has been reported regularly by several reporters (Isono *et al*., 1988, Immura *et al*., 1993, Seghal *et al*., 1993, Khalesi *et al*., 2006, Susithra *et al*., 2009, Mangamuri *et al*., 2012) [15, 12, 35, 20, 40, 25]. 52% of actinomycetes showed anticandida activity in the primary screening in our study, this was because of the selective isolation of mangrove actinomycetes by dry heat pretreatment (70 ºC for 15 min), nature of the source (mangrove soil) of actinomycetes and nutrient glucose broth supplied as optimized medium for better antibiotic production. The isolate M20 was selected based on its strong anticandida activity. The isolate M20 was effective in producing extracellular enzymes; it was very effective in utilizing chitin. Many researchers reported that *Streptomyces cacaoi* subsp. *cacaoi*, *Streptomyces cacaoi subsp asoensis* actively produced chitin synthetase inhibitors as antifungal agents (Isono *et al*., 1965, 1969, Suzuki *et al*., 1965, Chaudhary *et al*., 2009, 2013) [13, 14, 41, 5, 4]. 16sRNA sequencing results revealed that the isolate shared 98.6% similarity with the *Streptomyces cacaoi subsp. cacaoi.* Therefore the isolate M20 was designated as *Streptomyces cacaoi subsp. cacaoi*-M20. The sequence was submitted to Gene Bank with the accession No. KP872910. The methanolic partially purified yellow compound fraction (10mg) showed maximum anticandida activity (28mm) than the control clotrimazole (10mg) (12mm), the present observation was better than the results obtained by Susithra *et al*., (2009) [40] from *Streptomyces paraguyensis,* but, they used amphotericin as control. The presence of neutral and acidic polyoxins (230 nm), (270-290 nm) and Heptaene antifungal antibiotics (406-417 nm) are confirmed from the UV-Visible spectral analysis by the details given by Monisha khan, *et al*., (2011) [27] with the help of absorption spectra of reference antibiotics.

Conclusion

Repeated administration of antifungal drugs produces resistance and continuous use of other antifungal drugs may also produce resistant strains. *C. albicans* has been reported to develop resistance to antimycotic drugs. Hence, the search for new drugs against Candida infections is a major challenge to current research in Candidiasis. The present investigation aimed at searching an effective antibiotic to *C.albicans*. Totally 25 actinomycetes were isolated from soil sample of *Avicennia marina* and screened for anticandida activity. The most active isolate M20-*Streptomyces cacaoi subsp cacaoi* was selected. *Streptomyces cacaoi* subsp. *cacaoi* produce the pyrimidine and polypeptide nucleosides based antibiotics for controlling *Candida albicans* effectively; further investigations are needed to study about antifungal compounds elaboratively.

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