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Prevalence of *C.Albicans*in Clinical Specimens from Prayagraj Hospitals and Evaluation of Antifungal Potential in *Aloe Vera* (L.) Burm. F. Extract

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Abstract

*C.albicans*is one of the most harmful fungus causing expansive infections among human. It specially has its impact in places like hospitals owing to easy transferability propertyof fungus. One major infection caused by group of candida species is Candiasis. This study aimed to identify frequency of *C.albicans* in GTI cases in clinical samples of Allahabad region. Out of 150 GTI samples, *Candida albicans* were observed to have highest frequency at 56.81%, followed by *Candida tropicalis*at 17.29%. Isolated *C.albicans*were then characterized using ITS primers, thus sequence obtained were submitted to NCBI database. Ethanolic and aqueous extracts of Aole vera gel were assessed for antifungal activity, concluding remarkable antifungal potential (18.23±0.86 mm) against*C.albicans*which was found to be comparable toClotrimazole (21.00±0.20mm) and much higher than the aqueous extracts. In recent years, microbes have become resistant to many drugs at very fast rate, which is issue of concern for all, therefore we emphasized on traditional medicine, like *Aloe vera* (L.) Burm. f., to explore more antifungal agents from them.

Keywords: Candidiasis, antifungals, MIC, Clotrimazole, ITS

Introduction

One of the major issues in women is of Genital tract infection (GTI). Some common conditions are vulvitis, vaginitis, and cervicitis that subsequently leads topelvic inflammatory disease (PID)**(Dixon and Wasserheit, 1991). Few well known microorganisms which cause these infections are** *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Ureaplasmaurealyticum*, *Gardnerella vaginalis*and *Escherichia coli***(Sweet, 1987).** This study majorly focuses on the vaginal infection caused due to yeasts emphasizing on role of candida species. Some of its common prolems are irritation, discharge and itchiness of the vagina. As whole this condition is called vaginal candidiasis. Our study accentuates on Albicans and Non-albicans candida species and their incidence in GTI cases.A group of around 300 different species constitutes a genus of Candida. These varieties of species diverge from each other based on their characteristic features. The fungal species are part of the human microbiome especially reside in the genito-urinary tract, digestive tract, and skin. They are harmless to the human body until they exceed a given range. Candidiasis is a condition that arises when candida species show a rise in their number in the affected part. *C.albicans* is one of the most common

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species which is responsible for the condition when increases up to 85–95% from normal. Other Candida species also responsible are *C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, C. kefyr* and *C. lusitaniae.* Their roles have been reported in studies of **Sobel (2007), Mahmoudi*et al.* (2010), and Odds and Bernaerts (1994). Chowta*et al.* (2007)** have reported that there is an increase in the incidence of systemic candidiasis in the last two decades. Antifungal drugs have made their way into our lives to combat the effect of such fungal infestations **(Pereira-Cenci *et al.*, 2008)**. A major category of drugs that act against fungal infections are Azoles like fluconazole or ketoconazole, but their excessive usage is raising the problem of resistance in fungus against azoles **(Odds *et al*., 2003; Looi*et al*., 2005)**. Therefore, suitable alternates to these chemically synthesized drugs are in much demand, shifting attention towards plant and microbial sources for antifungal agents**(Oliveira *et al*., 2007; da Silva *et al*., 2008; Silva *et al*., 2008).** On the other hand, side effects caused by the usage of azoles **(Al-Mohsen & Hughes, 1998)** are also a major problem that is making users search for better replacements.

Traditional medicines are slowly emerging with new opportunities for researchers to develop products that have fewer side effects and low cost. The unexplored treasure of knowledge on these plants if blend with modern techniques, it will turn out to be very fruitful for living beings. In consideration to these benefits of plants over chemical compounds, this study explores role of *Aloe vera* (L.) Burm. f. extract against *C.albicans* which were obtained from clinical specimens. *Aloe vera* (L.) Burm. f., a member of Liliacea family has cactus like appearance. The gel of aloe-vera (L.) Burm. f.is colorless in appearance and very sticky in consistency. They are secreted by the parenchymatous cells of Aloevera leaves. The gel has chemical composition 1-2% bioactive components viz., aloin, aloe-emodin, aloemannan, flavonoids, aloesin, sterols, amino acids, and vitamins with 98–99% water **(Vogler *et al*., 1994)**. These gels have number of medicinal role in treatmentof the digestive tract problems, sunburn and wounds.Other reported properties of the aloe-vera gel are anti-inflammatory, antibacterial, antioxidant,immune-boosting and hypoglycemic properties**(Silver and Bostian, 1993; Vaghasiya and Chanda, 2007; Hamman, 2008)**. In this study, ethanolic and aqueous (hot and cold water) extracts of aloe vera gel was evaluated for their potential antifungal activity against *C.albicans*isolated frommucosal surface of genital tract infection. GTI samples were obtained from hospitals of Prayagraj and prevalence of *C.albicans* among other candida species as causative agent were determined.

Material and Methodology

Patients and sampling of clinical specimens

In this study, 250 mucosal surface swab samples were collected from female patients suffering from genital tract infection. Sample collection was done during the study period of August 2016 to September 2017 from patients of Hayes Memorial Mission Hospital and Jeevan Jyoti Hospital of Prayagraj. A Performa of patient's demographic details and a letter of consent were signed by all the participating patients. The letter of consent confirmed their willingness to participate in the study conducted for research purposes. Samples were collected and immediately transported

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to the laboratory to be evaluated for screening, morphological, biochemical, and molecular characterization.

Data Collection

Personal details as well as clinical history of the patients were recorded from the both the hospitals prior to study. These details of the participants helped in better understanding and correlating parameters with the clinical condition observed at present.

Screening based on morphological and biochemicalcharacteristics

Fungal species present in samples of swabs were characterized as Candida or non-candida species based on their morphological appearance on glucose-phosphate-proline (GPP) and Sabouraud Dextrose Agar(SDA) media for isolation of candida species **(Kulkarni and Nickerson, 1981)**. Growth patthern of fungal culture were observed on plates after incubation period of 3-5 day at 25^{o}C.*Candida albicans* species was differentiated from other candida species based on the colony and microscopic morphology **(St-Germain and Summerbell, 1996, Odds and Bernaert, 1994),** biochemical characteristics- chlamydospore formation, Sugar fermentation tests and Sugar assimilation tests.

Screening based on HiCrome™ Candida Differential Agar

HiCrome™ Candida Differential Agar helps for presumptive differentiation between different candida species. This differentiation is based on pigmentation produced by growing colony, which acts due to presence of chromogenic or fluorogenic hexosaminidase substrates into the growth medium. Each candida species act differently thus producing clear differentiation among them. *C.albicans*-light green colour(smooth colonies), *C.tropicalis*- blue to metallic blue colour(raised colonies), *C.glabrata*- cream to whitecolour(smooth colonies), *C.krusei*-purplecolour(fuzzy colonies), *C.parapsilosis*- pale pink colour.

Molecular characterization of *C.albicans*

Extraction of DNA

Extraction of fungal DNA was done using kit (Nucleo-pore Gdna Fungal Mini Kit (Cat. NP-7006D)). Briefly describing, 50-100 mg of fungal cells were obtained from culture broth by centrifugation at 8000 rpm for 5 minutes. To the pellets, 200µl of phosphate buffer saline was added and mixed well. Centrifuged the thrashing tube after addition of lysis buffer and transferred 400µl of supernatant to the shredder column placed on a collection tube. Binding buffer added twice and then pellet was washed with pre-wash buffer, followed by wash buffer and finally extracted DNA was eluted using 100µl of elution buffer.

Amplification of ITS1-5.8S-ITS2 region using PCR

Characterizations of the fungal isolates were done by amplifying DNA sequences representing the nuclear ribosomal internal transcribed spacer (ITS) region with primers (ITSF:

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TCCGTAGGTGAACCTGCGG; T_m: 55˚C and ITSR:TCCTCCGCTTATTGATATGC; T_m: 55˚C). Total 25-µl reaction mixture was prepared composed of100 µM dNTP, 0.1 µM forward and reverse primers, 1× PCR buffer added with 2.0 mM $MgCl_2$, 1 µl of template DNA sample, and 1 U of *Taq* polymerase (Qiagen). Temperature cycle: Initial denaturation- 96°C for 10 min, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension was at 72°C for 10 min, cycles: 30. Sizes of the amplified ITS segments estimated using molecular markers NEX-GEN DNA Ladder (1000 bp) and the gel was run at 100 volt for 45 minutes at RT. The PCR products were analyzed by staining gel with ethidium bromide and visualizing on Gel Doc System (Zenith, Gel Documentation System; Model No. Gel.LUMINAX-312). PCR products were then sequenced to obtain base arrangement of amplified fragment.

Extract preparation

Extraction of Aloe-vera gel was done to evaluate their potency of anti-fungal activity. For analysis, fresh leaves of aloe-vera were collected and thoroughly cleaned under running tap water. Then rinsed with sterile distilled water and dried with clean paper towel. Using a sterile blade, leaf was excised from centre exposing the gel like substance at the centre.Gently, gel was scraped using sterile spatula. The gel was dissolved at concentrations 0.5 gm/mlinto 10ml ethanol. Mixture was kept on shaker for 24hours, similar process was repeated for hot and cold water.

Maintenance of isolates: Pure fungal cultures were maintained on GPP or SDA media. To avoid any contaminants in the isolated fungus, isolates were on same medium, sub cultured regularly and stored at 4^oC. A master copy of each isolate was maintained separately which was not used for study.

Examination of Antifungal activity against *C.albicans*

Aloe vera extract was assessed for their anti-microbial activity against *C.albican* using Agar well diffusion methoddescribed by the Clinical and Laboratory Standards Institute **(NCCLS/ CLSI, 1997; Fothergill, 2011).**Muller-Hinton agar (MHA) media was sterilized and poured onto to plates; solidified plates were then spread with the *C.albican*suspension (0.1 ml), plates were allowed to absorb the suspension under aseptic condition. Wells were punctured with the borer and filled with aloe vera extract of different concentrations. Clotrimazole at concentration 10mg/ml was used as antifungal positive control. The plates were then incubated at 25°C for 48-72 hours. All the experiments were performed in triplicates, results were recorded as the diameter of the clear zone (in mm) surrounding the wells on the plate. Thus results were expressed as a zone of inhibition (mm) ± standard error of the mean.

MIC and MFC evaluation

Minimum inhibitory and minimum fungicidal concentrations were assessedto detect the lowest concentration of plant extract that prevents visible growth and lowest concentration required to

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achieve bactericidal and fungicidal killing respectively. MIC was done by broth microdilution method recommended by the Clinical and Laboratory Standards Institute/The National Committee for Clinical Laboratory Standards (CLSI/ NCCLS, 2002). Method could be briefly described as: extracts were serially diluted in range of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml in microtiter plate (96-well). Positive control (Clotrimazole) was also diluted simultaneously in range of 0.25 to 128 µg/mL.Culture innoculum was adjusted to 0.5 McFarland standards at 630nm wavelength. 0.1mL inoculums was added to each wells and incubated at 25°C for24–48 hours. Uninoculatedmedium was used as control (blank). All the set of experiments were prepared in triplicate. MFC was done by spreading 10 µL from the wells of the plate having no visible growth onto plates of SDA. Results were recorded as concentration of plates with no growth or colonies< 4 (corresponding 98% killing).

Statistical Analysis

The experiments performed in the study were done in triplicates, thus considering the handling error. The results' statistical significance was analyzed usingIBM SPSS Statistics 20

Result & Discussion

The study consisted of 250 women participants, based on their demographic data and clinical history obtained from hospital records, it was determined that (n=237/250) 94.8% participants were found to be HIV negative, (n=38/250) 15.2% pregnant women, (n=107/250) 42.8% had heart problems, (n=62/250) 24.8% had kidney problems. (n=138/250) 55.33% of participants were above age of 30. The samples of GTI were collected using sterile swab stick and directly spread on different media- SDA (Sabouraud Dextrose Agar), GPP (Glucose-phosphate-proline) and HiCrome™ Candida Differential Agar. Morphology of the grown colonies of fungus were studied on SDA and GPP media that helped to differentiate between candida and non-candida species. Out of 250 plates with clinical samples, 205 plates (82%) were observed to have candida species like appearance while only 45 plates (18%) showed other yeast presence and almost complete absence of any species of candida.

A total of 768 strains of candida were observed on candida positive plates. In this study, prevalence of some common candida species having role in GTI were studied. Candida species which are studied for the incidence were *C.albicans, C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis.* Around 70.31% (n=540/768) of total candida strains were from the above mentioned five candida species. Further differentiation among candida species *C.albicans, C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis*were based on pigmentation of colonies on HiCrome™ Candida Differential Agar. This media helped to identify each species based on the colony colour and appearance. The result and has been summarized in the table (*table 1*) and proportion of each species in samples are graphically shown in the figure (*Fig: 1*).

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Table 1: Colony appearance of five some common candida species on HiCrome™ Candida Differential Agar and their frequency observed in the study

S.No.	Species	Total number. of isolates	N (n/total)	Percentage (%)	Colony characteristics HiCrome™ Candida Differential Agar
1	*Candida albicans*	306	306/540	56.79	light green colour (smooth colonies)
2	*Candida tropicalis*	94	94/540	17.40	blue to metallic blue colour (raised colonies)
3	*Candida parapsilosis*	52	52/540	9.62	pale pink colour (matt appearance)
4	*Candida krusei*	63	63/540	11.66	purple colour (fuzzy colonies)
5	*Candida glabrata*	25	25/540	4.62	cream to white colour (smooth colonies)

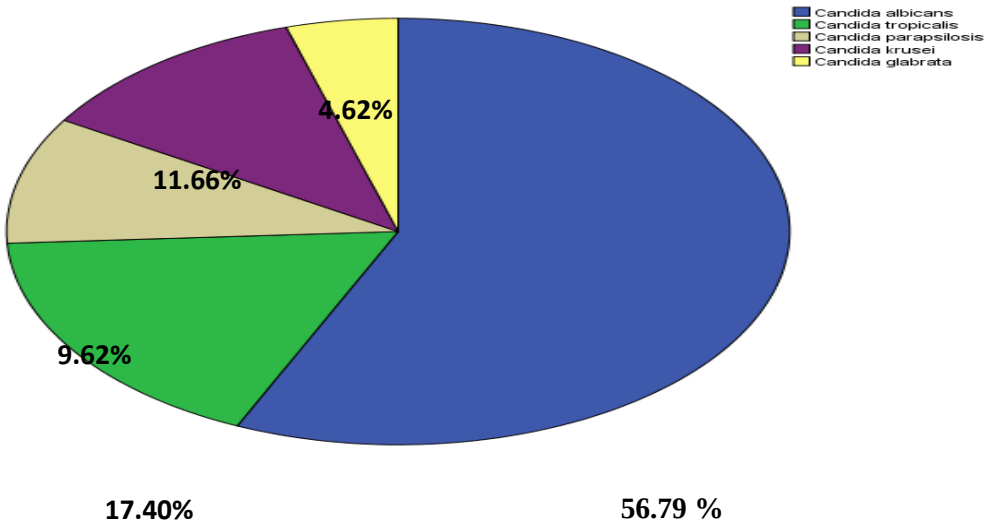

Fig 1: Prevalence of different species of Candida in clinical samples of UTI

The results showed highest frequency of *Candida albicans* (56.79%), followed by*Candida tropicalis* (17.40%) and*Candida krusei* (11.66%) while *Candida parapsilosis* (9.62%), and *Candida glabrata* (4.62%) showed low frequency. The results were observed in combination to other confirmatory tests of sugar assimilation test and chlamydospore formation. Sugar assimilation test helps to estimate the capacity of yeast to utilize particular carbohydrate as its only source of carbon in the medium while chlamydospore formation by culturing on corn meal agar media and then visualization under microscopy went in line with the results of HiCrome™ Candida Differential Agar media.

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C.albicans isolated and identified from above isolates were further characterized using molecular method of identification. ITS region of the fungus was amplified using set of primers (ITSF: TCCGTAGGTGAACCTGCGG and ITSR: TCCTCCGCTTATTGATATGC). Amplicon size of 900 bp was observed (in comparison to the ladder) as shown in the figure (fig 2 & 3). Genomic DNA purity was evaluated by the ratio of 260/280 nm for Samples to be in range of 1.689 and the concentration of DNA calculated to be in range of 7060 ng/µl. The concentration of DNA confirms its suitability for amplification.

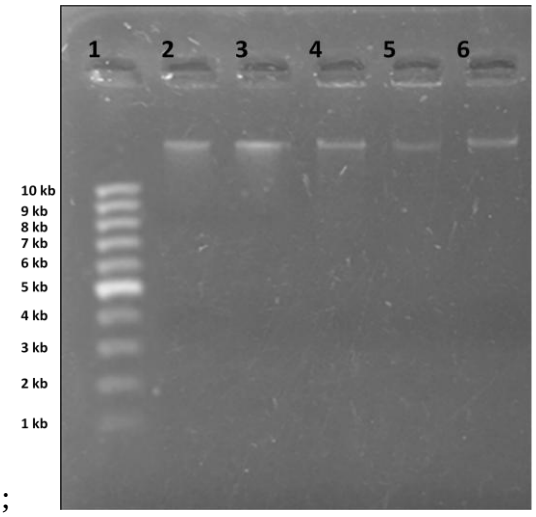

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Fig 2: Genomic DNA (0.8% Gel); Lane 1- 1 kb ladder; lane 2-sample 1; lane 3-sample 2; lane 4- sample 3; lane 5: sample 4; lane 6: sample 5

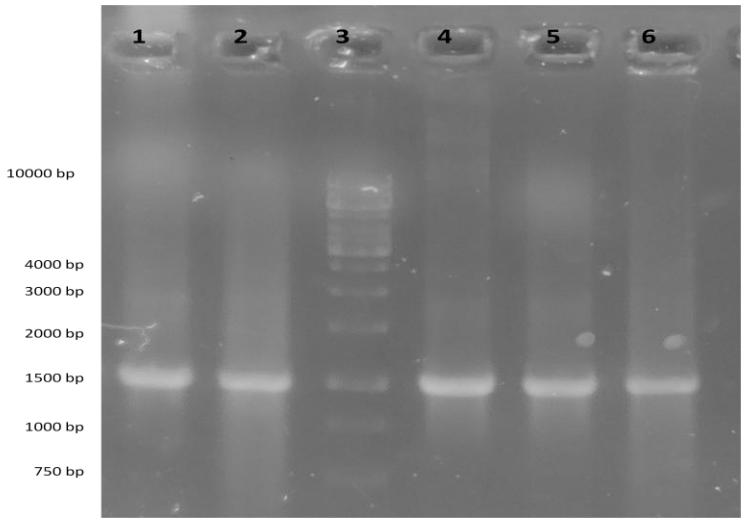

Fig 3: PCR (1.2% Gel); Lane 3- 1 kb ladder; lane 1-sample 1; lane 2-sample 2; lane 4- sample 3; lane 5: sample 4; lane 6: sample 5 (Approx size: 900bp)

The sequence obtained from sequencing was processed using a biological sequence alignment editor (BioEdit 7.2). The subsequent analysis had been done using NCBI-BLAST (National Centre for Biotechnology Information- Basic Local Alignment Search Tool)

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https://blast.ncbi.nlm.nih.gov/Blast.cgiand MEGA X (Molecular Evolutionary Genetics Analysis). These sequences were then submitted to NCBI.

Aloe vera extract were prepared in ethanol, hot and cold water. These extracts were dried and then assessed for their antifungal activity against *C.albicans*. This assay showed that ethanolic extract of aloe vera gel possess significant inhibitory action with zone of inhibition (18.23±0.86 mm) comparable to the positive control (21.00±0.20mm). Aqueous extracts showed weak inhibitory activity with low zone of inhibition, of 4.87±0.61 and 8.67±0.38 mm by cold and hot water extract respectively, against this fungus (shown in *table 2 and fig 4*).

Table 2: Antibacterial activity of Aloe vera extract against C.albicans

S.No.	Extract	ZOI (mm) ± SDEV
1	Ethanolic extract	18.23±0.86
2	Cold water extract	4.87±0.61
3	Hot water extract	8.67±0.38
4	Positive control	21.00±0.20

**ZOI: Zone of inhibition; SDEV: standard deviation; zone of inhibition are recorded excluding well diameter -6mm*

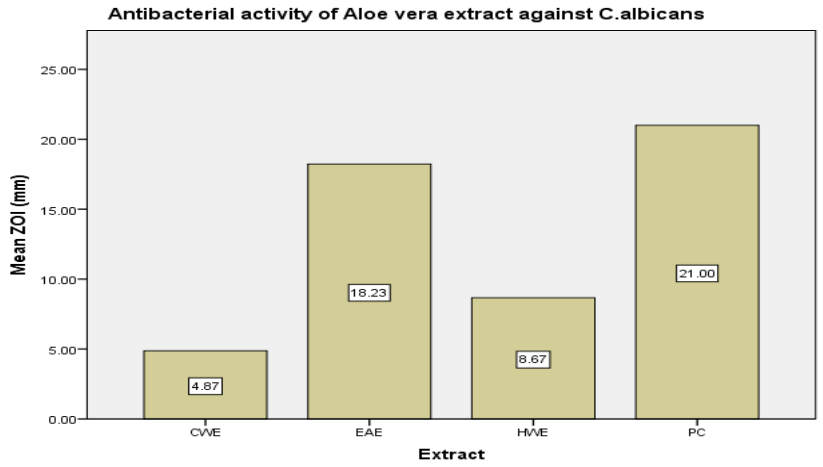

Fig 4: Zone of inhibition observed by aloe vera extract against C.albicans. EAE: ethanolic extract; CWE: cold water extract; HWE: hot water extract;PC: positive control; ZOI: Zone of Inhibition

The MIC and MFC determination of the extracts were done using micro-dilution method, results shown in the table 3. Six concentrations of the extract were prepared- 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml. Ethanolic extracts showed MIC at 25 mg/ml

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(as shown in *table 3*), while in aqueous extracts 100 mg/ml was observed to be their MIC point. MFC point of ethanolic extract was observed to be 100 mg/ml (*shown in table 3*).

Table 3: MIC and MFC of the Aloe vera extract against *C.albicans*

MIC						
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml
EAE	NT	NT	* NT	WT	MT	HT
CWE	*NT	WT	MT	HT	HT	HT
HWE	*NT	WT	HT	HT	HT	HT
MFC						
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml
EAE	*	*	-	-	-	-
CWE	*	-	-	-	-	-
HWE	*	-	-	-	-	-

EAE: ethanolic extract; CWE: cold water extract; HWE: hot water extract; NT: no turbidity; WT: weak turbidity; MT: moderate turbidity; HT: high turbidity; MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration) signified by (*)

Results observed were found to be in accordance to studies conducted on antifungal activity of aloe vera. Our study focused on the potential antifungal activity of aloe vera gel extracted using water and ethanol separately.

Conclusion

This study emphasizes to explore some natural sources as potential antifungal agents which could preferably replace drugs. The sides effects and resistant against broad spectrum of microbes are 2 major reasons which has led researchers to find better alternatives of drugs. Plants are most suitable candidate for this, therefore, in consideration to the seriousness of issue our study aimed to explore antifungal potential of the well known aloe vera gel. Ethanolic and aqueous extract of aloe vera gel showed inhibitory action against *C.albicans*. A deeper insight into its active constituents and purification may lead to better antifungal agents from it in near future.

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Conflicts of interest

There are no conflicts of interest

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