



# Antifungal activity of Phyto-extracts of *Piper longum*, *Aloe vera*, and *Withania somnifera* against human fungal opportunistic pathogen *Candida albicans*

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## ABSTRACT

The fungal infection Candidiasis is caused by *Candida* spp., most commonly *Candida albicans*. *Candida* is a dimorphic, opportunistic yet commensal organism that turns pathogenic to cause serious fungal infections that ranges from superficial mycoses to systemic infections that can cause death as commonly seen in patients with compromised immune system. The prevalence of AIDS, malignancies, increase in average life expectancy, invasive surgical interventions and the progress and sophistication of medical treatments have all contributed to the rising numbers of morbidity and mortality caused by candidiasis. Widespread usage of antifungal has rapidly led to the increasing cases of drug resistance which emerges as a threat to the antifungal therapy and therefore there is an urgent need for novel therapies against this pathogen. The use of plants as sources of pharmaceutical drugs that are used in traditional medicine has been carried out since ages. The fact that 80% of the medicines used worldwide have been directly or indirectly derived from plants, prompted us to take up this study. The present study is an effort to evaluate the antifungal activity of *Piper longum* (Pippali), *Aloe vera* and *Withania somnifera* (Ashwagandha) against human fungal pathogen, *Candida albicans*. These plants have previously been reported to have anti fungal activities mostly against plant and sometimes human fungal pathogens. GC-MS of the plant extracts was done to authenticate the extracts and the spectra results were as per the already known composition. Drug susceptibility testing was done using three different methodologies, namely toxicological end point determination by Minimum Inhibitory Concentration (MIC), spot assay and filter disc diffusion assay. In this study, the three plants have shown promising antifungal activity. Also, there was complete growth inhibition of *Candida albicans* when ethanolic extract was taken from the aged up /dried up fruit of Pippali. The ultimate aim has been to use these plant derivatives directly or as adjunct medicine with already known drugs to increase the efficacy by their synergistic activity.

**Key words:** antifungal, commensal, mycoses, secondary metabolites

## INTRODUCTION

Fungal infections are more common today than ever before. The most common organism implicated in these infections is *Candida*. Candidemias are mostly caused by *Candida albicans*, a dimorphic (Figure-1), commensal organism that turns pathogenic and also leads to soaring rate of nosocomial bloodstream infections (Hospital-Acquired Infections or HAI) resulting in increased morbidity and mortality (1, 2).

*Candida albicans*, a natural flora, can be found in various body parts of an individual (mouth, gastrointestinal tract and vagina) that grows into a deleterious infection depending upon medical condition of an individual(1, 2). Most commonly found infections are the oro-pharyngeal thrush and vaginal yeast infections in women. Patients having AIDS, extensive burn, pregnancy, birth control pills, long-term antibiotic therapy, steroid treatment, organ transplant, immunosuppressant, cancer treatments, genetic disorders, heart surgery, diseases due to endocrine deficiency, tuberculosis infections, diabetes and surgery (involving use of unsterilized needles or catheters) contribute greatly as predisposing factors for candidiasis (1, 2).

The common symptoms of candidiasis are:

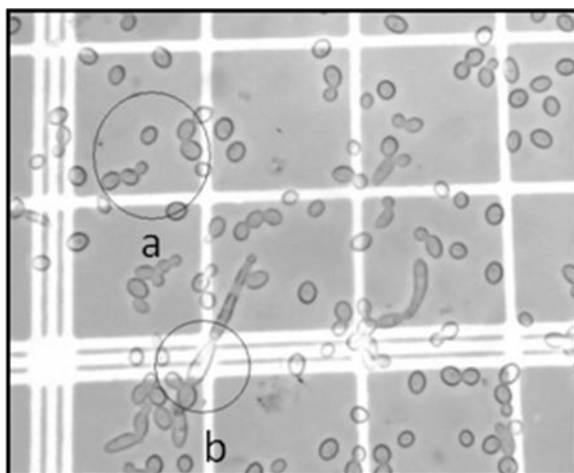
- Oral thrush which are cracks at the mouth's corner or creamy patches inside the mouth.
- Skin rashes in the form of patches or blisters in the groin region, between digits or under the breasts
- Vaginal itching and irritation accompanied by white cottage cheese like discharge (vaginal yeast infection).

Developments in transplantology, use of immunosuppressant, indwelling central venous catheters, corticosteroid therapy, cancer chemotherapy and surgical procedures (1, 2) are linked to severe systemic fungal infections. Availability of broad spectrum antifungal with minimum side effects has always been a limiting factor. Drug resistant strains in the biofilms-associated infections are further turning out to be a tremendous threat to the already limited arsenal against *Candida* infections. Given the complexities of *Candida albicans* life cycle, complex host-pathogen interactions, inadequate vaccines, existing problems of drug resistance and decreasing efficacy of the age-old drugs; there is rising need for novel therapies against this opportunistic pathogen. Renewed interest in treatment of Candidiasis through novel strategies has developed over the last several years. The predominant antifungal currently employed includes mouth washes, vaginal tablets, suppositories and creams. Miconazole (Monistat), clotrimazole (Gyne-Lotrimin) and ticonazole (Vagistat) are effective medicines against vaginal yeast infections, available in the form of creams and suppositories. In most cases fluconazole (Diflucan) is still the drug of choice. But all these medicines do have some restrictive factors associated with them.

Medicinal plants have been a reliable source of antimicrobial agents (3). Plant products generally used as traditional medicines are easily available at reasonable cost (4). Plants produce various secondary metabolites that have antimicrobial activity and pharmaceutical significance (5, 6). The potent effect of plant extracts on bacteria has been assessed globally (7, 8, 9, 10). Natural products or their synthetic derivatives as associated drugs may reduce dependence on conventional drugs and hence forms a smart alternative. These derivatives may be used as adjunct medicine or in combination with already known drugs to increase the efficacy by their synergistic activity.

The project was initiated to assess the potential of selected natural compounds using *in vitro* model system. *Candida albicans* CAF 2-1(WT) was used as the reference strain to assess pharmacodynamics of these natural products. The fruit of *Piper longum* or Pippali has been previously reported to be used against six phytopathogenic fungi, namely *Pyricularia oryzae*, *Rhizoctonia solani*, *Botrytis cineria*, *Phytophthora infestans*, *Puccinia recondita*, and *Erysiphe graminis* and had shown effect only in case of *Puccinia recondita* (11). In another study, the antifungal and antioxidant effect of leaf extract of *Aloe vera* was tested against *Aspergillus niger*, *Candida albicans*, *Cryptococcus neoformans*, *Penicillium*

*maneffei*, and *Phythium sp.* (12). But *Aloe vera* was found to be ineffective against *Candida albicans*. In yet another study, the extract of *Withania somnifera* (Ashwagandha) was also found to be very effective against *Aspergillus* infections (13). The fungicidal properties of the three plants prompted us to use them against *Candida albicans* CAF 2-1(WT/reference strain). The study may help us to develop novel therapeutic strategies and identify new antifungal drug targets for MDR reversal.



**Figure-I. (a) Blastospores-unicellular forms (b) Hyphal form of *Candida albicans*.**

#### METHODOLOGY

##### 1) Source:

Plants used in the study: *Piper longum* (Pippali longa), *Aloe vera* and *Withania somnifera* (Ashwagandha) were procured from Numero Uno Natural Herbs Ltd., Delhi – 110006.

All the chemicals used in the study were of analytical grade (Sigma Ltd.).

##### 2) Extraction and Isolation of plant extracts:

The dried leaves of *Aloe vera*, fruits (both fresh as well as dried/aged) of *Piper longum* and roots of *Withania somnifera* were milled separately into coarse powder using a mechanical grinder. The crude powder was also filtered using muslin cloth to obtain fine powder. The ground material was successively extracted using ethanol as solvent at the concentrations of 1g/ 4mL or 1g/3mL and kept for two days at room temperature on a shaker. The ethanol extract was filtered 2-3 times with a Millipore filter using nylon 66 filter membranes (0.2  $\mu$ m). The extracts were collected and stored in the refrigerator at 4°C. Varying concentrations of the crude extracts were subjected to antimicrobial studies.

##### 3) GCMS analysis of plant extracts:

GCMS analysis was performed using *QP-2010 Plus with Thermal Desorption System TD 20 with* Column DB-1.30 m 0.32 mm ID 0.25  $\mu$ m, Column Temperature 70 °C (1 min) @ 20 °C/min to 160 °C @ 2 °C/min 190 °C @ 15 °C/min to 320°C (5 min), He linear velocity, Pressure 47.5 kPa and Total flow 43.8 mL/min. Electron ionization (EI) with ionization energy of 70eV was used for GC/MS detection. Helium gas (99.999%) was used as the carrier gas at a constant column flow rate of 2.25 mL/min (purge flow 10.0 mL/mL) and injection volume was 1  $\mu$ l (split less injection mode) with Injection temperature 250°C. The parameters for MS were as follows: Ion-source temperature 230°C, interface temperature 270°C, scanning range- m/z 35 to 500, scanning interval 0.5 sec and SIM sampling rate 0.2 sec.

##### 4) *In vitro* fungicidal activity:

The activities of above extracts were evaluated using laboratory isolates of *Candida albicans* CAF2-1. *In vitro* culture of *Candida albicans* CAF2-1 was maintained in YEPD medium (Yeast extract, Peptone, Dextrose). Morphological studies and Cell counting was done using the microscope and hemocytometer. Determination of growth curve of the fungus (24 H) was done using Spectrophotometer at wavelength 600nm. Evaluation of antifungal activity was initially done by spot assay. In spot assay, serial dilutions of the culture were spotted onto YEPD plates in the absence (control) or presence of extracts. Recording the growth differences gave us the preliminary estimate of the potency of the extracts. For spot assay

analysis of Pippali we also used the older /aged/ dried up fruit (Black-brown) and the fresh fruit (Green) to check the differences in the activity depending on the age of the fruit.

Further evaluation of potent activity of plant-derived crude extracts was done using Kirby Bauer Disc Diffusion method by measuring the Zone of Inhibition of the pathogen in presence of these extracts. The crude ethanolic extracts were used for bioassay against *Candida albicans*. Sterile discs with 6mm diameter were loaded with different concentrations of the crude extracts and their potency tested against the fungus. The plates were incubated at 37 °C for 24 hours. Antimicrobial activity of each extract was evaluated by measuring the zone of inhibition. All experiments were repeated in at least three replicates and results were recorded. Inhibitory effect of solvent ethanol was verified by using ethanol alone and it did not show any inhibitory effect at indicated concentration. MIC was determined in accordance with the recommendations of the Clinical and Laboratory Standard Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS).

## RESULTS

The results of the study provide important scientific basis for the use of plant extracts in the development of antifungals against *Candida albicans*.

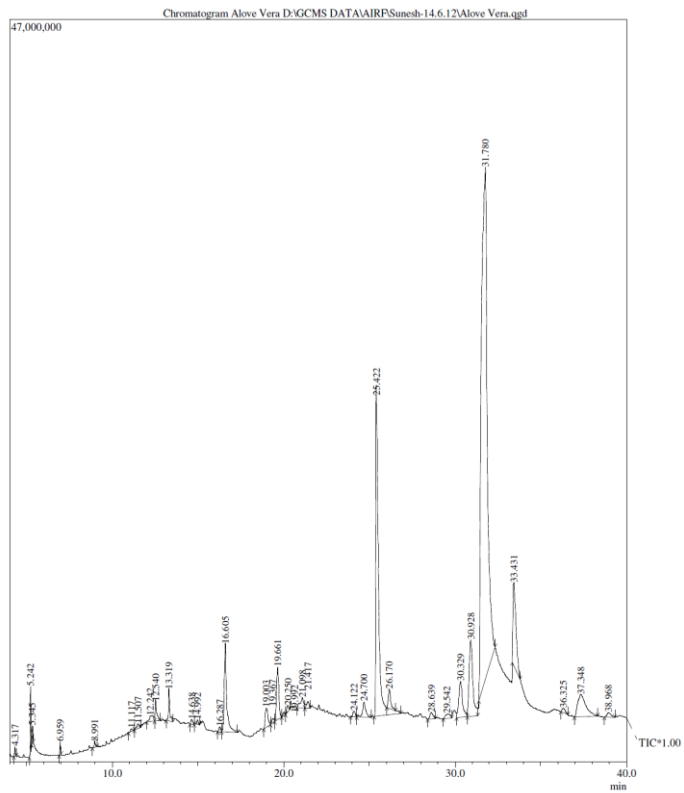
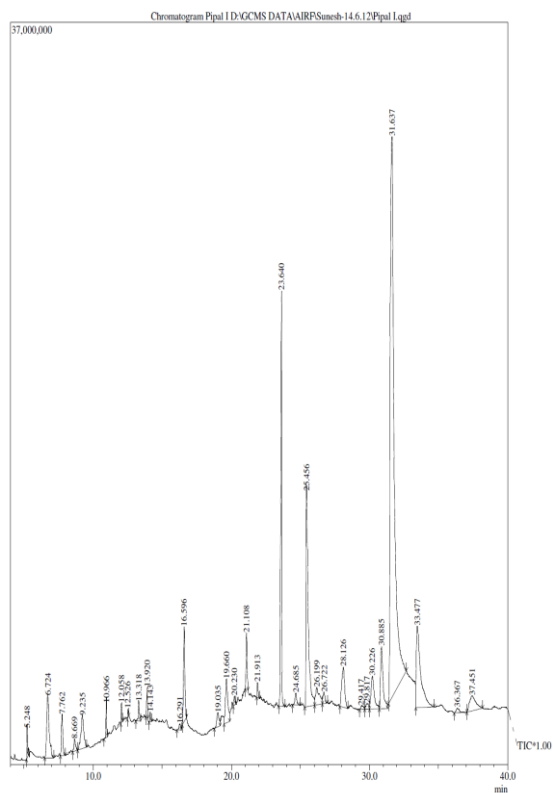
GCMS-spectra recorded different peaks as per the composition of plant extracts, supported by the mass spectra results. The molecular ion peaks corresponded to the molecular weight of the components which are present in the extract. In GCMS analysis, 10 phytochemical compounds were recognized in the ethanolic extract of *Piper Longum* and 9 phytochemical compounds were recognized in the ethanolic extract of *Aloe Vera* (Figures- IIa and IIb; Table I and II). The identification is based on the peak area, molecular weight, molecular formula using the database of National Institute Standard & Technology (NIST) and WILEY 8. LIB. These phytochemicals have previously been reported in the extracts of *Piper longum* and *Aloe vera* (14, 15). This validated the dependability of the extracts used in the study.

The spot assay was subsequently carried out to check the antifungal activity of the extracts. The fruit-derived materials of *Piper longum* or Pippali has been previously reported to be used against six fungi, namely *Pyricularia oryzae*, *Rhizoctonia solani*, *Botrytis cineria*, *Phytophthora infestans*, *Puccinia recondita*, and *Erysiphe graminis* and had shown effect only in case of *Puccinia recondita* (11). This is the first report of Pippali extract being used against human fungal pathogen *Candida albicans in-vitro*. (Figures- III and IV). As seen in Figure-III, there is marked inhibition in the growth of *Candida* in all the concentrations (1g/3mL or 1g/4mL). The inhibition is also seen, whether 750 µL is used or 1000µL of Pippali extract is used. However, the older /aged/ dried up fruit has been found to give much better fungicidal results (Figure-IV).

In previous studies, the anti-fungal activity of *Aloe vera* leaf extract has also been tested against *Aspergillus niger*, *Candida albicans*, *Cryptococcus neoformans*, *Penicillium maneffei*, and *Phythium* sp. But *Aloe vera* was found to be ineffective against *Candida albicans* (12). However, in this study encouraging results have been obtained with *Aloe vera* leaf extract against *Candida albicans* CAF2-1 (Figure-IV). The difference in the result so obtained from the previous study seems to be in the method of extraction. In the previous study, the preparation of plant- ethanolic extract was done at 90°C, while the same has been done at room temperature (24 °C) in the present study. Since the protocols followed in both are almost similar so we can decipher that the active component is lost (or degraded) in the ethanolic extract if extraction is done at higher temperature.

In another study, the extract of *Withania somnifera* (Ashwagandha) was also found to be very effective against *Aspergillus* infections (13). In this study fungicidal property has also been observed with roots of Ashwagandha against *Candida albicans* (Figure-IV)

In order to directly check the growth inhibition, various concentrations of ethanolic extract of *Piper longum* or Pippali was added to the culture medium and the growth curve was obtained. The results showed marked inhibition in growth of *Candida albicans* (Figure-V).



(a)

(b)

**Figure-II. GCMS-Chromatogram of (a) *Piper longum* (Pippali) and (b) *Aloe vera*.**

**Table I. GCMS analysis of *Piper longum* (Pippali)**

S.No.	R. Time	Compound	MW	Molecular Formula	Peak Area%
1.	6.724	Pentadecane	212.47	C <sub>15</sub> H <sub>32</sub>	3.13
2.	7.762	Caryophyllene (β-Bisabolene)	204.35	C <sub>15</sub> H <sub>24</sub>	1.16
3.	9.235	Heptadecane	240.47	C <sub>17</sub> H <sub>36</sub>	1.91
4.	21.108	Phytol	296	C <sub>20</sub> H <sub>40</sub> O	1.28
5.	23.640	Hexadecene	224	C <sub>16</sub> H <sub>32</sub>	9.33
6.	25.456	Hexadecanoic acid	256	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	9.53
7.	28.126	Piperine	285	C <sub>17</sub> H <sub>19</sub> O <sub>3</sub> N	2.39
8.	31.637	Oleic acid (cis-9-octadecenoic acid)	282	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	44.85
9.	33.477	9,12-Octadecadienoic acid	280	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	7.06
10.	37.451	Eicosane	282	C <sub>20</sub> H <sub>42</sub>	1.91

**Table II. GCMS analysis of *Aloe vera*.**

S.No.	R. Time	Compound	MW	Molecular Formula	Peak Area%
1.	6.959	Salicylic acid	138	C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>	0.41
2.	16.605	9, 12-octadecadienoic acid, methyl	294	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	3.88
3.	19.661	9-octadecenoic acid, ethyl ester	310	C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>	2.10
4.	21.098	Phytol	296	C <sub>20</sub> H <sub>40</sub> O	0.61
5.	25.422	n-Hexadecanoic acid	256	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	15.6
6.	30.928	Octadecanoic acid, ethyl ester	284	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	5.00
7.	31.780	Oleic acid (cis-9-octadecenoic acid)	282	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	53.85
8.	33.431	9,12-Octadecadienoic acid	280	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	4.43
9.	37.348	Eicosane	282	C <sub>20</sub> H <sub>42</sub>	3.22

Plant Extract	Sample I (1ml Extract)	Sample II (750 ul Extract)
Control		
EtOH (Absolute)		
PIP-I (1g/4ml EtOH)		
PIP-II (1g/4ml EtOH) (made from Fine powder)		
PIP-III (1g/3ml EtOH)		
PIP-IV (1g/3ml EtOH) (made from fine powder)		

**Figure-III**

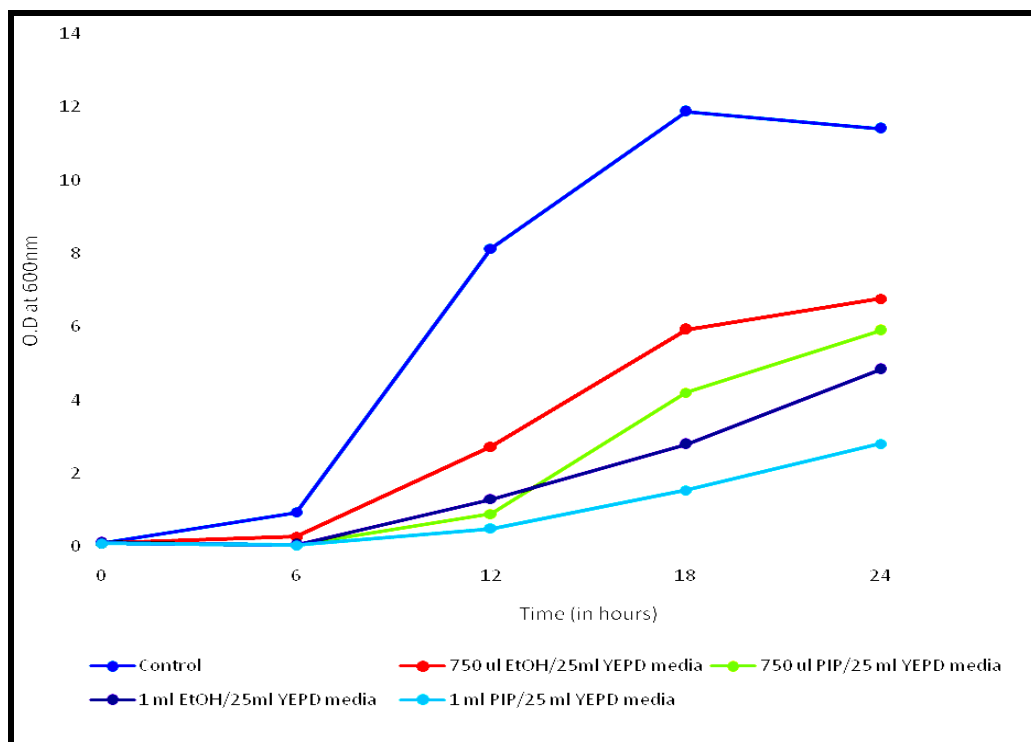
**Spot Assay-I: Growth Inhibition of *Candida albicans* using *Piper longum* (Pippali) fresh fruit extract. PIP-I and PIP-III-unfiltered Pippali fruit extract (crude powder); PIP-III and PIP-IV-filtered Pippali fruit extract (fine powder).**

Plant Extract (1g/4mL EtOH)	Sample I (1 mL)	Sample II (750 µL)	Sample III (500 µL)
Control			
EtOH			
<i>Aloe vera</i>			
<i>Piper longum</i>			
<i>Withania somanifera</i>			

**Figure-IV**

**Spot Assay-II: Growth Inhibition of *Candida albicans* using *Aloe vera*, *Piper longum* (Pippali-aged fruit) and *Withania somnifera* (Ashwagandha) extracts at the conc. 1g/4mL EtOH**





**Figure-V. Effect of *Piper longum* (Pippali) on the growth of *C. albicans* (reference strain)**

#### DISCUSSION

Nature includes many medicinal plants and herbs that can be used for treatment of various diseases and remains unexplored. Few are aware that most of the medicines that are commonly prescribed are from plant extracts or their synthetic derivatives. Medicinal plants help cure many diseases like malaria, diabetes, glaucoma, arthritis, heart disease, thyroid disorders and skin conditions. The research undertaken in this project is one such endeavor. In this study, *Candida albicans*, an opportunistic fungal pathogen, has been used as the test organism to assess effective activity of medicinal plant extracts. Encouraging results have been obtained with *Piper longum* (Pippali), *Aloe vera* and *Withania somnifera* (Ashwagandha). In-depth studies of potent compounds from the present study may also provide clue to their mechanism of action and might help to develop novel therapeutic strategies and identify new antifungal drug targets for MDR (multi drug resistance) reversal in this organism and other such fungal pathogens of clinical interest. It will be interesting to evaluate these derivatives as adjunct medicine or in combination with already known drugs to increase the efficacy by synergistic activity.

#### CONCLUSION

Increase in fungal pathogens, invasive fungal infections, limited therapeutic options and emergence of MDR together build a burden on patients with compromised immunity. Due to increasing prevalence in various patient groups, *Candida* spp. has gained remarkable importance and among all *Candida* spp., *Candida albicans* is most frequently isolated from blood and tissue samples of affected patients. Search for novel therapeutic alternatives has emerged due to clinical needs for novel antifungal agents with broad spectrum activity and minimal toxic effects on the host. Plant products have been used since ages in traditional medicines. Due to their safe human use, in recent times there has been lot of efforts for developing therapeutic options using molecules from plant sources. This study is also a similar effort to check the antifungal effects of selected plant materials. In this study, *Piper longum* (Pippali), *Aloe vera* and *Withania somnifera* (Ashwagandha) were found to show potent anti-fungal activity. This study merits further investigation in order to characterize the effects of these three plant sources showing antifungal activity in order to characterize the mechanism of action for further translation into clinical use and overcoming the challenges of fungal therapeutics.



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