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Research Article

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Anticandidal activity of some plant extracts

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ABSTRACT

Candidiasis, especially by *Candida albicans* is the most prevalent disease over the years. To control the infection, several synthetic drugs and their formulations have been applied. Although antifungals are quite effective in treating candidiasis, long term use has been reported to have side effects. Nevertheless, it has other drawbacks such as efficiency as well as cost, recurrence of the infection, emergences of resistant strains etc. Thus, plant based natural compounds are being investigated for their antifungal activity. In the present study, five different plant extracts assessed exhibited retardation of growth and protease production (molar concentration) in *C. albicans*. The mycelia form of the organism showed growth resistance to tested plant extracts than the yeast extract form which conferred the higher pathogenicity of the mycelia form. The minimum inhibitory concentration (MIC) of each plant extract was experimentally evidenced with the oil obtained from the seeds of *Pongamia glabrata* showed the MIC values at the lowest concentration (20-30 µL/mL), followed by seed oil of *Azadirachta indica* and *Ricinus communis*. The order of candidostatic efficacy of the various oils was observed to be: *Pongamia* > *Azadirachta* > *Ricinus* > *Eucalyptus* > *Curcuma*. These findings have paved the way for further investigation of plant based antifungal agents and their clinical appropriateness for the treatment of Candidiasis.

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INTRODUCTION

It is well evident that the appropriate yeast *Candida albicans*, a unicellular fungus, causes superficial or deep infections in immune-deficient hosts or when the host immunity is impaired or the host is treated with certain therapeutics (Molina *et al.*, 1992). The frequency of the disease has been increased remarkably due to health conditions like diabetes, HIV, chemotherapy as well as regular use of antibiotics (Akinyemi *et al.*, 2005). Mycoses are the most ubiquitous and most difficult of all microbial infections which is largely due to limited therapeutic agents available and the lack of reliable diagnostic methodologies (Zervos & Meunier, 1993). Moreover, traditional treatment modalities are unable to treat *Candida* infection when different sites of infection (mucosal, cutaneous, sub-cutaneous and systemic) and drug-resistant fungal strains are concerned (Mathews, 1994; Sati & Joshi, 2011). Although, antifungal agents like fluconazole, echinocandins, and amphotericin B are effective in treating *C. albicans*, long-term antifungal use and recurrent infection have been reported (Arendrup & Patterson, 2017).

Since the beginning of history, plants have been treasured for their medical utilities. Plant produced compounds are a safer and more effective substitute for synthetically produced antimicrobial agents and hence, are of great interest. However, hundreds of plant species have been analysed for antimicrobial properties (Doddanna *et al.*, 2013; Haba *et al.*, 2014; Ebrahimy *et al.*, 2015; Soares *et al.*, 2015; Varadarajan *et al.*, 2015; Al-Abdalall, 2016; Soliman *et al.*, 2017) but the vast majority have not yet been adequately evaluated. Studies on their anti-candidal activities are still in their infancy. Moreover, none of these plant extracts has been approved by regulatory agencies for human use either because of a lack of information regarding their efficacy, toxicity or lack of defined chemical structure.

The antimicrobial properties of plant extracts have attracted great interest in at the recent times. It is unequivocally known that the plant extracts are easily assimilated, side effect free and are indigenously used as drugs in many parts of the world. This aspect of the plant extracts is yet to be sufficiently available with few or restricted reports. Herein, the current study evaluates the effectivity and mechanism of action of locally available plant products that inhibit the microbe *C. albicans* because these crude extracts are being used for fungal ailments since ancient ages. Different plant extracts or natural oils were tested to develop an ancillary method to deal with the menace of Candidiasis.

MATERIALS AND METHODS

Starch, casein powder, Monopotassium phosphate (KH_2PO_4), Dipotassium phosphate (K_2HPO_4), Magnesium sulphate

heptahydrate epsomite ($MgSO_4.7H_2O$) and yeast extract were purchased from Merck. Distilled water was used to prepare all the solution and media.

Methodology of Protease Test

For the quantitative test of protease enzyme, a synthetic medium was selected with the following constituents: starch, 1.0 g; Casein powder, 10.0 g; KH_2PO_4, 0.7 g; K_2HPO_4, 0.3 g; $MgSO_4.7H_2O$, 0.5 g and yeast extract 1.0 g in 1 litre of distilled water and the pH of the medium was adjusted to 6.0. The protease assay was performed according to the methods of Anson (1938) with slight modification here. To 1.0 mL of 1% (w/v) substrate solution, (casein, being the most suitable one) in 0.1 M citrate phosphate buffer (pH-3.8), 0.2 mL of enzyme solution (culture supernatant) was added. The reaction mixture was kept at 30± 2 °C for one hour, after which the activity was stopped by the addition of 1 mL of 20% TCA. After a period of 20 minutes, the reaction mixture was centrifuged at 10,000 rpm for 10 minutes in order to remove the unhydrated proteins. To 1 mL of supernatant, 5.0 mL of 0.275 M sodium carbonate, 2.0 mL of distilled water and 0.5 mL of 1:1 diluted Folin-ciocalteu reagent were added. The intensity of the blue colour developed after 20 minutes that was measured in Erma photoelectric Tris colorimeter using a red filter (660 nm). All other conditions remain the same, a control was run in which TCA was added before the addition of the enzyme. Protease activity units were subsequently calculated by matching the readings against a standard curve prepared by plotting the colour values of tyrosine. 1 unit of proteolytic activity was considered as the amount of enzyme which liberated 1 µmol equivalent of tyrosine under experimental conditions.

Antifungal Activity

The morphological variants of *Candida albicans* were isolated from different sources like skin lesions and soils of localities of the patients suffering from the disease. They were further ascertained by certain confirmatory methods like Chlamydospore formation (Figure 1c). Finally, two variants i.e., yeast and mycelia forms (Figures 1a and 1b) were taken to analyse their growth and biochemical aspects.

To evaluate the effect of various plant extracts, the fungal isolates were grown in modified Lilly and Barnett's broth (Lilly & Barnett, 1951) with the following constituents: 1.0 g starch, 10.0 g casein powder, 0.7 g KH_2PO_4 (Monopotassium phosphate), 0.3g K_2HPO_4 (Dipotassium phosphate), 0.5 g $MgSO_4.7H_2O$ (Magnesium sulphate heptahydrate, epsomite) and 1.0 g yeast extract in 1 L of water. The pH of the medium was adjusted to 6.0. The medium (5 mL) was dispensed into culture tubes and then sterilized at 12 lb/in^2 steam pressure.

The various plant extracts tested here, were the oil of *Azadiracta indica*, *Pongamia glabrata*, *Ricinus communis*, *Eucalyptus globulus* and the crude extracts from the rhizomes of *Curcuma longa*. The oils were dissolved in acetone water (1:1) and were added in different concentrations (i.e., 1, 2, 3, 5, 7, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µL/mL) to the culture tubes containing 5 mL aliquots of modified Lilly & Barnett's synthetic broth. Finally, the volume was made up to 10 mL by the addition of sterilized distilled water. Apart from the triplicates, one control was run without any plant extract for each isolate of the organism. In order to mix the oil thoroughly in the media, incubation was done under shaking conditions at 30± 2°C (Anson, 1938).

RESULTS

Different concentrations of plant extract were studied in different days of incubation such as 1, 3, 5, 7, 9, 11, 13 and 15 days. The optimum growth and protease production were found after 9th and 11th days of incubation in yeast and mycelial forms of C. albicans respectively. After 11 days of incubation, the pattern in growth and protease production declined.

All the plant extracts tested showed Candidostatic properties on the isolates of the organism under *in vitro* conditions. However, the oil obtained from the seeds of *P. glabrata* showed the minimum inhibitory concentration (MIC) values at the lowest concentration (i.e., 20-30 µL/mL) (Figure 2), followed by seed oil of *A. indica* and *R. communis* (Figures 3 and 4).

R. communis and *E. globulus* oil showed Candidostatic property at higher concentrations like 60-70 µL/mL and 90-100 µL/mL respectively (Figures 4 and 5). In all cases, the retardation of growth of the isolates of the organism was linked to decline in the enzyme protease production. However, *C. longa* extracts neither affected the enzymatic activity nor growth of the isolates up to 100 µL/mL (Figure 6). The order of candidostatic efficacy of the various oils was observed to be: *P. glabrata* > *A. indica* > *R. communis* > *E. globulus* > *C. longa*.

DISCUSSION

In the last few years, different plant derived compounds have been studied for the regulation of processes involved in fungal virulence related mechanisms such as adhesion, hypha formation or secretion of extracellular proteases and other hydrolases (Guevara-Lora *et al.*, 2020).The infection caused by *C. albicans* depends partly on the site of disorder and partly on the individual patient. However, in the pre-antibioticera, relapse of the disease frequently occurred. It was difficult to eradicate the micro-organism, particularly with the granulomatous form and in the case of systemic involvements, the outcome was often fatal.

It has been observed that various factors influence the *in vitro* MIC values like changes in size, medium composition, pH of the media, incubation temperature and incubation time (Odds, 1994). It is well known that MIC is the most simple, suitable and dependable comparative indicator among the various measurements of determination of susceptibility of bacteria and fungi *in vitro* (Odds, 1994). However, Venugopal and Venugopal (1994) studied the anti-dermatophytic activities of the aqueous and ethanolic extracts of *Azadirachta* leaves and used about 86 clinical isolates of *Trichophyton* and *Microsporum*

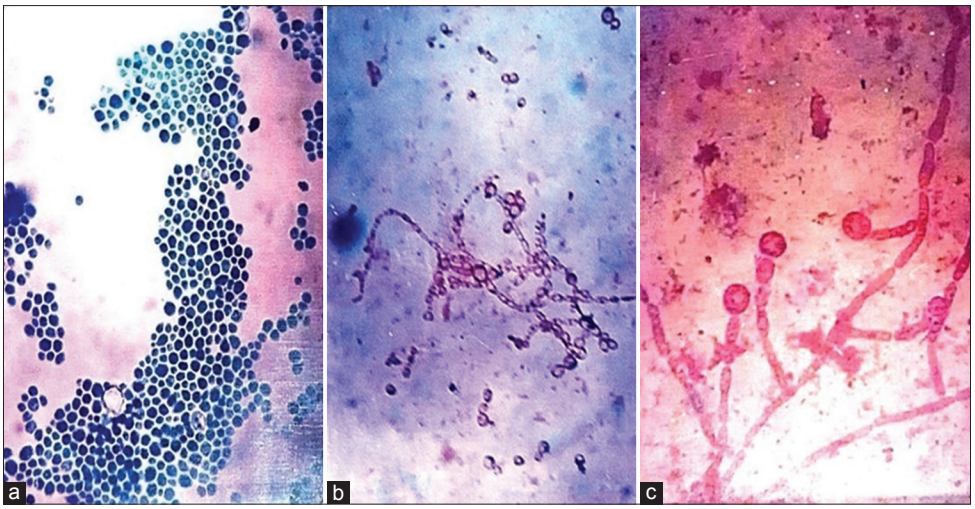

Figure 1: a) Unicellular form of *C. albicans* isolates b) Mycellial form of *C. albicans* isolates and c) Induction of Chlamydospores in *C. albicans*

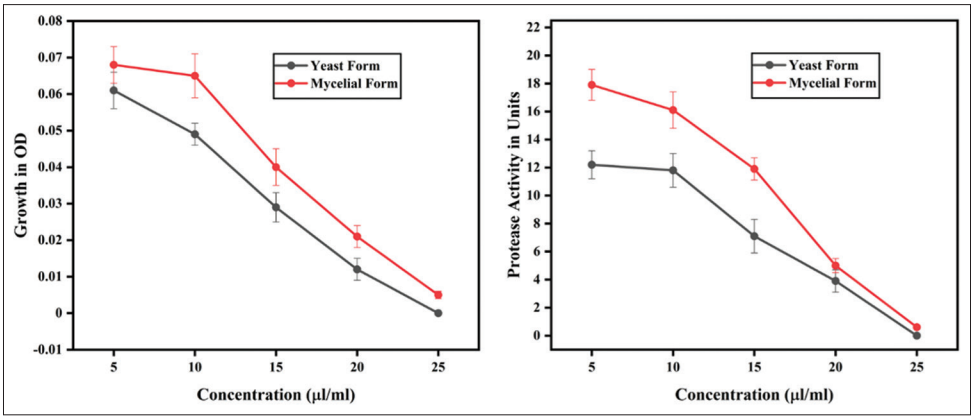

Figure 2: Effect of *Pongamia glabrata* on Growth and Protease Production

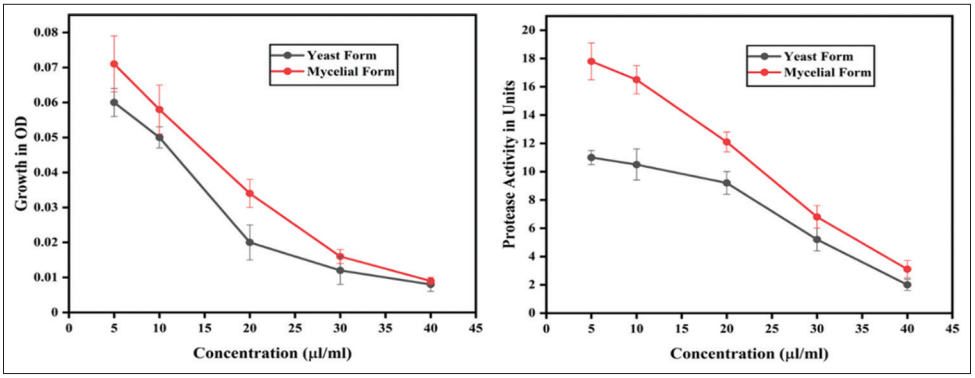

Figure 3: Effect of *Azadirachta indica* on Growth and Protease Production

species to determine the minimal inhibitory concentration. Several workers have demonstrated that 63% of the botanical species showed antifungal properties against *C. albicans*. A paste made of *A. indica* and *C. longa* used to treat 814 people with scabies cured 97% of them within 3-5 days of treatment (Lans, 2007). Reports are also available regarding the exploitation of naturally occurring compounds from plants and microbes in cure of diseases (Sati & Joshi, 2011).

The results indicate that oil extracted from the seeds of *P. glabrata*, *A. indica*, *R. communis* (Figures 2-4) have therapeutic value (Table S1). Use of such oils might be preventing the organism to come in contact with water and thereby impairing the growth of the pathogen. The alkaloids present in *Pongamia* oil is karanjin; *Azadirachta* are azadirachtin and nimbidin; *Ricinus* is ricinoleic acid that probably contribute to their antimycotic properties.

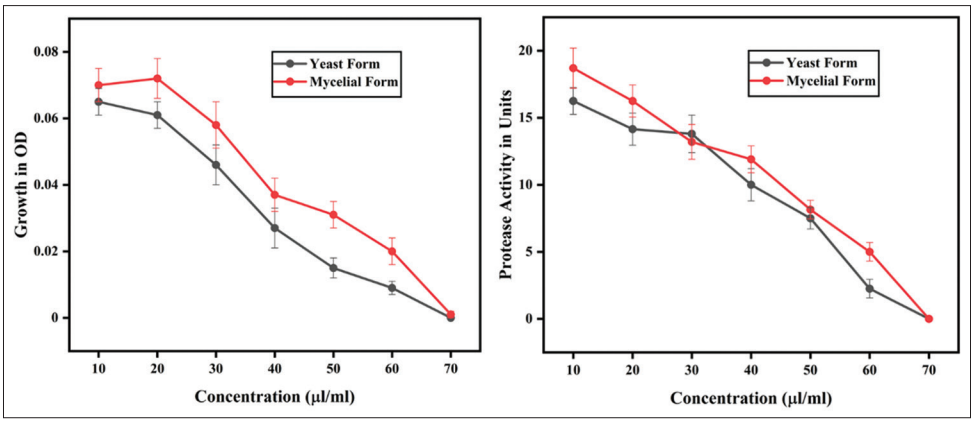

Figure 4: Effect of *Ricinus communis* on Growth and Protease Production

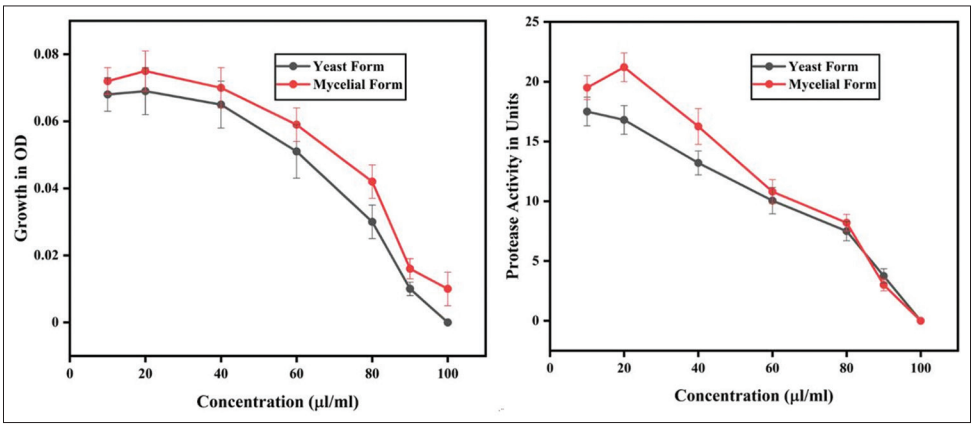

Figure 5: Effect of *Eucalyptus globulus* on Growth and Protease Production

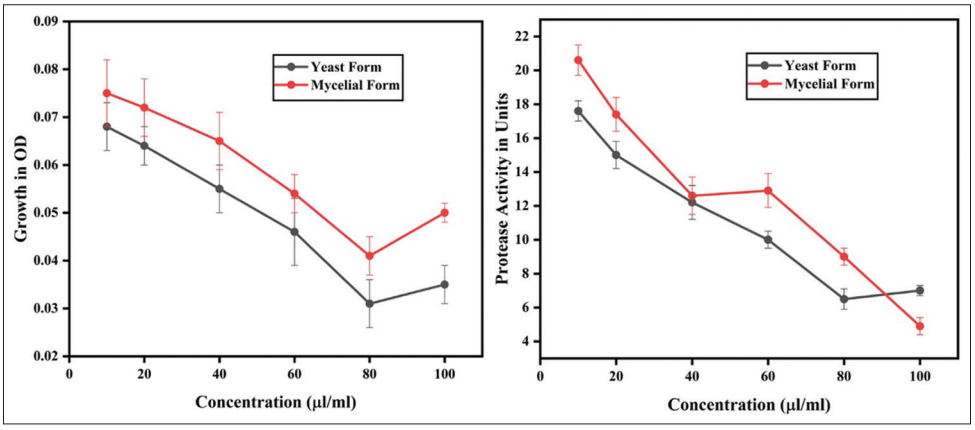

Figure 6: Effect of *Curcuma longa* on Growth and Protease Production

P. glabrata is a mangrove plant of the family Fabaceae, greatly known for its medicinal properties. Its bark is used in piles; leaves for rheumatic pain; seeds for hypertension, bronchitis, whooping cough, skin diseases and rheumatic arthritis (Ballal, 2006); the crushed seeds and leaves are used for antiseptic properties; and roots are used for cleaning gums, teeth, ulcers and gonorrhoea. The fungicidal activity of various parts of *P.* was found in the order of seed > root > bark > leaf against *C. albicans* (More & Baig, 2013). Thus, the plant extracts of *Pongamia* have immense potential to be developed as antifungal agents that can be used in the treatment of fungal infections (Dahikar & Bhutada, 2017; Usha, 2017).

A. indica is a tree of the family Meliaceae that has been used traditionally for the treatment of many diseases and dentistry. The leaf extract and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, anti-hyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antioxidant, antimutagenic and anticarcinogenic properties (Subapriya & Nagini, 2005). About more than 140 structurally

and chemically variable biologically active compounds have been isolated from different parts of the plant. Quercetin and (beta) - setosterol are the polyphenolic flavonoids purified from leaves are known to have antifungal and antibacterial properties. The results of Dasgupta *et al. (*2004*)* suggested that the tree contained at least 35 biologically active principles. The different types of leaf extract of *Azadirachta* effectively suppressed the mycelial growth of six fungal pathogens including *C. albicans* and results were similar to the findings of Mahmoud *et al.* (2011). The effect of leaf extract of *Azadirachta* on the adhesion, cell surface hydrophobicity and biofilm formation may affect the colonization of *C. albicans* (Polaquini *et al.*, 2006).

R. communis is an oil yielding plant of the family Euphorbiaceae. The oil is used in the production of surfactants, coatings, greases, cosmetics, and pharmaceuticals. Various parts of the plant possess antimicrobial, antidiabetic, and anti-inflammatory activity (Jeyaseelan & Jashothan, 2012). Results of Suurbaar *et al. (*2017*)* revealed that the various extracts of leaves of *R. communis e*xhibited growth inhibition of 5 micro-organisms including *C. albicans*.

The plant contains several compounds such as steroids, saponins, alkaloids, flavonoids, tannins, phenols, phytates, oxalates and glycosides that contribute to the antimicrobial properties of castor. Since it contains many compounds that are toxic to animals, human beings and micro-organisms, it is evident that the anti-microbial properties are shown by the whole plant (Rashmi *et al.*, 2019). The major toxic protein, ricin can kill human beings at extremely low conc. It inhibits protein synthesis by acting mainly on eukaryotic ribosomes. Thus, fungi are more susceptible to ricin than bacteria (Suurbaar *et al.*, 2017) several investigations suggested the antimicrobial activity of *R. communis* on strains of *C. albicans* (Poonam & Pratap, 2012; Bhaumik *et al.*, 2014).

E. globulus, a tall, ever green plant of the family Myrtaceae, has been used traditionally for the treatment of diabetes (Ahlem *et al.*, 2006; Eidi *et al.*, 2009).It has antihyperglycemic, anti-inflammatory (Vigo *et al.*, 2004), anti-bacterial and anti-fungal properties (Sartorelli *et al.*, 2007). Thus, *E. globulus* oil is used as an anti-microbial element in different kinds of creams, soaps and toothpastes (Lis-Balchin *et al.*, 2000). Several *in vitro* studies confirmed anti-bacterial (Salari *et al.*, 2006) and anti-fungal (Agarwal *et al.*, 2010) effects of *E. globulus* but there is no evidence about its *in vivo* anti-microbial effects. However, results of M. Bokaeian (Bokaeian *et al.*, 2010) showed considerable inhibitory effect on the growth of *C. albicans* in normal and diabetic rats. Many constituents have been investigated such as monoterpenes (Sartorelli *et al.*, 2007), tannins (Barry *et al.*, 2001), alkaloids, and phenols (Ahmad & Beg, 2001). However, the anti-microbial effect of *E. globulus* could be due to a synergism between the above-mentioned constituents. Elaissi *et al. (2012)* identified 18 major components of *E. globulus* oil that contribute to its antibacterial properties.

C. longa belongs to the family Zingiberaceae, globally demanded in recent years due to the active ingredient curcumin, having a wide range of beneficial properties such as anti-inflammatory, antioxidant, antitumor, chemopreventive and chemotherapeutic activities besides the antimicrobial properties (Gupta *et al.*, 2013). Besides as a spice, food preservative, flavouring and colouring agent, (Bhawana *et al.*, 2011, Hani & Shivakumar, 2014) *Curcuma* is used for liver diseases, jaundice, rheumatoid arthritis, eye infections and dental pain (Hoseini *et al.*, 2010). Studies revealed that it has anti-inflammatory (Sa *et al.*, 2010), anti-cancer (Sa *et al.*, 2010; Lu *et al.*, 2013), hepatoprotective, anti-allergic, wound healing, anti-spasmodic and anti-HIV properties (Gupta *et al.*, 2015). The mode of action of curcumin may involve alteration in the morphology of the hyphae which may appear severely collapsed, plasma membrane disruption, mitochondrial destruction, lack of cytoplasm, folding of the nuclear membrane and thickened cell wall (Murugesh *et al.*, 2019) due to chitin accumulation on the outer layer of the cell wall (Huang *et al.*, 2016). Many comparative analyses of curcumin and antifungal antibiotics such as amphotericin B, fluconazole and nystatin (Sharma *et al.*, 2010; Babaii & Zamaninejad, 2016) suggested a significant inhibitory effect of curcumin. The anti-fungal activity of Curcumin on the strains of *C. albicans* has been studied (Martins *et al.*, 2009). *Curcuma* extract did not have any effect on the inhibition of *C. albicans in vitro* (Nosratzehi *et al.*, 2019). However, Chen *et al.* (2018) suggested that curcumin can modulate the pathogenicity of *C. albicans* and thus a potential antimicrobial natural compound.

It has been demonstrated that the essential oil evidenced good fungicidal activity against all *Candida* species such as *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (Sati & Joshi, 2011). Again, oil and oil products are multicomponent mixtures composed of mainly hydrocarbons. The interaction of lipophilic hydrocarbons, with membrane lipids and proteins must affect the thickness and fluidity of the phospholipid bilayers, as well as the activity of membrane enzymes and transport proteins (Sikkema *et al.*, 1995). This in turn must have led to the malfunction of cell membranes, in particular to the leakage of protons and other intracellular ions. These changes in the structure and permeability of membranes may influence the energy state and homeostasis of cells, leading eventually to a decrease in their viability (Fomchenkov *et al.*, 1998).

However, it has been found that there is a wide range of synthetic products or antifungals to treat Candidiasis but in very rare cases they can completely cure the patients but have a lot of side effects. The majority of clinically used antifungals have various draw backs in terms of toxicity, efficacy as well as cost and their frequent use has also led to the emergence of resistant strains. Due to the reasons cited, the use of synthetic fungicides in agriculture in recent years has also been highly reduced and attention has now been diverted to plant products which are cheaper, nearer and more effective in the treatment of human ailments. Finding plants with antifungal potential has been a difficult task (Souza *et al.*, 2010) because whenever such activities are observed, many other substances are simultaneously found with high levels of toxicity (Silva *et al.*, 2020). This investigation demonstrated that compounds inhibitory to *C. albicans* are present in certain plants. The mycelial form of the organism came up with greater resistance

to the tested plant extracts than the yeast form which proved the higher pathogenicity of mycelial form. This has also been observed by several workers. The plant products tested in this experiment not only retarded the growth of the isolates but also inhibited protease production.

In the study, a maiden attempt has been taken in this part of the world to know the efficacy of certain plant products on the growth and protease production of *C.albicans* under *in vitro* conditions. The retardation of growth under the influence of plant extracts is directly proportional to the protease production. It shows that the plant extracts might have directly contributed to the inhibition of the extrusion of protein digesting enzymes of the pathogen. The exploration of bioactive substances present in plants that fight drug resistant *Candida* strains is highly recommended to offer new effective antifungal therapies and further assessment of this aspect can solve the nagging skin disease to a great extent.

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SUPPLEMENTARY TABLE

Table S1: Effect of *Pongamia glabrata, Azadirachta indica, Ricinus communis, Eucalyptus globulus and Curcuma longa* on Growth and Protease Production

Name of Plant	Conc. (μL/mL)	Growth in OD		Protease Activity in units	
		Yeast	Mycelial	Yeast	Mycelial
Pongamia glabrata	5	0.061±0.005	0.068±0.005	12.2±1	17.9±1.1
	10	0.049±0.003	0.065±0.006	11.8±1.2	16.1±1.3
	15	0.029±0.004	0.04±0.005	7.1±1.2	11.9±0.8
	20	0.012±0.003	0.021±0.003	3.9±0.8	5±0.5
	25	0	0.005±0.001	0	0.6±0.01
Azadirachta indica	5	0.06±0.004	0.071±0.008	11±0.5	17.8±1.3
	10	0.05±0.003	0.058±0.007	10.5±1.1	16.5±1
	20	0.02±0.005	0.034±0.004	9.2±0.8	12.1±0.7
	30	0.012±0.004	0.016±0.002	5.2±0.8	6.8±0.8
	40	0.008±0.002	0.009±0.001	2±0.4	3.1±0.6
Ricinus communis	10	0.065±0.004	0.07±0.005	16.25±1	18.7±1.5
	20	0.061±0.004	0.072±0.006	14.15±1.2	16.25±1.2
	30	0.046±0.006	0.058±0.007	13.8±1.4	13.8±1.4
	40	0.027±0.006	0.037±0.005	10±1.2	11.9±1
	50	0.015±0.003	0.031±0.004	7.5±0.8	8.15±0.7
	60	0.009±0.002	0.02±0.004	2.25±0.7	5±0.7
	70	0	0.001±0.001	0	0
Eucalyptus globulus	10	0.068±0.005	0.072±0.004	17.5±1.2	19.5±1
	20	0.069±0.007	0.075±0.006	16.8±1.2	21.2±1.2
	40	0.065±0.007	0.07±0.006	13.2±1	16.25±1.5
	60	0.051±0.008	0.059±0.005	10.05±1.1	10.8±1
	80	0.03±0.005	0.042±0.005	7.5±0.8	8.2±0.7
	90	0.01±0.002	0.016±0.003	3.75±0.6	3±0.5
	100	0	0.001±0.005	0	0
Curcuma longa	10	0.068±0.005	0.075±0.007	17.6±0.6	20.6±0.9
	20	0.064±0.004	0.072±0.006	15±0.8	17.4±1
	40	0.055±0.005	0.065±0.006	12.2±1	12.6±1.1
	60	0.046±0.007	0.054±0.004	10±0.5	12.9±1
	80	0.031±0.005	0.041±0.004	6.5±0.6	9±0.5
	100	0.035±0.004	0.05±0.002	7±0.3	4.9±0.5