***Original Research Article***

# *In vitro* Anti-candidal Activity of Extracts of Some Selected Medicinal Plants on Clinical Isolate of *Candida albicans* and its Standard ATCC 10231 Strain


**ABSTRACT**

**Aim:** Extracts of four different medicinal plant parts such as stem bark of *Murayya koenigii* (Curry leaf plant), fruit peel of *Punica granatum* (Pomegranate), cloves of *Allium sativum* (Garlic) and rhizome of *Zingiber officinale* (Ginger) were used to test their anti-candidal activity on clinical isolate of *Candida albicans* and its ATCC 10231 strain.

**Methodology:** Culture test, germ tube test and chlamydospore formation test were used for the confirmation of *C. albicans* isolates. Disc diffusion method was evaluated for anti-candidal activity. Secreted aspartyl proteinase (SAP) enzyme was assayed spectrophotometrically using bovine serum albumin as substrate.

**Results:** Out of ten isolates of *Candida albicans* made in the laboratory, isolate 5 showed good growth as compared to its standard culture ATCC 10231. Optimum temperature and pH was found to be 35 - 40ºC and pH 3.5 – 4, respectively. The garlic extract exhibited better anti-candidal activity on clinical isolate 5 and ATCC 10231 than the other plant extracts, as the zone of inhibition was found to be 28 mm for isolate 5 and 30 mm for ATCC 10231. Allicin, a highly unstable molecule was found to be a major ingredient of *Allium sativum*, and its concentration was found to be 214 µg/mL.

**Conclusion:** Allicin has not only exhibited good anti-candidal activity but also destroyed the Secreted Aspartyl Proteinase (SAP), a key enzyme responsible for the candidiosis in humans.



**Keywords:** Anti-candidal activity; *Candida albicans*; Disc diffusion; Secreted aspartyl proteinases


## 1. INTRODUCTION

The World Health Organization (WHO) has recognized the potential utility of traditional remedies and strives to preserve the primary health care involving medicinal plants. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years. One of the most efficient ways of finding new bioactive compounds is collecting data on the use of medicinal plants in traditional pharmacopeia.

*Candida* spp. constitutes one of the major groups of pathogenic fungi, and the incidence of infections has increased dramatically over the past three decades causing increase in morbidity and mortality in immune-compromised patients (Naglik et al., 2003; Dolan et al., 2009). *Candida albicans* is an ubiquitous,

most common fungal pathogen of humans and etiologoical agent of candididosis. It has ranked fourth place in most commonly encountered nosocomial pathogens in blood stream infections (Smith 2005). Secreted aspartyl proteinase (SAP), most significant extracellular enzyme produced by *C. albicans*, plays vital role in invading, colonizing and causing damage to the host tissue. Under suitable predisposing conditions when the host has compromised this colonized site provides the base for candidal proliferation, invasion and in some instances dissemination (Naglik et al., 2003).

The increase in incidence of fungal infections worldwide especially due to *Candida* spp. and emergence of antifungal resistance among clinical isolates especially against the conventionally used azole antifungals, poses an important challenge to the clinician. Much attention has been paid to plant derived antifungal compounds based on the knowledge that plants have their own defense system (Fontenelle, 2007). Since ancient times, mankind has used plants to treat common diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Rois and Reico, 2005). However, in the absence of a scientific research base such practices may induce serious adverse effects in patients. Medicinal plants possess many bioactive properties, usually anti-microbial, anti-oxidant, anti-cancer and anti-inflammatory. They also represent a rich source of anti-microbial agents. Therefore, medicinal plants extracts and their derived products offer considerable potential for the development of new agents effective against infections currently difficult to treat (Machado et al., 2005).

Researchers worldwide have literally found thousands of phyto-chemicals having *in vitro* inhibitory effects on all types of microorganisms. These *in vitro* screening programs using the ethno-botanical approach are important in validating the traditional use of herbal remedies and for providing leads in the search for new active substances (Gurib-Fakim, 2006). These plants extracts and their derived compounds are subjected to animal and human *in vitro* studies to determine their effectiveness in whole organism systems; this includes toxicity studies as well as an evaluation of their effects on normal microbiota.

In the present study, *Murayya koenigii* (Linn) Spreng, a member of family Rutaceae, is a deciduous to semi-evergreen aromatic tree found throughout India. Traditionally it is used as an analgesic, febrifuge, stomachic, carminative and for the treatment of dysentery and skin eruptions (Arulselvan and Subramanian, 2007). This is commonly known as curry leaf, used as spice due to the aromatic nature of leaves. *Punica granatum* is commonly known as pomegranate, native to the region from northern India to Iran. It is a fruit bearing deciduous shrub or small tree which belongs to the family Lythraceae. *Zingiber officinale* (Ginger), a medicinal plant that has been widely used all over the world, for a wide array of unrelated ailments includes arthritis, cramps, rheumatism, sprains, dementia, fever and against infectious diseases (Ali et al., 2008) were selected and studied against *Candida albicans* clinical isolate and a standard *C. albicans* ATCC 10231 strain. *Allium sativum* (Garlic) is a common spice belongs to Liliaceae family commonly used for flavouring and has been traditionally popular with strong folkloric awareness. It is rich in sulphur- based compounds which contribute to the characteristic odour (Jabar and Al-Mossawi, 2007). Clove of garlic is rich in allicin, a highly unstable molecule. The main aim of the present study is to

examine the *in vitro* anti-candidal activity of extracts of medicinal plants, especially the *Allium sativum*, and also try to destroy SAP, a pathogenic factor of *C. albicans*.

## 2. MATERIALS AND METHODS

### 2.1 *Candida albicans* strains

Ten isolates of *Candida albicans* were collected from different dental hospitals, of patients suffering from oral thrush. *C. albicans* isolated from oral candidiosis patients by using oral swabs. Sterile cotton swabs were wiped on thrush present in oral cavity. The swabs were aseptically streaked on Sabouraud's dextrose agar (SDA) plates. Samples were cultured on SDA in an aerobic atmosphere at 37°C for 48 h. Suspected yeast colonies were subcultured for identification purpose. Presumptive identification methods (Lee *et. al.,* 1999) used to identify the colonies for further studies. The standard culture of *Candida albicans* ATCC 10231 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India.

### 2.2 Identification test for *Candida albicans*

Culture test, germ tube test and chlamydospore formation test were used for the confirmation of *C. albicans* isolates.

#### 2.2.1 Culture test

The suitable samples were cultured on plain SDA and also SDA containing chloramphenicol and cycloheximide. SDA plates were incubated at 37°C and examined twice a week to look for growth of cream coloured pasty colonies suggestive of *Candida* species. Plates incubated for one week and observed for growth.

#### 2.2.2 Germ tube test

A rapid presumptive identification of *C. albicans* could be made by placing the organism in serum and observing germ tube formation. A single colony was inoculated into serum at 39°C. After 2 - 4 h, wet mount was prepared and examined under the microscope to look for the presence of germ tube.

#### 2.2.3 Chlamydospore formation

All *Candida* isolates were tested for the production of chlamydospores in corn meal agar with Tween 80. The *Candida* strains were inoculated in corn meal agar (CMA) and then incubated at 25°C. After 72 h, the plates were examined under the microscope for the presence of chlamydospores.

## 2.3 Growth of *Candida albicans* at different intervals of time

A loopful of culture from slant was taken and inoculated into the Sabourad Dextrose (SD) broth medium and incubated for 48 h at 28°C. After attaining log phase, the culture was taken and transferred into broth medium and the absorbance (OD) was measured at 540 nm at regular intervals of time up to 48 h.

## 2.4 Collection of plant material and preparation of extracts

### *2.4.1 Collection of plant materials*

Stem bark of *Murayya koenigii*, *fruit* peel of *Punica granatum*, cloves of *Allium sativum* and rhizome of *Zingiber officinale* (Fig 1), were procured from the local market/ area and used in the present study.

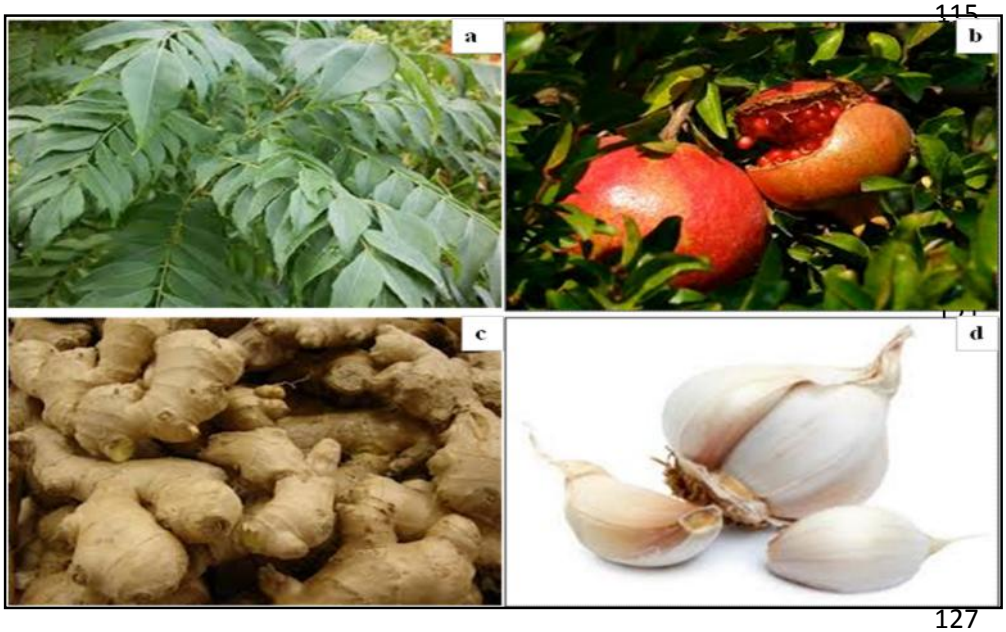


**Fig 1: a= *Murayya koenigii* (Stem bark); b= *Punica granatum* (Fruit peel); c= *Zingiber officinale* (Rhizome) d= *Allium sativum* (cloves) were selected for the study.**

### *2.4.2 Plant extracts by soxhlation*

All plant materials were shade dried, powdered and subjected to solvent extraction by soxhlation. Solvent extraction of different plant materials is represented in a schematic diagram (Fig. 2). These extracts were used in the anticandidal assay.

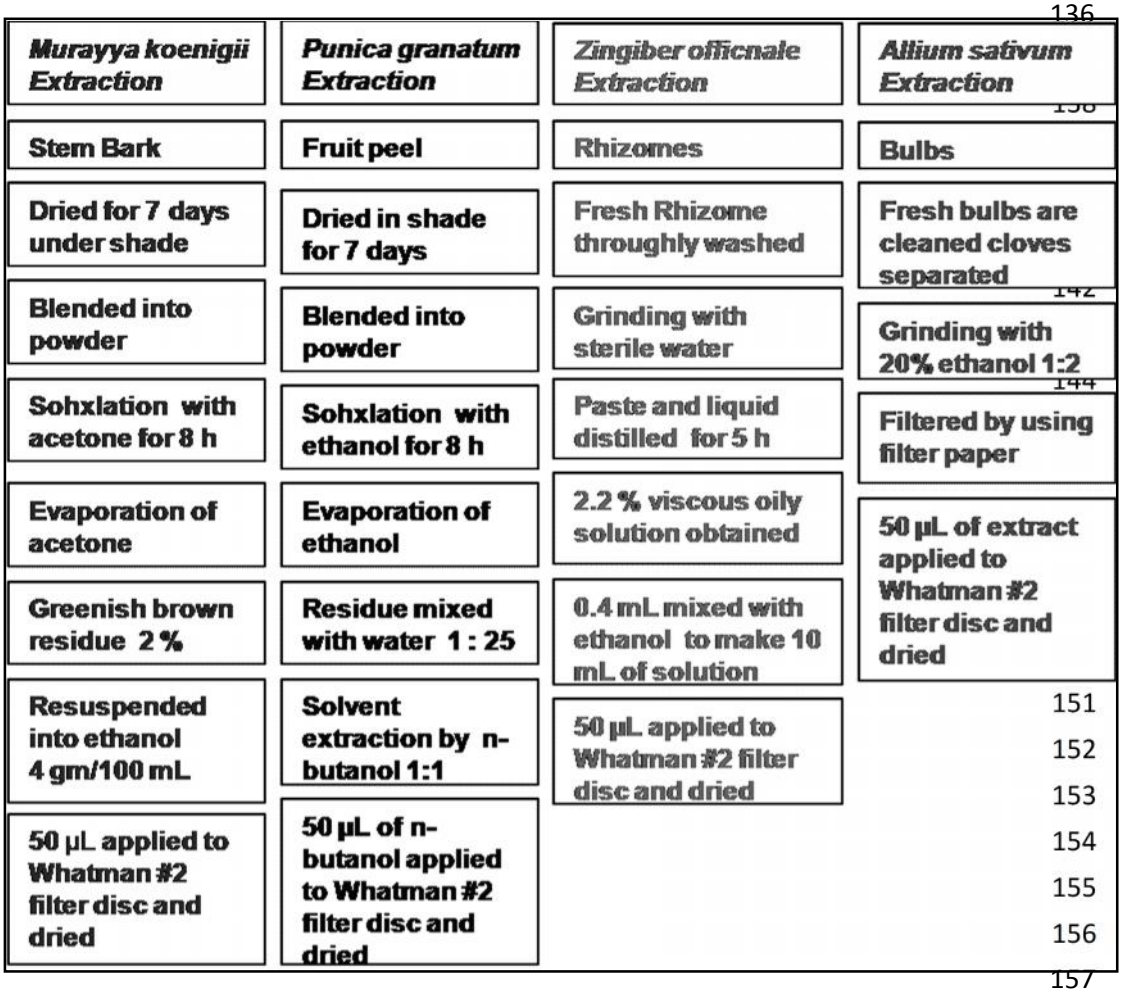


**Fig 2: Schematic representation of solvent extraction of plant extracts**

## 2.5 Anti-candidal assay using Disc-diffusion method

Anti-candidal activity of plant extracts was tested using Disc diffusion method (Bauer *et al*., 1966; NCCLS, 2000). *Candida* strains were maintained on Potato dextrose agar (PDA) medium. A loopful of culture from a slant culture was inoculated into the potato dextrose broth and incubated at 28ºC for 48 h. About 0.1 ml of this culture each was evenly spread on the PDA plates. Sterile discs of Whatmann No. 2 filter paper of about 6 mm in diameter were impregnated on the surface of the media. 50 µl each of the plant extracts were applied on to the discs, and incubated for 48 h at 28ºC. Results were recorded by measuring the diameter of the zone of inhibition around the discs.

## 2.6 Secreted Aspartyl Proteinase (SAP)

SAP production was according to method of Kwon-Chung et al. (1985). Log phase cultures of *C. albicans* were grown on Yeast extract, Peptone, Dextrose (YEPD) broth. The culture was centrifuged at 3000 rpm

for 10 min. and the cell pellet was washed with sterile distilled water, and $10^6$ washed cells were then inoculated into flasks (125 ml) containing 20 ml of bovine haemoglobin (BH) broth and incubated on a rotary shaker at 37°C for a total of 65 h. Culture (1 ml) was drawn periodically and centrifuged at 3,000 rpm in a clinical centrifuge for 10 min at 25°C to obtain the culture supernatant. The culture supernatant was the source of the SAP.

### 2.6.1 Enzyme (SAP) assay

SAP activity was determined spectrophotometrically following the digestion of bovine serum albumin (BSA) as the substrate described by Morrison et al. (2003). It (0.2 ml) was mixed with substrate (0.8 ml of 1% BSA in 0.025 M sodium citrate buffer, pH 2.5) and incubated at 37°C for 3 h. The reaction was halted by the addition of 2.0 ml of 5% trichloro acetic acid (TCA) resulting in precipitation of BSA and the enzyme. The tubes were kept at 4°C overnight and centrifuged at 2000 rpm for 20 min. Proteolysis was determined by measuring the absorbance of the soluble peptides at 280 nm. For control, substrate was added to the culture supernatant and immediately treated with TCA. The absorbance of controls was subtracted from test samples to obtain values for enzyme activity. The experiments were repeated four times and mean (±SD) of the readings was recorded.

## 2.7 Sample preparation for separation of allicin in ethanolic extract of *Allium sativum*

Fresh garlic cloves (20 g) of each sample were peeled, chopped and blended with 100 ml of 20% ethanol. The ethanol extract is filtered through filter paper. Quantification of allicin in the ethanol extract was according to Eagling and Sterling (2000), using internal standard method. Ethyl p-hydroxybenzoate, used as internal standard.

### 2.7.1 Preparation of internal standard solution

An internal standard solution with a concentration of 0.2 mg cm-3 was prepared. For this, approximately 80 mg of ethyl p-hydroxybenzoate (Sigma-Aldrich) was added to 8 $cm^3$ of methanol and the solution was shaken until it dissolved. Then, 360 cm3 of Milli-Q water at 353 K was added. Finally, 32 $cm^3$ of Milli-Q water at room temperature was added and the solution was shaken. After performing the first HPLC analysis, it was observed that the concentration of this internal standard solution was much higher than the allicin concentration of the samples.

### 2.7.2 Quantification of allicin

Allicin quantification in garlic extract samples was performed using ethyl p-hydroxybenzoate as the internal standard. The allicin concentration in the garlic samples was calculated using the equation below.

$$C_{Allicin} = \frac{C_{IS} \times Area_{Allicin} \times V_{IS}}{m_{Sample} \times Area_{IS}}$$

Where $C_{Allicin}$ is the allicin concentration (mg.g-1 sample), $C_{IS}$ is the concentration of the internal standard (mg.cm-3), $Area_{Allicin}$ is the area of the allicin peak, $Area_{IS}$ is the area of the internal standard peak, $V_{IS}$ is the volume of the mixture of the internal standard and the sample and $m_{Sample}$ is the mass of the sample that was analyzed on wet basis.

## 3. RESULTS AND DISCUSSION

*Candida* spp. constitute one of the major groups of pathogenic fungi, causing candidiasis, potentially life-threatening diseases that range from superficial to systemic mycoses. Last few decades the incidence of fungal infections by *C. albicans* and other related human opportunistic yeast species has increased dramatically due to their rise in the number of immune-compromised such as HIV infections many of whom are surviving longer with supportive therapy, and increased numbers of transplant recipients as well as cancer patients undergoing aggressive chemotherapy (Dolan *et al.,* 2009). Ten isolates were obtained from different dental hospitals of orally infected patients. Germ tube test, chlamydospore test and culture test were carried out to confirm the identification of *C. albicans*. Out of ten clinical isolates, the 5$^{th}$ isolates exhibited 90% germ tube formation at 2$^{nd}$ h (Table 1 & Fig 3), culture and chlamydospore formation (Fig 4a & 4b). Based on the preliminary results, the 5$^{th}$ isolate was selected for further studies, and ATCC 10231 was used as standard reference. The growth curves of both strains were monitored and growth was increased with the incubation period and started declining at decline phase.

**Table 1. Germ tube test**

| Isolates | GT at 1$^{st}$ h | GT at 2$^{nd}$ h |
|---|---|---|
| 1 | 58 | 60 |
| 2 | 48 | 51 |
| 3 | 56 | 55 |
| 4 | 45 | 50 |
| 5 | 50 | 90 |
| 6 | 65 | 53 |
| 7 | 43 | 49 |
| 8 | 50 | 55 |
| 9 | 46 | 52 |
| 10 | 46 | 58 |
| ATCC 10231 | 70 | 90 |

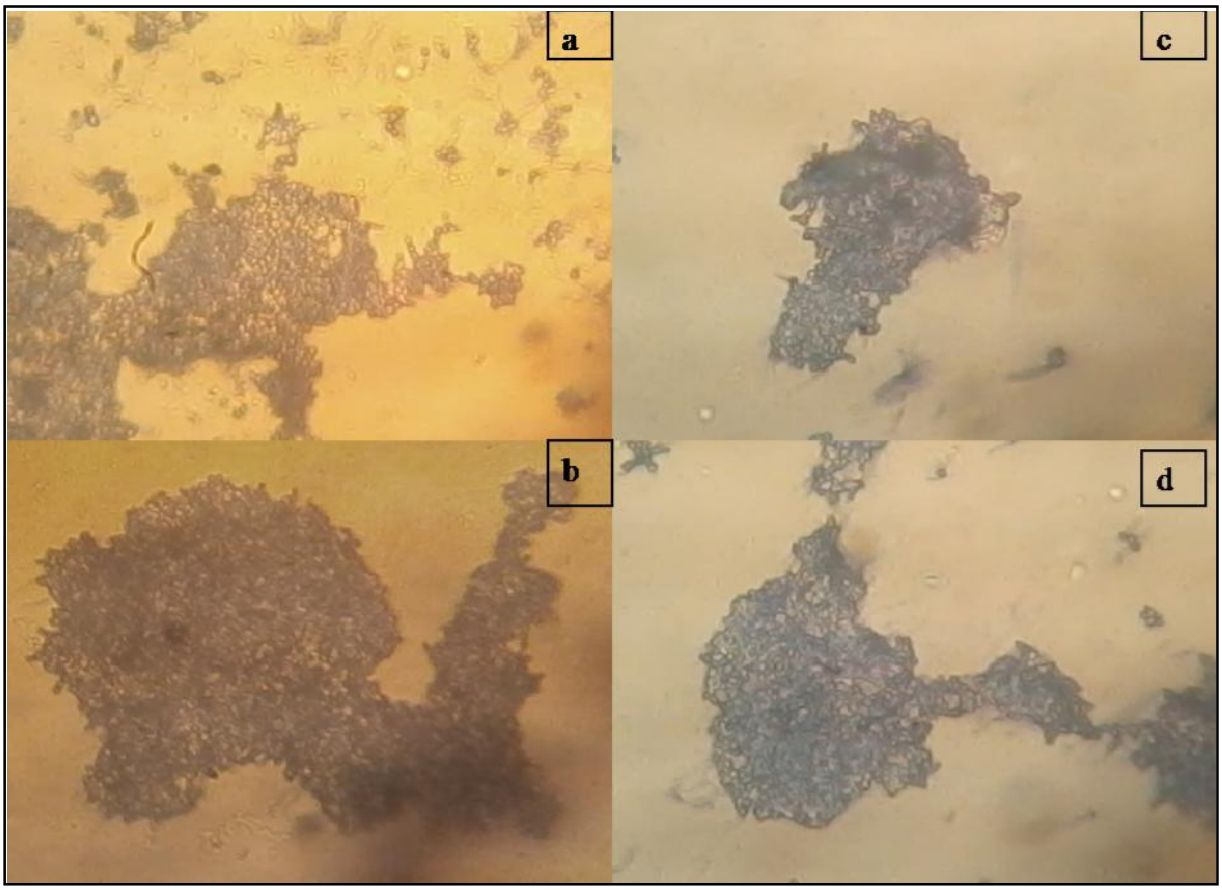


**Fig 3: Schematic representation of identification, confirmation tests and growth parameters of *Candida albicans* with standard ATCC 10231.**

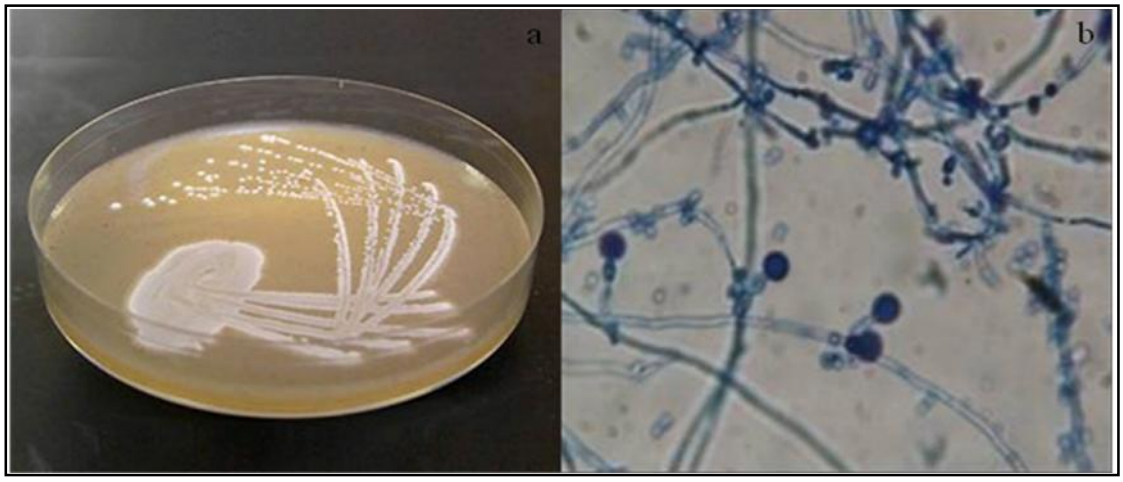


**Fig 4: (a) Culture test of clinical isolate strain of *Candida albicans*; (b) Chlamydospore formation test clinical isolate strain of *Candida albicans***

Optimization of growth conditions for isolate 5 has been studied by using various temperatures and pH conditions. The optimum temperature and pH was found to be 35 - 40ºC and pH 3.5, respectively (Table 2 & 3).

**Table 2. Effect of temperature on growth of *C. albicans***

| Temperature | ATCC 10231 | Isolate 5 |
|---|---|---|
| 20 | 1.12 | 1.09 |
| 25 | 1.51 | 1.84 |
| 30 | 1.78 | 1.79 |
| 35 | 1.89 | 1.92 |
| 40 | 1.81 | 1.89 |
| 45 | 1.55 | 1.67 |
| 50 | 0.74 | 0.80 |
| 55 | 1.05 | 0.10 |
| 60 | 0.01 | 0.02 |
| 65 | 0.01 | 0.02 |

**Table 3. Effect of pH on growth of *C. albicans***

| pH | ATCC 10231 | Isolate 5 |
|---|---|---|
| 2 | 0.24 | 0.25 |
| 2.5 | 0.65 | 0.69 |
| 3 | 1.32 | 1.38 |
| 3.5 | 1.95 | 1.98 |
| 4 | 1.86 | 1.85 |
| 4.5 | 1.37 | 1.42 |
| 5 | 0.71 | 0.76 |
| 5.5 | 0.32 | 0.40 |
| 6 | 0.18 | 0.21 |
| 6.5 | 0.11 | 0.13 |
| 7 | 0.03 | 0.05 |
| 7.5 | 0.01 | 0.02 |
| 8 | 0.01 | 0.01 |

The plant materials selected in the present study were shown to possess good anti-candidal activity. Preparation of plant extracts were by soxhlation method with slight modifications. Disc-diffusion (Zone of inhibition) was employed for *in vitro* anti-candidal activity tests. The zone of inhibition of the clinical isolate

was in the following order; *Allium sativum* (28 mm) > *Punica granatum* (22 mm) > *Murayya koyeingii* (16 mm) > *Zingiber officinale* (15 mm) (Fig 5). For ATCC 10231 strain the order of inhibition was as follows; *Allium sativum* (30 mm) > *Punica granatum* (20 mm) > *Murayya koyeingii* (19 mm) > *Zingiber officinale (*12 mm) (Table 4). Flucanzole was used as a positive standard (experimental) control and exhibited zone in the range of 25 - 30 mm and ethanol was used as negative standard control.

**Table 4: *In vitro* anti-candidal activity by Disc- diffusion method**

| S. No | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| ATCC 10231 | 30 | 19 | 12 | 20 | 30 | - |
| Isolate 5 | 28 | 16 | 15 | 22 | 25 | - |

1=*Allium sativum;* 2=*Murayya koenigii*; 3=*Punica granatum*; 4=*Zingiber officinale;* 5= Flucanazole (40 µg/mL) standard; 6= Ethanol (negative standard control). All experiments were carried out in triplicates and the values expressed are mean value of the zone of inhibition (diameter in mm) of the plates.

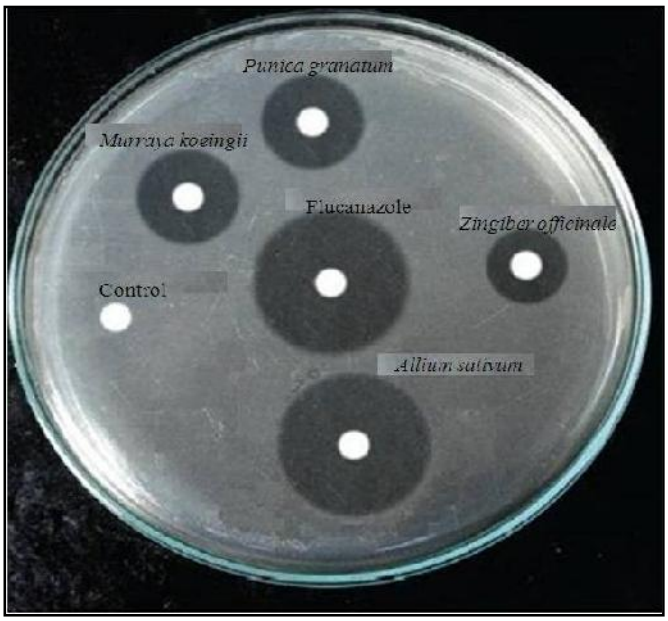


**Fig 5: *In vitro* anti-candidal activity by Disc- diffusion method**

The Secreted Aspartyl Proteinases were found to be both in isolate 5 and in ATCC 10231 (Fig 6). Allicin which is partly characterized from the cloves of *Allium sativum* were further subjected to anti-candidal activity. Allicin exhibited zone of inhibition in both isolate 5 and ATCC 10231. Upon allicin treatment, SAP was destroyed in both Isolate 5 and ATCC 10231 (Fig 6).

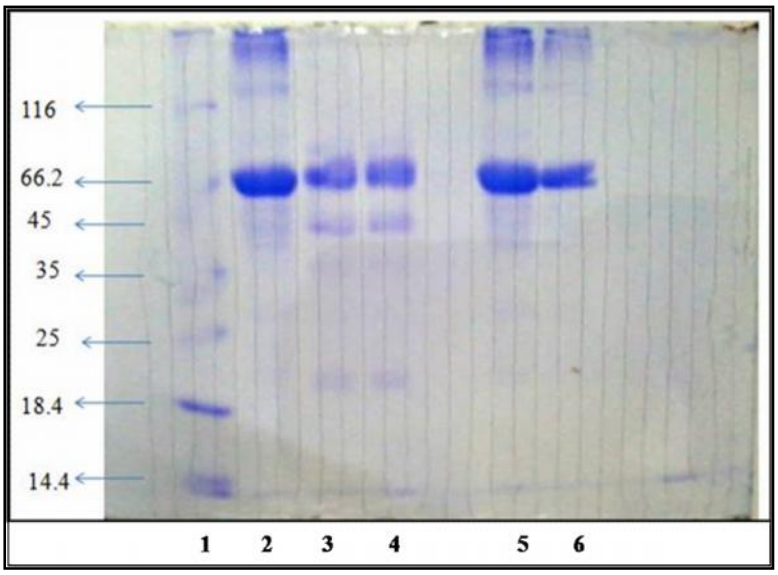


**Fig 6. SDS- PAGE of SAP enzyme of both clinical isolate and ATCC 10231 strains. 1= Molecular marker; 2= Bovine serum albumin; 3= clinical isolate; 4= ATCC 10231; 5 & 6 represents SAP after treatment with allicin.**

Allicin quantification in garlic extract samples was performed using ethyl p-hydroxybenzoate as the internal standard. The allicin concentration in the garlic extract samples was found to be 214 µg/mL and quantified by using HPLC chromatogram. The retention time for allicin was found to be 6 and 9 for the internal standard (Table 5 & Fig 7).

**Table 5. Allicin quantification using HPLC chromatogram**

| **Sample** | **Ethanol extract of garlic** |
|---|---|
| Conc. of internal standard (mg cm-3) | 0.2 |
| Retention of allicin (min) | 6.50 |
| Peak area of allicin | 1,051,669 |
| Retention of internal standard (min) | 9.22 |
| Peak area of internal standard | 4,903,720 |
| Allicin concentration (µg $g^{-1}$sample) | 214 |

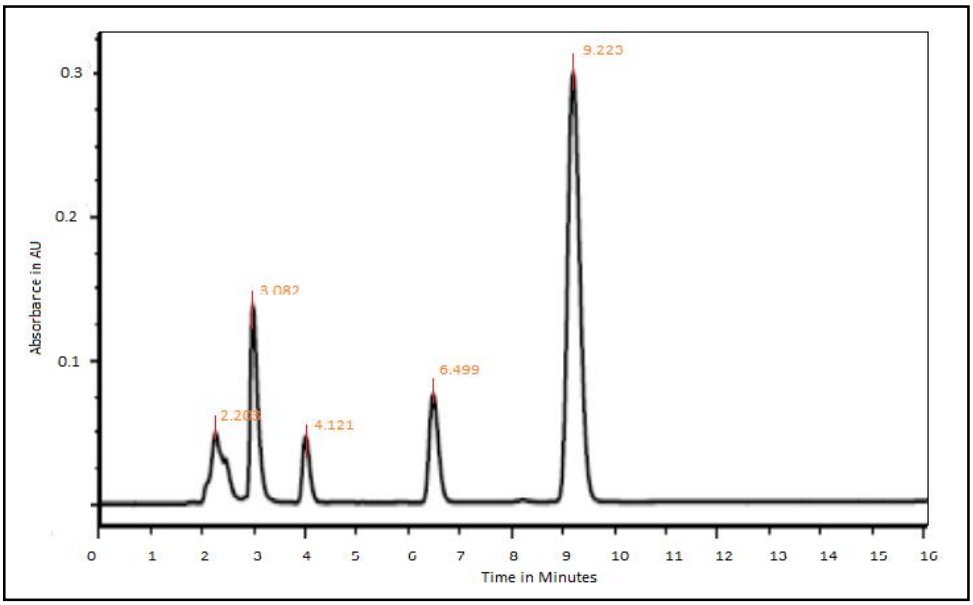


**Fig 7: HPLC chromatogram of allicin quantification**

The present study shows the effect of ethanolic extracts of *Allium sativum* cloves on *C. albicans* and on its SAP. The 20% ethanol extract showed high yield of allicin and also was stable for more time than the other % ethanol solvent extractions. The data on other ethanol percentages are not presented. These results are in accordance with the Chang *et al.* (2013). Allicin has antibacterial, antiviral, antiparasitic and anti hypertensive and hepatoprotective effects (Cai *et al.,* 2007). Allicin was found to have important anticandidal activity owing to its interaction with thiol groups of proteins and aminoacids, especially with the latter, and allicin forms s-allyl derivatives. By these reactions SH-compounds inhibit the antibiotic properties of extract derived allicin and authentic allciin (Ogita *et al.,* 2007).

*Candida* species have developed an effective battery of putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease, and overcome host defenses (Naglik, 2003). The virulence factors expressed or required by *Candida* species, and in particular *C. albicans*, to cause infections may well vary depending on the type of infection (*i.e*., mucosal or systemic), the site and stage of infection, and the nature of the host response (Ferreira *et al.,* 2010). The destruction of SAP by allicin needs further specific study with pure samples of SAP and allicin. Because the ethanol extract may contain other compounds and enzymes other than allicin and allinase that may take part in destruction of SAP. It was shown, however, that total enzyme was destroyed by ethaolic extract of *Allium sativum*, which was confirmed by SDS-PAGE (10% gel) electrophoresis.

## 4. CONCLUSION

From the results, it has been concluded that 20% ethanol extract of *Allium sativum* yields high percentage of allicin production and also stable for more time. Allicin produced not only exhibited anti–candidal activity but also destroyed SAP, key virulence factor of *Candida albicans*. Further studies on *in vivo* testing and other key virulence factors are in progress in our laboratory to test the pathogenicity of the *Candida albicans* and also the efficacy of plant extracts.

## CONSENT

Not applicable

## ETHICAL APPROVAL

Not applicable

## REFERENCES


1. Ali, BH, Blunden G, Tanira MO, Nemmar A. Some phytochemical, pharmacological and toxicological properties of ginger (*Zingiber officinale* Roscoe): A review of recent research. Food Chem. Toxicol. 2008; 46: 409-420.
2. Arulselvan P, Subramanian SP. Beneficial effects of *Murraya koenigii* leaves on antioxidant defense system and ultra structural changes of pancreatic beta–cells in experimental diabetes in rats, Chem Biol Interact. 2007; 165 (2): 155-164.
3. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol. 1996; 45: 493-496.
4. Cai Y, Wang R, Pei F, Liang BB. Antibacterial activity of allicin alone and in combination with β-lactams against *Staphylococcus* sp. and *Pseudomonas aeruginosa*. J. Antibiotics. 2007; 60: 335-338.
5. Chen IN, Chang CC, Ng CC, Wang CY, Shyu YT. Antioxidant and antimicrobial activity against of *Zingiberaceou*s plants in Taiwan. Plants Foods Hum Nutr. 2008; 63:15-20.
6. Cheriti A, Belboukhari M, Belboukhari N, Djeradi H. Phytochemical and biological studies on *Launaea cass*. Genus (Asteraceae) from Algerian Sahara. Curr Top Phytochem. 2012; 11: 67-80.
7. Dolan K, Montgomery S, Buchheit B, Didone L, Wellington M, Krysan DJ. Antifungal activity of tamoxifen: *in vitro* and *in vivo* activities and mechanistic characterization. Antimicrob Agents Chemother. 2009; 53(8):3337-46.
8. Eagling DR, Sterling SJ. A cholesterol-lowering extract from garlic. Rural Industries Research and Development Corporation, Australia Tech. Rep.2000; 1-14.
9. Ferreira C, Silva S, Faria-oliveria F, Pinho E, Henriques M, Lucas C. *Candida albicans* virulence and drug-resistance requires the O-acyltransferase Gup1p. BMC Microbiol. 2010; 10: 238.

10. Fontenelle RO, Morais SM, Brito EH, Kerntopf MR, Brilhante RS, Cordeiro RA,Tomé AR, Queiroz MG, Nascimento NR, Sidrim JJ, Rocha MF. Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. J Antimicrob Chemother. 2007; 59 (5): 934–940.
11. Gurib-Fakim A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine. 2006; 27: 1–93.
12. Halberstein R. Medicinal plants: historical and cross-cultural usage patterns. Annals of Epidemiology. 2005; 15: 686.
13. Jabar MA, Al-Mossawi. Susceptibility of some multiple resistance bacteria to garlic extract. Afr J Biotechnol. 2007; 6: 771-775.
14. Kwon CKJ, Lehman D, Good C, Magee PT. Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. Infect. Immunol. 1985: 49:571-575.
15. Lee KH, Shin WS, Kim D, Koh CM. The presumptive identification of *Candida albicans* with germ tube induced by high temperature. Yonsei Med J. 1999; 40: 420-424.
16. Machado TB, Leal ICR, Kuster RM, Amaral ACF, Kokis V, Silva MG, Santos KRN. Brazilian phyto-pharmaceuticals evaluation against hospital bacteria Phytotherapy Research (2005); *19*: 519-525.
*17.* Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJG, Verweij PE, Cuenca-Estrella M, Rodriguez-Tudela JL. A New *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of cyp51A alterations. Antimicrob Agents Chemother. 2007; *51: 1897-1904.*
18. Mendonca-Filho RR. Bioactive Phytocompounds: New approaches in the Phytosciences. In: Modern Phytomedicine: Turning Medicinal Plants into Drugs. 2006; Ahmad, I., F. Aqil and M. Owas (Eds.). Wiley-VCH, Weinheim, Germany
19. Morrison CJ, Hurst SF, Reiss E. Competitive binding inhibition enzyme-linked immunosorbent assay that uses the secreted aspartyl proteinase of *Candida albicans* as an antigenic marker for diagnosis of disseminated candidiasis. Clin. Diagn. Lab. Immunol.2003; 10:835-848.
20. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev. 2003; 67(3):400-28.
21. National Committee for Clinical Laboratory Standards (NCCLS). Approved standard M7-A5. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically 5th edn. 2000. Wayne, PA
22. Ogita A, Nagao Y, Fujita K, Tanaka T. Amplification of vacuole targeting fungicidal activity of antibacterial antibiotic polymyxin B by allicin, an allyl sulfur compound from garlic. J. Antibiot (Tokyo).2007; 60: 511-518.
23. Rois JL, Recio MC. Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology. 2005; 100; 80–84.

24. Singh RP, Chidambara Murthy KN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. J Agric Food Chem. 2002; 50(1):81-86.
25. Verghese S, Padmaja P, Asha M, Elizabeth SJ, Anitha A, Kundavi KM, Jayanthi N, Varma, T. Prevalence, species distribution and antifungal sensitivity of vaginal yeasts in infertile women. Ind J Pathol Microbiol. 2001; 44: 313-314.