Original Research Article 1 In vitro Anticandidal Activity of Extracts of Some 2 Medicinal Plants on Clinical Isolate Selected of 3 Candida albicans and its Standard ATCC 10231 Strain 4

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12

6 ABSTRACT

7 Aim: Extracts of four different medicinal plant parts such as stem bark of Murayya koenigii (Curry leaf 8 plant), fruit peel of Punica granatum (Pomegranate), cloves of Allium sativum (Garlic) and rhizome of 9 Zingiber officinale (Ginger) were used to test their anti-candidal activity on clinical isolate of Candida 10 albicans and its ATCC 10231 strain.

11 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. 13 Secreted aspartyl proteinase (SAP) enzyme was assayed spectrophotometrically using bovine serum 14 albumin as substrate.

15 Results: Out of ten isolates of Candida albicans made in the laboratory, isolate 5 showed good growth as 16 compared to its standard culture ATCC 10231. Optimum temperature and pH was found to be 35 -40°C 17 and pH 3.5 – 4, respectively. The garlic extract exhibited better anti-candidal activity on clinical isolate 5

18 and ATCC 10231 than the other plant extracts, as the zone of inhibition was found to be 28 mm for isolate

19 5 and 30 mm for ATCC 10231. Allicin, a highly unstable molecule was found to be a major ingredient of

20 Allium sativum, and its concentration was found to be 214 µg/mL.

21 Conclusion: Allicin has not only exhibited good anti-candidal activity but also destroyed the Secreted 22 Aspartyl Proteinase (SAP), a key enzyme responsible for the candidiosis in humans.

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24 Keywords: Anti-candidal activity; Candida albicans; disc diffusion; Secreted aspartyl proteinases

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26 **1. INTRODUCTION**

27 The World Health Organization (WHO) has recognized the potential utility of traditional remedies and 28 strives to preserve the primary health care involving medicinal plants. Although some of the therapeutic 29 properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the 30 empirical findings of hundreds and thousands of years. One of the most efficient ways of finding new 31 bioactive compounds is collecting data on the use of medicinal plants in traditional pharmacopeia.

32 Candida spp. constitutes one of the major groups of pathogenic fungi, and the incidence of infections has

33 increased dramatically over the past three decades causing increase in morbidity and mortality in

34 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).

41 The increase in incidence of fungal infections worldwide especially due to Candida spp. and emergence 42 of antifungal resistance among clinical isolates especially against the conventionally used azole 43 antifungals, poses an important challenge to the clinician. Much attention has been paid to plant derived 44 antifungal compounds based on the knowledge that plants have their own defense system (Fontenelle, 45 2007). Since ancient times, mankind has used plants to treat common diseases and some of these 46 traditional medicines are still included as part of the habitual treatment of various maladies (Rois and 47 Reico, 2005). However, in the absence of a scientific research base such practices may induce serious 48 adverse effects in patients. Medicinal plants possess many bioactive properties, usually antimicrobial, 49 antioxidant, anticancer and anti-inflammatory. They also represent a rich source of antimicrobial agents. 50 Therefore, medicinal plants extracts and their derived products offer considerable potential for the 51 development of new agents effective against infections currently difficult to treat (Machado et al., 2005).

Researchers worldwide have literally found thousands of phytochemicals 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.

58 In the present study, Murayya koenigii (Linn) Spreng, a member of family Rutaceae, is a deciduous to 59 semi-evergreen aromatic tree found throughout India. Traditionally it is used as an analgesic, febrifuge, 60 stomachic, carminative and for the treatment of dysentery and skin eruptions (Arulselvan and 61 Subramanian, 2007). This is commonly known as curry leaf, used as spice due to the aromatic nature of 62 leaves. Punica granatum is commonly known as pomegranate, native to the region from northern India to 63 Iran. It is a fruit bearing deciduous shrub or small tree which belongs to the family Lythraceae. Zingiber 64 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 65 66 diseases (Ali et al., 2008) were selected and studied against Candida albicans clinical isolate and a 67 standard C. albicans ATCC 10231 strain. Allium sativum (Garlic) is a common spice belongs to Liliaceae 68 family commonly used for flavouring and has been traditionally popular with strong folkloric awareness. It 69 is rich in sulphur- based compounds which contribute to the characteristic odour (Jabar and Al-Mossawi, 70 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*.

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74 2. MATERIALS AND METHOD

75 **2.1 Candida albicans strains**

76 Ten isolates of Candida albicans were collected from different dental hospitals, of patients suffering from 77 oral thrush. C. albicans isolated from oral candidiosis patients by using oral swabs. Sterile cotton swabs 78 were wiped on thrush present in oral cavity. The swabs were aseptically streaked on Sabouraud's 79 dextrose agar (SDA) plates. Samples were cultured on SDA in an aerobic atmosphere at 37°C for 48 h. 80 Suspected yeast colonies were subcultured for identification purpose. Presumptive identification methods 81 (Lee et. al., 1999) used to identify the colonies for further studies. The standard culture of Candida 82 albicans ATCC 10231 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, 83 India. Schematic representation of confirmation tests and growth parameters of Candida albicans of both 84 clinical isolate along with standard ATCC 10231 is represented in Fig 1. 85

86 2.2 Identification test for Candida albicans

87 Culture test, germ tube test and chlamydospore formation test were used for the confirmation of *C.*88 *albicans* isolates.

89

90 **2.2.1 Culture test**

91 The suitable samples were cultured on plain SDA and also SDA containing chloramphenicol and 92 cycloheximide. SDA plates were incubated at 37°C and examined twice a week to look for growth of 93 cream coloured pasty colonies suggestive of *Candida* species. Plates incubated for one week and 94 observed for growth.

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97 **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.

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102 2.2.3 Chlamydospore formation

All *Candida* isolates were tested for the production of chlamydospores in corn meal agar with Tween 80.

104 The *Candida* strains were inoculated in corn meal agar (CMA) and then incubated at 25°C. After 72 h, the

105 plates were examined under the microscope for the presence of chlamydospores.

106

107 2.3 Growth of *Candida albicans* at different intervals of time

108 A loopful of culture from slant was taken and inoculated into the Sabourad Dextrose (SD) broth medium 109 and incubated for 48 h at 28°C. After attaining log phase, the culture was taken and transferred into broth 110 medium and the absorbance (OD) was measured at 540 nm at regular intervals of time up to 48 h.

111

2.4 Collection of plant material and preparation of extracts

113 2.4.1 Collection of plant materials

114 Stem bark of *Murayya koenigii*, *fruit* peel of *Punica granatum*, cloves of *Allium sativum* and rhizome of 115 *Zingiber officinale* (Fig 1), were procured from the local market/ area and used in the present study.



- 128
- 129 Fig 1: a= Murayya koenigii (Stem bark); b= Punica granatum (Fruit peel); c= Zingiber
- 130 *officinale* (Rhizome) d= *Allium sativum* (cloves) were selected for the study.
- 131 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.



158

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

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161 **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 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.

169 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 10⁶ 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.

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178 2.6.1 Enzyme (SAP) assay

179 SAP activity was determined spectrophotometrically following the digestion of bovine serum albumin 180 (BSA) as the substrate described by Morrison et al. (2003). It (0.2 ml) was mixed with substrate (0.8 ml of 181 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 182 183 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 184 185 added to the culture supernatant and immediately treated with TCA. The absorbance of controls was 186 subtracted from test samples to obtain values for enzyme activity. The experiments were repeated four 187 times and mean (±SD) of the readings was recorded.

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189 **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.

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195 **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 cm³ of methanol and the solution was shaken until it dissolved. Then, 360 cm3 of Milli-Q water at 353 K was added. Finally, 32 cm³ 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.

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- 203

204 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.

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

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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.

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213 3. RESULTS AND DISCUSSION

Candida spp. constitute one of the major groups of pathogenic fungi, causing candidiasis, potentially lifethreatening diseases that range from superficial to systemic mycoses. Last few decades the incidence of fungal infections by *Candida 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 5th isolate exhibited 90% germ tube formation at 2nd hour (Table 1 & Fig 3) and also culture and chlamydospore formation (Fig 4a & 4b). Based on the preliminary results, the 5th isolate was selected for further studies, and ATCC 10231 was used as standard reference.

225

226 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

227 228



229

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*

244

245 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 (Table

- 247 2 & 3).
- 248

249 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

250

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

рН	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				

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253

254 The plant materials selected in the present study were shown to possess good anti-candidal activity. 255 Preparation of plant extracts were by soxhlation method with slight modifications. Disc-diffusion (Zone of 256 inhibition) was employed for in vitro anti-candidal activity tests. The zone of inhibition of the clinical isolate 257 was in the following order; Allium sativum (28 mm) > Punica granatum (22 mm) > Murayya koyeingii (16 258 mm) > Zingiber officinale (15 mm) (Fig 5). For ATCC 10231 strain the order of inhibition was as follows; 259 Allium sativum (30 mm) > Punica granatum (20 mm) > Murayya koyeingii (19 mm) > Zingiber officinale 260 (12 mm) (Table 4). Flucanzole was used as a positive standard (experimental) control and exhibited zone 261 in the range of 25-30 mm and ethanol was used as negative standard control.

262

263 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	-				
264								_			
265											
266											
267											
268	1=Allium sativur	m; 2=Mura	ayya koen	igii; 3=Pı	ınica gra	anatum; -	4=Zingi	ber offic	cinale; 5	= Flucana	zole (40
269	µg/mL) standard	l; 6= Etha	nol (negat	tive stand	ard cont	rol). All e	experim	ents we	ere carrie	ed out in tr	iplicates
270	and the values e	expressed	are mean	value of t	he zone	of inhibiti	ion (diaı	neter in	mm) of	the plates.	
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275	NAU	urraya koeingi	i	1999							
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287 Fig 5: In vitro anti-candidal activity by Disc- diffusion method

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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 μ g/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).

313

314 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



329 **Fig 7: HPLC Chromatogram of allicin quantification**

330

331 The present study shows the effect of ethanolic extracts of Allium sativum cloves on C. albicans and on 332 its SAP. The 20% ethanol extract showed high yield of allicin and also was stable for more time than the 333 other % ethanol solvent extractions. The data on other ethanol percentages are not presented. These 334 results are in accordance with the Chang et al. (2013). Allicin has antibacterial, antiviral, antiparasitic and 335 anti hypertensive and hepatoprotective effects (Cai et al., 2007). Allicin was found to have important 336 anticandidal activity owing to its interaction with thiol groups of proteins and aminoacids, especially with 337 the latter, and allicin forms s-allyl derivatives. By these reactions SH-compounds inhibit the antibiotic 338 properties of extract derived allicin and authentic allciin (Ogita et al., 2007).

339 Candida species have developed an effective battery of putative virulence factors and specific strategies 340 to assist in their ability to colonize host tissues, cause disease, and overcome host defenses (Naglik, 341 2003). The virulence factors expressed or required by Candida species, and in particular C. albicans, to 342 cause infections may well vary depending on the type of infection (*i.e.*, mucosal or systemic), the site and 343 stage of infection, and the nature of the host response (Ferreira et al., 2010). The destruction of SAP by 344 allicin needs further specific study with pure samples of SAP and allicin. Because the ethanol extract may 345 contain other compounds and enzymes other than allicin and allinase that may take part in destruction of 346 SAP. It was shown, however, that total enzyme was destroyed by ethaolic extract of Allium sativum, 347 which was confirmed by SDS-PAGE (10% gel) electrophoresis.

348

349 **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*.

353

- 354 CONSENT
- 355 Not applicable
- 356
- 357 ETHICAL APPROVAL
- 358 Not applicable
- 359

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