



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

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### **Authors' contributions**

*This work was carried out in collaboration between all authors. Authors NV and KHR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author OVSR managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.*

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### **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 chlamyospore 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

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to be 35 - 40°C 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 [1,2]. *Candida albicans* is a ubiquitous, most common fungal pathogen of humans and etiological agent of candidosis. It has ranked fourth place in most commonly encountered nosocomial pathogens in blood stream infections [3]. 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 [1].

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 [4]. 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 [5]. 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 [6].

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 [7]. 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 [8]. 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 [9]. *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 [10] 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 [11]. 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 candidosis 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 sub cultured for identification purpose. Presumptive identification methods [12] 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 chlamyospore 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 Chlamyospore formation

All *Candida* isolates were tested for the production of chlamyospores 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 chlamyospores.

### 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.

#### 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.

### 2.5 Anti-candidal Assay Using Disc-diffusion Method

Anti-candidal activity of plant extracts was tested using Disc diffusion method [13,14]. *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 28°C 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 28°C. Results were recorded by measuring the diameter of the zone of inhibition around the discs.



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

<b><i>Murayya koenigii</i> Extraction</b>	<b><i>Punica granatum</i> Extraction</b>	<b><i>Zingiber officinale</i> Extraction</b>	<b><i>Allium sativum</i> Extraction</b>
Stem Bark	Fruit peel	Rhizomes	Bulbs
Dried for 7 days under shade	Dried in shade for 7 days	Fresh Rhizome thoroughly washed	Fresh bulbs are cleaned cloves separated
Blended into powder	Blended into powder	Grinding with sterile water	Grinding with 20% ethanol 1:2
Sohxlation with acetone for 8 h	Sohxlation with ethanol for 8 h	Paste and liquid distilled for 5 h	Filtered by using filter paper
Evaporation of acetone	Evaporation of ethanol	2.2 % viscous oily solution obtained	50 µL of extract applied to Whatman #2 filter disc and dried
Greenish brown residue 2 %	Residue mixed with water 1 : 25	0.4 mL mixed with ethanol to make 10 mL of solution	
Resuspended into ethanol 4 gm/100 mL	Solvent extraction by n-butanol 1:1	50 µL applied to Whatman #2 filter disc and dried	
50 µL applied to Whatman #2 filter disc and dried	50 µL of n-butanol applied to Whatman #2 filter disc and dried		

Fig. 2. Schematic representation of solvent extraction of plant extracts

## 2.6 Secreted Aspartyl Proteinase (SAP)

SAP production was according to method of Kwon-Chung et al. [15]. 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. [16]. 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 ( $\pm$ 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 [17], using internal standard method. Ethyl p-hydroxy benzoate, used as internal standard.

### 2.7.1 Preparation of internal standard solution

An internal standard solution with a concentration of 0.2 mg cm<sup>-3</sup> was prepared. For this, approximately 80 mg of ethyl p-hydroxybenzoate

(Sigma-Aldrich) was added to 8 cm<sup>3</sup> of methanol and the solution was shaken until it dissolved. Then, 360 cm<sup>3</sup> of Milli-Q water at 353 K was added. Finally, 32 cm<sup>3</sup> 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<sup>-1</sup> sample),  $C_{IS}$  is the concentration of the internal standard (mg.cm<sup>-3</sup>),  $Area_{Allicin}$  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 [2,18,19,20]. Ten isolates were obtained from different dental hospitals of orally infected patients. Germ tube test, chlamyospore test and culture test were carried out to confirm the identification of *C. albicans*. Out of ten clinical isolates, the 5<sup>th</sup> isolates exhibited 90% germ tube formation at 2<sup>nd</sup> h (Table 1 and Fig. 3), culture and chlamyospore formation (Fig. 4a and 4b). Based on the preliminary results, the 5<sup>th</sup> 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.

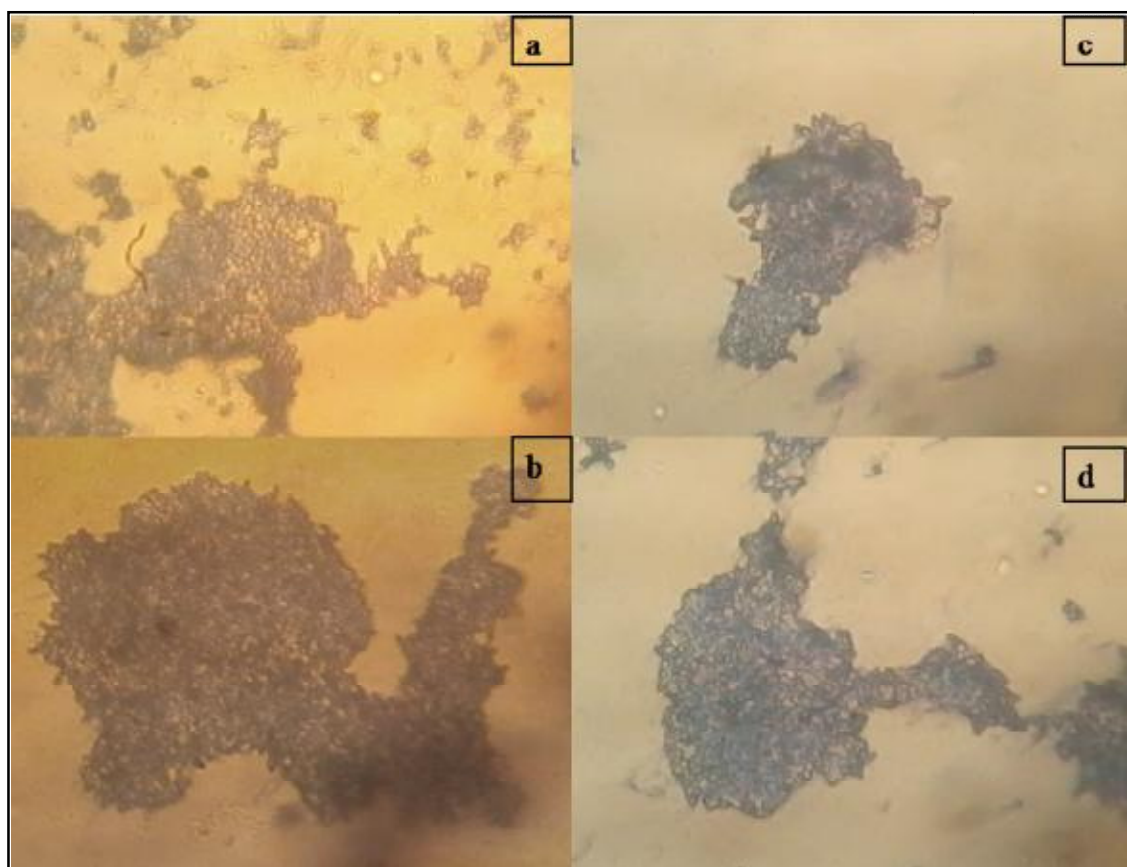
Optimization of growth conditions for isolate 5 has been studied by using various temperatures and pH conditions. The optimum temperature and pH was found to be 35 - 40°C and pH 3.5, respectively (Tables 2 and 3).

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).

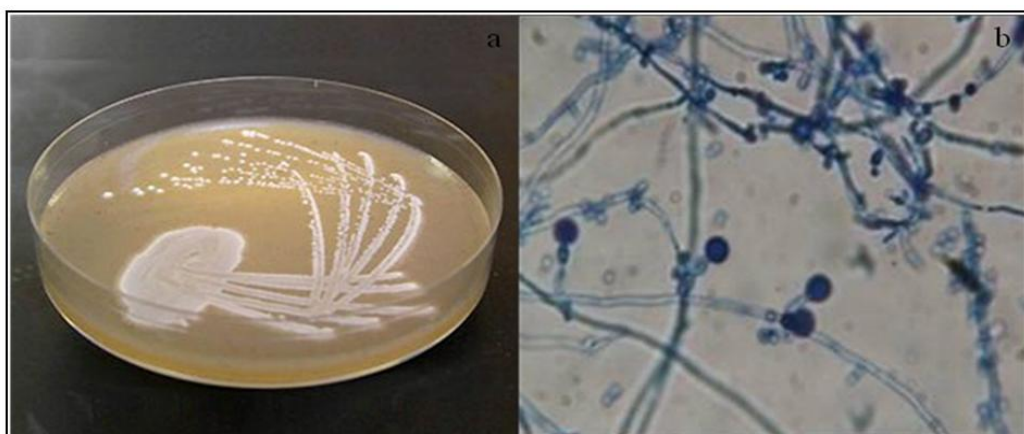
Flucanazole was used as a positive standard (experimental) control and exhibited zone in the range of 25 - 30 mm and ethanol was used as negative standard control.

**Table 1. Germ tube test**

Isolates	GT at 1 <sup>st</sup> h	GT at 2 <sup>nd</sup> 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) Chlamyospore formation test clinical isolate strain of *Candida albicans***

**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 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).

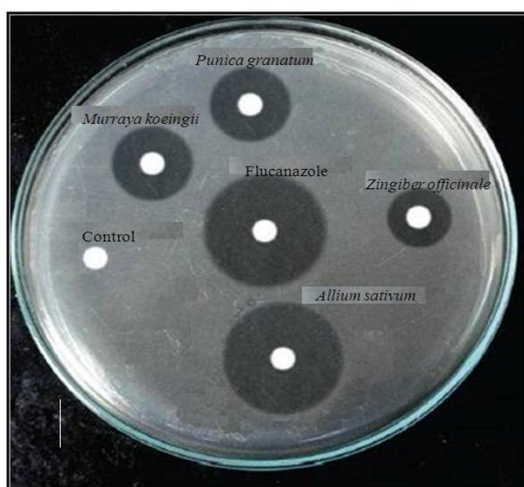
Allicin quantification in garlic extract samples was performed using ethyl p-hydroxybenzoate as the internal standard. The allicin concentration in the garlic extract samples was found to be 214 µg/mL and quantified by using HPLC chromatogram. The retention time for allicin was found to be 6 and 9 for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

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 [21]. Allicin has antibacterial, antiviral, antiparasitic and anti hypertensive and hepatoprotective effects [22]. 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 allicin [23].

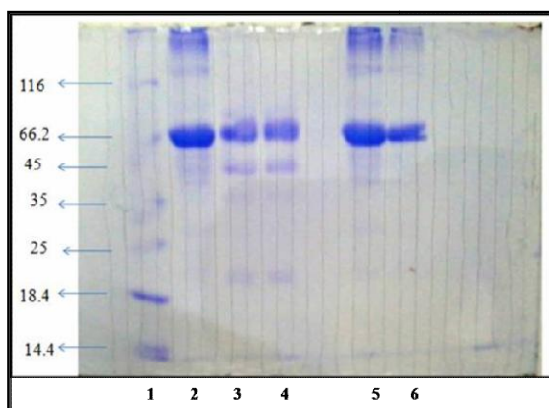
**Table 4. In vitro anti-candidal activity by Disc- diffusion method**

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**



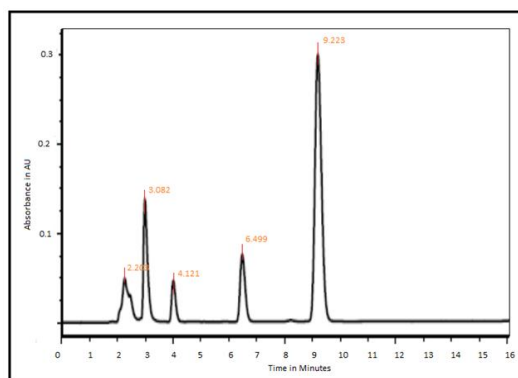
**Fig. 6. SDS- PAGE of SAP enzyme of both clinical isolate and ATCC 10231 strains. 1= Molecular marker; 2= Bovine serum albumin; 3= clinical isolate; 4= ATCC 10231; 5 & 6 represents SAP after treatment with allacin**

*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 [1,24,25]. 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 [26]. The destruction of SAP by allacin needs further specific study with pure samples of SAP and allacin. Because the ethanol extract may contain other compounds and enzymes other than allacin 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.

**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 <sup>-1</sup> sample)	214



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



#### 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

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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