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Antifungal activity of clove and cinnamon on oral candida species

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Abstract
Pathogens are the key agents for infections and food poisonings which are threatening public health all over the world. The effectiveness of numerous synthetic antimicrobial agents, that are regularly used to alleviate diseases in clinical aspects as well as to increase shelf-life, safety of food products in industries have been weakened by developing drug resistance. Therefore, to overcome this problem there is an essential need to discover analogous agents which intern shows the antimicrobial activity. Based on these lines we made an attempt to evaluate *invitro* antifungal efficiency of methanol and acetone crude extracts of clove and cinnamon on four oral pathogenic fungi (*Candida albicans, Candida tropicalis Candida parapsilosis and Candida ontarieonsis*) isolated from diabetic subjects. It has been proved that (5mg/ml) acetone extracts of cinnamon were more susceptible than that of methanol and enthusiastically the clove extracts synergistically inhibited the oral Candida species. Therefore, the active principle of spices has a great potential to be developed as new and safe antimicrobial drugs.

Keywords: *Candida albicans*, clove, cinnamon, ethanol, acetone and plant extracts micro dilution method

Introduction
Fungi are ubiquitous; they seems to be able to survive in almost all habitats and plays a innumerable role in the ecosystems [Buckley, 2008] [1]. The advent of recombinant DNA technology and large scale genomics analysis has placed yeasts and filamentous fungi in the forefront of contemporary commercial applications. The fungal white biotechnology includes biodiversity of fungi from different habitats, including extreme environments i.e., high temperature, low temperature, salinity, and pH) and associated with plants (epiphytic, endophytic, and rhizospheric) and their industrial applications in diverse sectors. Apart from this some fungi that contaminate food can also be harmful to human health.

In spite of the benefits created by fungi, Some food-borne illnesses could also be due to fungi or their byproducts, such as poisoning by mushrooms or mycotoxins. Some food born fungal pathogens are *Alternaria*, *Aspergillus*, *Candida*, *Fusarium*, and mucormycetes [Brenier-Pinchart *et. al.*, 2006; Pitt and Hocking, 2009] [2, 3]. Worldwide, an estimated 600 million food borne diseases occur yearly [WHO, 2015] [4]. In contrast, some fungi identified as human pathogens such as *Aspegillus, mucar, pencillium* and Candida. Approximately 300 of the estimated 1.5 million fungal species on earth are known to cause illnesses ranging from allergic reactions to life-threatening invasive infections [Hawks worth, 2001] [5]. However, these pathogens primarily infect immunocompromised persons by establishing several clinical manifestations in general, invasive fungal infections (IFIs) in particular cause devastating illnesses that result in considerable mortality, yet quantifying their public health burden is challenging [Vallabhaneni *et al.*, 2015] [6].

In order to cure several fastidious pathogenic infections people consuming high loads of antimicrobial drugs which disturb the human immune system by developing drug resistance. Antibiotic resistant microorganisms can increase mortality rates because they can survive and recover through their ability to acquire and transmit resistance after exposure to antibiotic drugs, which are one of the therapies to infectious diseases [Marchese *et al.*, 2016] [7]. Antibiotic resistant bacteria threaten the antibiotic effectiveness and limit the therapeutic options even for common infections [Paphitou 2013] [8]. Therefore, much attention should be paid to natural products, which could be used as effective drugs to treat human diseases, with high efficacy against pathogens and negligible side effects.

Plants have been a valuable source of natural products to maintain human health, especially with more intensive studies in the last decade for natural therapies. Spices have been used for not only flavor and aroma of the foods but also to provide antimicrobial properties (Nanasombat *et al.*, 2002, and Agaoglu *et al.*, 2007) [9, 10]. Instantly to overcome the burden of chemical, synthetics as well as antimicrobial agents scientists looking to work on the plant products. Moreover by observing the development of drug resistance there is an urgent need to go for alternative therapeutics such as plant products to cure number of pathogenic infections with limited time. Hence the present study deeply concentrating on the use of spices (plant products) as alternative therapeutics.

Methodology
Preparation of plant extracts
Dried buds of cloves (*Syzygium aromaticum*) and bark of cinnamon (*Cinnamomum verum*) were purchased from local retail markets and transported to the Laboratory. The obtained material was washed with sterile distilled water and air dried then individually ground into a fine powder using a mortar and pistil.

Methanol or Acetone extracts

100 grams of cloves and cinnamon powder was soaked individually in 400 ml of methanol or acetone in sterile bottles with constant agitation overnight at 20℃ in a temperature controlled bio-shaker. The extracted fractions were separated using sterilized cheesecloth and filtered through Whatman filter paper (No. 2). All the extracts were then concentrated using a rotary vacuum evaporator at 40℃, and the concentrated extracts were diluted to desired concentration using 10 % DMSO as solvent, sterilized by filter (0.45µm), and kept at - 20℃ until use.

Isolation and identification of pathogenic Candida strains

Collected oral samples from a total of 125 diabetic patients who were regularly visiting at Rajiv Gandhi Institute of Medical Sciences, Kadapa, andhra Pradesh, India in this study after under signed a consent form. Before collecting the sample from the patients we explained clearly and asked them to rinse their mouth with sterile distilled water and collect it in to sterile sample container and transported to the laboratory for further analysis.

Antifungal screening

All extracts were dissolved to a concentration of 1, 2.5 and 5 mg/ml. A modification of the NCCLS proposed method (M27-P) broth microdilution test was performed [EspinelIngroff and Pfaller, 1995] [11]. Four milliliters of sterile saline were added to approximately 400 µl of 24 h old Candida cultures. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 McFarland standard solution. From the prepared stock culture of candida, a 1:1000 dilution with broth was prepared. One hundred microlitres of broth were added to each well of a 96-well microplate. Organic solvent extracts 25 µl of the extracts were added to 175 µl broth and serially diluted. Three replicates were prepared for each extract. All the wells were then filled with 100 µl of stock yeast culture. Amphotericin B was used as a reference for this experiment and the following controls were prepared: wells containing broth only, fungal strain with no extract, and serial dilutions of Amphotericin B with the fungi at the recommended inhibitory concentrations. The plates were then read at 630 nm in an ELISA reader, covered with parafilm and incubated at 37^0C overnight, where after their absorbance was reread.

Ethical committee clearance

The present descriptive study was approved by the Institutional ethical committee (1841/Go/Reg/S/CPCSEA: DATED 18/11/2015.

Statistics

All the results obtained from the present study was analyzed by using correlation and test of significance.

Results and Discussion

In the present study, out of 125 samples tested from both diabetic and non-diabetic patients [67 male and 58 female (Smokers (24) and non-smokers (101)] with age range from 21 to 75 years. Among the total cases 22.4% showed Candida infection from which 8 Females cases and 20 Males cases. From the total samples we isolated 70 different yeast species including; 22 (31.4 %) *Candida albicans* and 48 (68.5%) non-*Candida albicans*. These organisms further conformed by 18S rRNA sequence analysis and also by using PCR based markers RAPD and ISSR's. Among the total isolated cultures, we selected only predominant isolates from *Candida albicans (Candida albicans-1* and *non-Candida albicans* (*Candida tropicalis, Candida parapsilosis and Candida ontarioensis)* [Fig 1] for further study.

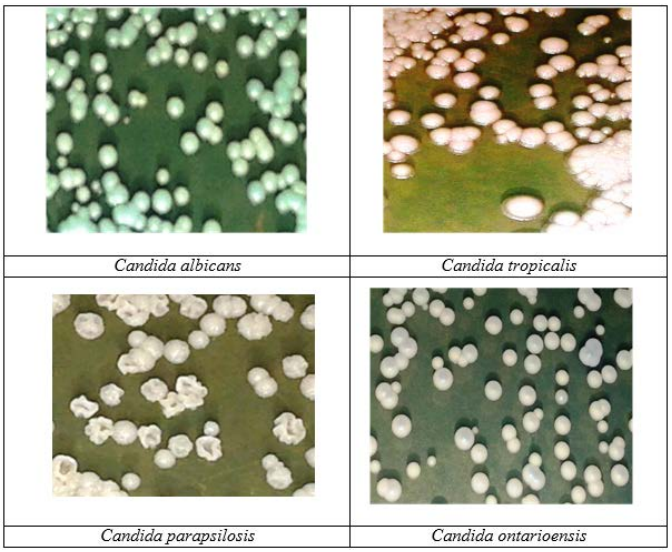

Fig 1: Culture characteristics of the test organisms on CHROM agar medium.

A review of national, regional, and worldwide studies showed that, throughout the years, the incidence of *C. albicans* has declined from 70% to 50% and these infections have been replaced by non-Candida albicans [NAC]. The most important predisposing condition for increasing incidence of NAC infections are ever-expanding population with mucosal or cutaneous barrier disruption, quantitative or qualitative dysfunction of neutrophils or of cell-mediated immunity and metabolic disorders. In this regard, the present study was conducted to determine the prevalence of NAC in oral infections of diabetic subjects. In present investigation we found that 22(32.3%), strains were belongs to the *Candida albicans* and remaining 48 strains were the non albicans. Among the NAC group *C. tropicalis* (47.9%), *C.ontariensis* (20.8%) *C. parapsilosis* (16.6%) and dipodascus (14.5%). Similar to our study Oufi, Z *et.al.*, 2020 analyzed 144 urine samples from diabetic and non-diabetic subjects and found that the incidence of *C.glabrata* (29.9%), then *C.tropicalis* (13.8%), whereas, *C.krusei* (=Pichia kudriavzevii) displayed the least incidence (9.7%). Esmailzadeh *et al.*, 2017 worked on 400 diabetic patients, and found the frequencies of the *Candida* species were as follows: *C. albicans* (46.4%), *C. glabrata* (42.8%), *C. kefyer* (7.2%) and *C. krusei* (3.6%).

Antifungal activity of clove and cinnamon ethanol and acetone extracts

Different concentrations of crude Clove and cinnamon extracts have been used in the present investigation and the results were showed in fig.2.

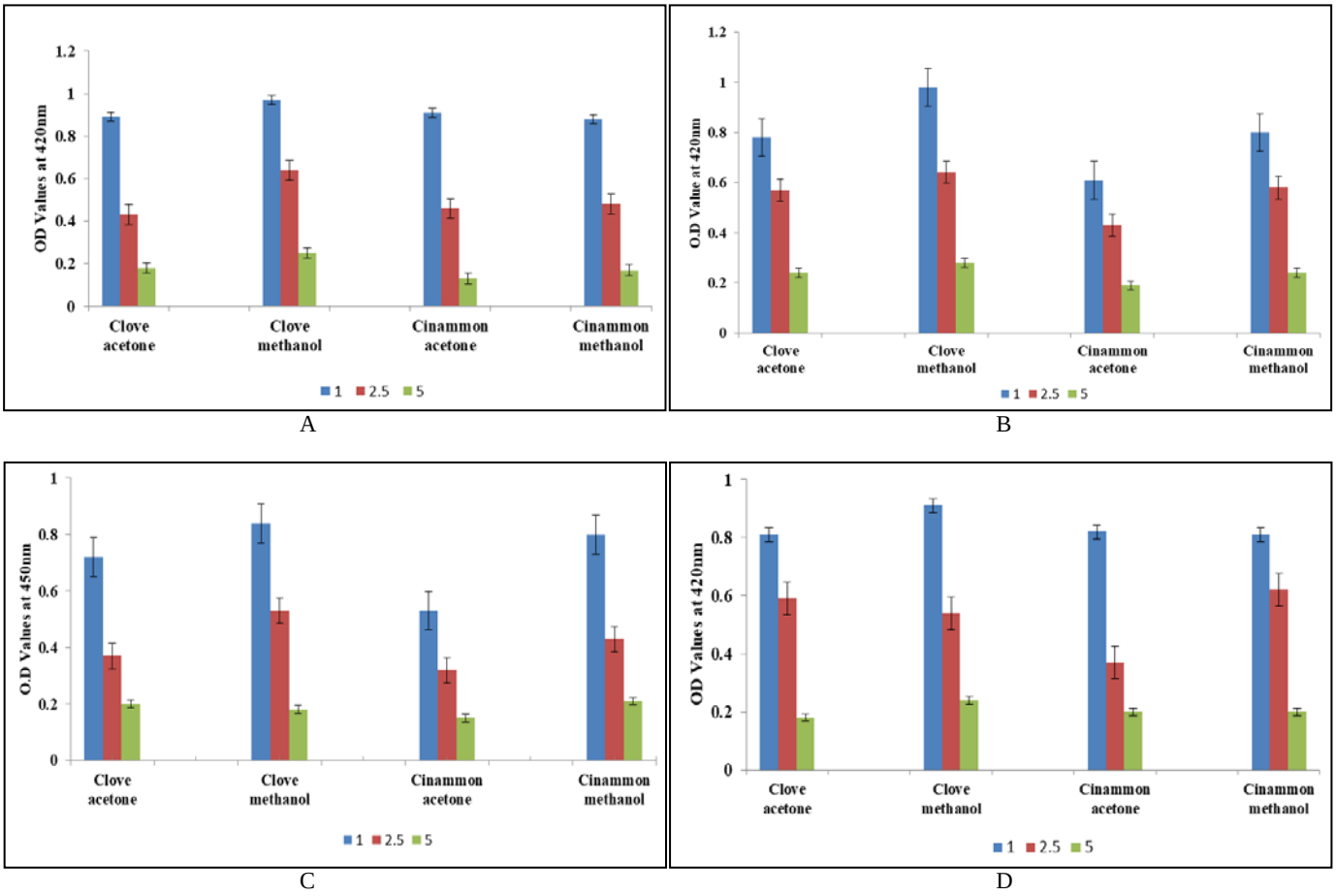

Fig 2: *In vitro* susceptibility of different test organisms to acetone and ethanol extracts of clove and cinnamon at different concentrations. A) *Candida albicans* B) *Candida tropicalis* C) *Candida parapsilosis* D) *Candida ontarioensis.*

From the fig.2 it was noticed that *C.albicans* susceptibility *pattern at different concentrations of crude extracts of clove and cinnamon. C.albicans* showed maximum susceptibility at 5mg/ml concentration of both clove and cinnamon with acetone extracts than the alcohol extracts. *Candida albicans* tested for susceptibility with 5mg and 2.5mg concentration of the plant extracts and the correlation values were observed. P<0.05 significant level was obtained with two extract comparisons of spice extracts *viz* Clove acetone versus Cinnamon acetone and Clove methanol versus Cinnamon acetone r=0.9924, 0.9973.

C.tropicalis was also tested with the same lines and the results showed similarly at 5mg/ml concentration with acetone extracts of clove & cinnamon but cinnamon extracts have wide range of susceptibility than the clove. *Candida tropicalis* showed susceptibility at 5mg and 2.5mg/ml (Fig: 2) for these observations the correlations was performed. Distinct for all these concentrations of extracts of Clove acetone extract versus Clove Methanol extract the correlation coefficient r=0.9991 with the significance P<0.05 was tabulated similarly other concentration of Clove acetone versus Cinnamon methanol, Clove methanol versus Cinnamon acetone, Clove methanol versus Cinnamon methanol and Cinnamon acetone versus Cinnamon methanol reported the correlation of r= 0.9959, 0.9991, 0.9910 and 0.9957 (P<0.05). But in contrast to these results, the highest significance was shown by Clove acetone versus Cinnamon acetone with correlation coefficient r= 1.000 (P<0.001).

Candida parapsilosis expressed its susceptibility range with cinnamon acetone extracts (5mg/ml) than clove acetone, clove methanol and cinnamon methanol. Similarly *Candida parapsilosis* was also done the same tests and observed P<0.05 and recorded correlation r=0.9985. At the same time we tested the *C.ontarioensis* a gentle pathogen has susceptibility pattern with minimal difference at 5mg/ml con. of clove (acetone &methanol) and cinnamon (acetone & methanol). Traditional spices Cinnamon and Clove with the above said combinations and noted the significance at P<0.05 (r=0.9977) with Clove acetone versus Cinnamon methanol. No significance with other extracts.

Since the overall observations of susceptibility ranges intimated that non albicans Canada was an aggregates dominant pathogen of oral infections and moreover it has been suppressed by using natural spices extracts of clove and cinnamon (acetone &methanol). The proximate reason for using spices usage obviously is to enhance food palatability. But the ultimate reason is most likely that these spices could help to clean the foods and eliminate pathogens, thereby contribute to the good health longevity, reproducibility of taste for the people who enjoy with their flavours. Since from several years numerous investigations conducted to inhibit the microorganisms in foods by spices and their extracts. Many of

them have significant antimicrobial activity. Similar to our results Nassen *et al.*, proved the water extracts of clove &cinnamon both *invitro* and *invivo* and found that the clove extracts are empowered than other extracts. Another supports was conformed that among the various powders tested clove has good range of susceptibility [Kaung *et al*., 2011]. Dilek Keskin and Sevil Toroglu 2011 [16]; Shan *et al*, 2009 and Ziwei *et al*, 2003 reported their results with ethanol extracts of clove showed considerable difference in susceptibility pattern against various bacteria and fungi tested. Never the less, the present investigation has been marked that the antifungal activity and susceptibility changes recorded successfully by using cinnamon and clove extracts.

Conclusions

The present work mainly focused on the antifungal activity of the spices used against the oral Candida species. From this study we conclude that the oral infections of diabetic patients were influenced by predominantly Candida species particularly by non albicans species (*Candida tropicalis*, *Candida parapsilosis*, *Candida ontarioensis*) and moreover these non albicans species are also responsible for various food born infections. So interestingly we take-up this problem and tested for the susceptibility for the crude acetone and ethanol extracts of clove and cinnamon and found very significant susceptible pattern for acetone extracts.

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