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Preliminary studies on the antifungal activities of ethanol and hot water extracts of local spices against fluconazole-resistant *Candida albicans* isolates recovered from clinical samples

Akaihe CL, Chidebelu PE and §Nweze EI

Department of Microbiology, University of Nigeria, Nsukka, Nigeria.

§Corresponding author: Prof Emeka I. Nweze. Email: emeka.nweze@unn.edu.ng;

Abstract

This study evaluated the antifungal effect of local spices namely *Capsicum annum* var. shombo, *Capsicum annuum* var. atarugu, *Capsicum annuum* var. Nsukka yellow pepper, *Zingiber officinale* and *Allium sativum* against fluconazole-resistant *Candida albicans* isolates. Hot water and ethanol were used as solvents for extraction. The seed and pericarp extracts from *Capsicum annuum* alone showed no inhibitory effect against the test organisms. Notwithstanding, whole *Capsicum annuum* fruit extracts showed variable levels of inhibition against the tested isolates. The minimum inhibitory concentration (MIC) results of hot water extract showed that *Capsicum annuum* var. atarugu had the lowest MIC of 6.25 mg/ml while *Capsicum annuum* var. yellow pepper showed the highest MIC of 100 mg/ml. *Zingiber officinale* showed MIC range of 3.125 mg/ml to 50 mg/ml and Allium sativum an MIC range of 12.5 mg/ml to 50 mg/ml for both hot water extract and ethanol extracts. The least MIC was obtained with *Zingiber officinale* ethanol extract with an MIC of 3.125 mg/ml. Our finding shows that these spices may be potential sources of antifungal agents.

Key words: ***Capsicum annum, Zingiber officinale, Allium sativum,*** Antifungal activity, Nsukka

INTRODUCTION

Spices are plant substances that are generally used to enhance flavor, which include leaves (coriander or mint), flower bud (clove), fruits (black pepper), bark (cinnamon), and rhizomes (turmeric) (Pavithra *et al*., 2016). The delightful flavour and health benefits of spices have made them important ingredients in food processing. Among the spices includes pepper, ginger and Garlic. Pepper has natural antioxidants such as flavonoids, tannins and phenols and are increasingly attracting because they prevent disease, promote health as well are anti-aging substances (Tamizhazhagan *et al*., 2017). It occupied a unique position due to its

characteristic pungency and flavor. Pepper have found positions in preparations of numerous medicines due to their beneficial pharmacological properties like; anti-leukemic, anti-diabetic, antitumor, cytotoxic, laxative, purgative and immune-modulatory property (Soetarno *et al.,* 2009). *Zingiber officinale* has been reported to exhibit anti-inflammatory, antipyretic, antimicrobial, hypoglycemic, anti-migraine, anti-schistosomal, antioxidant, hepatoprotective, diuretic and hypo-cholesterolemic (Muhsin and Hussein, 2014). Phytochemical studies showed the presence of pungent principles, such as gingerol, On the other hand, the pungency of dry *Zingiber officinale* is mainly due to the presence of shagaols (such as 6-shagaol), which are considered as dehydrated forms of gingerols. The concentrations of gingerols in the dry ginger are slightly reduced from that of fresh *Zingiber officinale*, while the concentration of shagaols increases (Wail and Emad, 2018). *Allium sativum* can be considered as a national product of many centralized Asian countries. *Allium sativum* follows to the family *Alliaceae*. Plants of *Allium sativum* can be developed in a close proximity to each other, giving up proper area to enhance the maturation of the bulbs, and are simply developed in vessels of appropriate deepness. According to Muhsin and Hussein (2014), *Allium sativum* have beneficial cardiovascular properties. This study evaluated the antifungal effects of hot water and ethanol extracts of *Capsicum annuum* variety shombo, *C. annuum* variety atarugu, *C. annuum* variety Nsukka yellow pepper, *Zingiber officinale* and *Allium sativum* respectively on fluconazole-resistant *Candida albicans* isolates.

MATERIALS AND METHODS

Plant material

Local spices cultivated in Nsukka area namely: *Zingiber officinale*, *Allium sativum* and three different varieties of *Capsicum annuum* (*Capsicum annuum* variety shombo, *Capsicum annuum* variety Nsukka yellow pepper and *Capsicum annuum* variety atarugu) were collected from a local market (specifically Ogige market) in Nsukka, Nigeria. All the spices used were without blemish, fresh and firm.

Test Microorganisms

All the seven test microorganisms (fluconazole-resistant *Candida albicans* with isolate numbers; 45, OR20, 71, 75, 59, 53, 57) were all obtained from stock cultures in the Mycology laboratory, Department of Microbiology, University of Nigeria, Nsukka. All the microorganisms were maintained at 4^{o}C on Sabouraud Dextrose Agar (SDA) slants.

Preparation of Extracts

About 2 kg of the respective *Capsicum annuum* samples and 1 kg of *Zingiber officialle* and *Allium sativum* respectively were washed. The backs of rhizomes of *Zingiber officialle* were peeled off while the cloves of *Allium sativum* were separated and the leathery cover removed and washed with distilled water respectively. Then, the respective samples were sliced and dried under room temperature for 1 week except garlic with very high moisture content that stayed up 3-4 weeks to dry. About 2 kg of the respective pepper samples were washed and the pericarp and seed separated and dried separately. They were ground using electric blender to fine powdered.

Ethanol extraction was carried out using the method described Alo *et al (*2012)., by dissolving the sample in solvent in the ratio of 1:5 in a 1000 ml conical flask. The set up was left for 48 hours with intermittent shaking of the flask. The hot aqueous extraction was also done using the same extraction procedure of 1 g of sample in 5 ml of solvent, the respective equivalent volumes of distilled water was introduced into the sample in a conical flask of 1000 ml capacity. The extraction was done using a water bath at 90^{o}C for 1 hour. After extraction, it was allowed to cool to room temperature. Thereafter, the respective sample-solvent mixtures were filtered with Whatman No.1 filter paper and the respective filtrates were collected in a beaker. The stock solution was concentrated using a rotary evaporator to approximately 70%. The extracts were scrapped using spatula and stored in the sample bottle.

Standardization of Extracts

The extracts were dissolved in 10% dimethylsulphoxide (DMSO) by weighing out 0.5 g of the respective samples into a test tube, then, 0.5 ml of DMSO was introduced into the test tube containing the sample and thereafter 4.5ml of distilled water was added to the mixture to dissolve it.

Standardization of the test microorganisms

The isolates were sub-cultured into Mueller-Hinton broth overnight. About 3 ml of sterile broth was introduced into a sterile test tube and the pure isolate of the test organism was introduced. The turbidity was compared with the turbidity of 0.5 Mc-Farland standard. This turbidity gives approximate yeast cells of 1 x 10^6 cells/ml. This turbidity was used to carry out inhibition zone diameter (IZD). The inoculum for MIC was standardized by introducing 1 ml of the standardized microorganism (i.e. one with turbidity of 0.5 McFarland) into 99 ml of sterile Mueller-Hinton broth to give about 0.5- 1 x 10^4 cells/ml. Then two-fold serial dilutions were done to give 5 x 10^3 cells/ml.

Determination of antimicrobial activity of the respective ethanol and hot aqueous extracts

About 0.5 g of each of the extract was dissolved in 5 ml of solvent (0.5 ml DMSO and 4.5 ml of distilled water). This dilution gives a stock concentration of 100 mg/ml (i.e. 500 mg in 5 ml of solvent). The stock was further serial diluted (twofold) down to four concentrations of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml. The standardized inoculum was streaked on the surface of the Mueller-Hinton agar using a sterile swab stick and the plates were left for 10 minutes for the cells to settle. Thereafter, wells of approximately 6 mm in diameter were made on the surface of the agar medium using a sterile cork borer. The plates were labeled at the bottom of each well according to the concentrations of the extract to be introduced. Each well was filled with the extract according to the way it was labeled using a sterile pipette and fluconazole was used as a control for the cultures. This experiment was carried out in duplicates. About 30 minutes pre-diffusion time was observed before incubating the plates aerobically at 37^{o}C for 24 hours. Thereafter, the sensitivity of the organisms to different extracts were measured and recorded.

Determination of the minimum inhibitory concentration (MIC) of the extracts

Broth dilution protocols based on Clinical Laboratory Standard Institute (CLSI) approved reference document as previously described by Josep *et al*. was used to determine MIC values for *Candida albicans.* Overnight cultures of the test organisms were prepared by sub-culturing the isolates in sterile Mueller-Hinton Broth (MHB) and incubating at 37^{o}C. The following day, the cultures were standardized to 0.5 Mc-Farland turbidity standard which is equivalent to 1x 10^6 cells/ml based on the CLSI-M27A3 protocol. This turbidity was adjusted to turbidity standard of 0.5-1x10^3 cells/ml by adding 1 ml of the standardized inoculum into 99 ml of sterile MHB followed by 2- fold serial dilution with the diluted extract solution in Eppendorf tubes. The *Candida albicans* growth controls (positive controls) was prepared in Eppendorf tube containing sterile MHB without the test extracts respectively. Purity control tests were prepared by using MHB only. The set up was incubated at 37^{o}C for 36-48 hours. At the end of incubation period, the tubes were observed for turbidity and the least tube without any turbidity was read and recorded as the MIC.

RESULTS

Inhibition zone diameter of hot water extracts of *Zingiber officinale, Allium sativum* and *Capsicum annuum*.

Results from the agar well sensitivity test indicated that the test isolates were susceptible to some of the tested extracts and their inhibition zone diameter increased gradually as the concentration of extracts increased. The seed extract of *Capsicum annuum* variety shombo, *Capsicum annuum* var. Nsukka yellow pepper and *Capsicum annuum* var. atarugu respectively showed no inhibition around the wells of the extract when tested against fluconazole-resistant *Candida albicans* isolates. Also, when the pericarp extract of *Capsicum annuum* variety shombo, *Capsicum annuum* var. Nsukka yellow pepper and *Capsicum annuum* var. atarugu were tested against the same fluconazole resistant *Candida albicans* isolates respectively, no inhibition was seen around the wells of the extract for both ethanol and hot water extracts even at concentration of 100 mg/ml. The *Zingiber officinale* inhibited all the tested isolates (fluconazole-resistant *Candida albicans* isolates). The highest inhibition zone diameter (IZD) of 9.5 ± 0.5 was obtained when tested against isolate number 53 and the least IZD of 7.0 ± 0.0 against isolate number 71. At concentrations of 12.5 mg/ml and 6.25 mg/ml, no inhibition was observed. Hot water extract from *Allium sativum* inhibited all the tested isolates with the highest IZD of 8.5 ± 0.5 against isolate number 75 and the least IZD of 7.0 ± 0.0 against isolate numbers OR20 and 71 at concentration of 100 mg/ml respectively. The hot water extracts from

seed and pericarp of all the *Capsicum annuum* varieties tested respectively, did not show any inhibition against the fluconazole-resistant *Candida isolates*. Hot water extract of *Capsicum annuum* var. Nsukka yellow pepper inhibited all the tested isolates with the highest IZD of 7.5 ± 0.5 and least IZD of 7.0 ± 0.0. Also, hot water extract of *Capsicum annuum* var. atarugu (seed and pericarp) inhibited all the test isolates with the highest IZD of 8.5 ± 0.5 and the least IZD of 7.0 ± 0.0. The hot aqueous extracts from whole *Capsicum annuum* var. shombo (seed and pericarp) showed its highest IZD of 7.5 ± 0.5 at a concentration of 100 mg/ml.

Inhibition zone diameter of ethanol extracts of *Zingiber officinale*, *Allium sativum* and *Capsicum annuum.*

Ethanol extracts of *Zingiber officinale* displayed relatively high activity against the fluconazole-resistant *Candida albicans* isolates. The highest IZD of 9.5 ± 0.5 was obtained with a concentration of 100 mg/ml and the least IZD of 7.0 ± 0.0 at a concentration of 12.5 mg/ml. The concentration, 6.25 mg/ml of the extract could not inhibit the tested isolates. Ethanol extracts of *Allium sativum* inhibited all the tested isolates at a concentration of 100 mg/ml with highest IZD of 8.5 ± 0.5 and the least IZD of 7.0 ± 0.0. Ethanol extracts from the seed of *Capsicum annuum* and pericarp of *Capsicum annuum* varieties tested respectively did not inhibit the fluconazole-resistant *Candida albicans* isolates. *Capsicum annuum* var. atarugu displayed the highest IZD of 8.5 ± 0.5 at a concentration of 100 mg/ml. The IZDs of *Capsicum annuum* var. Nsukka yellow pepper and *Capsicum annuum* var. shombo is 8.5 ± 0.5 and 7.5 ± 0.5 respectively.

Minimum Inhibitory Concentrations (MICs) of the test extracts

The different varieties of *Capsicum annuum* hot water extracts tested had similar MIC results ranging from 6.25 - 100 mg/ml. *Zingiber officinale* extract had MIC range of 12.50 – 25.00 mg/ml while *Allium sativum* extract had MIC range of 12.50 – 50.00 mg/ml. The least MIC of hot aqueous extracts was obtained from *Zingiber officinale* with a geometric mean MIC of 10.25 mg/ml as shown in Table 1.

For ethanol extracts, the MIC ranged from 3.125 – 50.00 mg/ml. The least MIC was obtained from ethanol extracts of *Zingiber officinale* with an MIC of 3.125 mg/ml against *Candida albicans* 59. *Capsicum annuum* var. atarugu and shombo exhibited similar MIC of 25.00mg/ml while *Capsicum annuum* var. Nsukka yellow pepper had an MIC of 12.50 mg/ml which is a bit lower that the MIC of the former as shown in Table 2.

DISCUSSION

It has been established that some local spices such as *Capsicum annuum*, *Zingiber officinale* and *Allium sativum* have both antibacterial and antifungal effect against microorganisms including *Candida albicans* (Muhsin and Hussein, 2014; Anikwe *et al*, 2017). The special interest in the consumption of these spices since time immemorial can be attributed to their inherent flavors, colors and source of pungency as well as bioactive compounds with nutritional, nutraceutical and pharmaceutical importance. Different solvents such as water and ethanol were routinely used in extracting bioactive compounds from these medicinal plants. In this study, hot water and ethanol were used as solvents for extraction. Greater inhibitory effect was observed at higher concentration, which is in line with the findings of Sharandeep *et al*., 2017. Ethanol extracts showed relatively higher inhibition when compared to hot water extracts. This may be due to the ability of ethanol to extract more of the polar and some non-polar bioactive compounds and secondary plant metabolites which are believed to exert antimicrobial effect on the test organism. This agrees with the findings of Wail and Emad (2018).

It has been established that the antimicrobial potency of *Zingiber officinale* essential oils, extracts and oleoresins is as a result of their chemical composition.(Bellik, 2014; Par *et al*., 2008), the solvent used for the extraction (Naji *et al*., 2010) and the method used to obtain the extract (Singh *et al*., 2008; Mesomo *et al*., 2013). The presence of phenolic compounds like eugenol, shogaols, zingerone, gingerdiols, gingerols, and others in addition to their synergistic relationship with other compounds such as β-sesquiphellandrene, cis-caryophillene, zingiberene etc. are mainly responsible for the antimicrobial activity (Singh *et al*., 2008).

Table 1: Minimum Inhibitory Concentration (MIC) for hot water extracts in mg/ml

Isolate number	YP (S&F)	AT (S&F)	SH (S&F)	*Zingiber officinale*	*Allium sativum*
45	12.50	12.50	6.25	25.00	25.00
OR20	100.00	12.50	25.00	12.50	50.00
71	6.25	12.50	12.50	12.50	12.50
75	6.25	25.00	12.50	12.50	12.50
59	12.50	12.50	12.50	12.50	12.50
53	50.00	25.00	25.00	12.50	50.00
57	100.00	6.25	25.00	25.00	50.00
MIC_{50}	12.50	12.50	12.50	12.50	25.00
Range	6.25-100.00	6.25-25.00	6.25-25.00	12.5-25.00	12.5-50.00
Geometric Mean MIC	22.64	13.80	15.24	11.26	25.00

Key: YP (S&P): *Capsicum annuum* var. yellow pepper (seed and pericarp); AT (S&P): *Capsicum annuum* var. atarugu (seed and pericarp); SH (S&P): *Capsicum annuum* var. shombo (seed and pericarp).

Table 2: Minimum Inhibitory Concentration (MIC) for ethanol extracts in mg/ml

Isolate number	YP (S&F)	AT (S&F)	SH (S&F)	*Zingiber officinale*	*Allium sativum*
45	12.50	6.25	-	12.50	50.00
OR20	-	-	-	25.00	25.00
71	12.50	-	-	12.5	12.5
75	50.00	25.00	50.00	6.25	50.00
59	12.50	25.00	-	3.125	25.00
53	-	25.00	-	12.50	12.50
57	25.00	25.00	25.00	12.50	25.00
MIC_{50}	12.50	25.00	25.00	12.50	25.00
Range	12.50-25.00	6.25-25.00	25.00-50.00	3.125-25.00	12.50-50.00
Geometric mean MIC	18.95	18.95	35.36	10.25	25

Key: –; No inhibition around the wells of the test extract. No MIC was done for them; YP (S&F): *Capsicum annuum* var. yellow pepper (seed and pericarp); AT (S&F): *Capsicum annuum* var. atarugu (seed and pericarp); SH (S&F): *Capsicum annuum* var. shombo (seed and pericarp).

Interestingly, most of these compounds are insoluble in water and this may explain why our hot water extracts displayed lower antimicrobial activity when compared to ethanol extract with pronounced antifungal effects (Supreetha *et al*., 2011; Atai *et al*., 2009; Wail and Emad, 2018**).** The findings of Wenhui *et al*., suggests that the antioxidant ability of ginger extract is as a result of hydroxyl groups and suitable solubilizing side chains. Yamamoto-Ribeiro *et al.*, reported that the hydrophobic nature of ginger essential oil is responsible for the disintegration of fungal cell wall, hampering ergosterol biosynthesis. Certain compounds of ginger essential oil bind to the ergosterol, thereby disrupting the integrity of the membrane and function of some membrane-bound proteins leading to osmotic imbalance and consequently cell death **(**Bendaha *et al*., 2011**).** These antimicrobial properties of *Zingiber officinale* extracts may explain why in this work, it showed the highest antifungal effect amongst all the tested extracts.

In our study, *Allium sativum* ethanol and hot water extracts showed anti-candida effects. It has been reported that aqueous extract of *Allium sativum* has the potency to inhibit the synthesis of some macromolecular components such as protein and nucleic acid synthesis in *Candida albicans*, but the major anti-candida effect is the inhibition of lipid synthesis (Jeyabalan, 2014). Adams *et al*., reported that *Allium sativum* extract has the ability to inhibit hyphal formation which is a fundamental virulent factor. The recent *in vitro* study conducted by Kumar *et al.,* showed that *Allium sativum* has the antifungal efficacy to inhibit the growth of *Candida albicans.* In contrast to our own study, most of these experiments were done using antifungal susceptible strains of *Candida albicans*. Even though in our experiment, *Allium sativum* extracts exhibited anti-candida effect on the fluconazole-resistant strains tested, more studies need to be conducted to support this finding. The compound capsiacinoids is the main source of pungency in peppers which includes capsaicin, dihydrocapsiacin and others. Capsiacin was the most abundant (Sunil *et al*., 2012).

Studies have shown that the seed of *Capsicum annuum* contains more protein of up to 20.88 % when compared to the pericarp with 14.13 % (Jana *et al*., 2014). Also, analysis of the protein content of *Capsicum annuum* seed extract confirmed the proteins as antimicrobial peptides (AMP) which strongly inhibited the growth of all the yeast strains tested and showed morphological alteration like pseudohyphae formation in *Candida albicans* (Vieira Bard *et al*., 2015). Furthermore, another study confirmed the ability of the AMP from whole *Capsicum annuum* fruit to permeate membranes of all the fungi tested and also induced the endogenous production of reactive oxygen species (ROS) (Dos Santos *et al*., 2017). According to Tomi *et al.,* their results revealed high phenolic contents in *Capsicum annuum* extract. Studies have implicated phenols to exhibit high antioxidant activity connected to the prevention of certain diseases in human body (Ohikhena *et al*., 2018; Zhuang *et al.,* 2012). The *Capsicum annuum* fruit extracts were able to display strong antioxidant activity due to the presence of phenol with redox properties that enables it to adsorb and scavenge free radicals (Tomi *et al.,* 2019; Ohikhena *et al*., 2018). However, the extracts from *Capsicum annuum* seed showed no inhibition at all as well as the extracts from the pericarp of *Capsicum annuum*. This maybe because the *Candida albicans* isolates used in this study were already resistant to the antifungal drug, fluconazole. Interestingly, extracts from whole *Capsicum annuum* fruit samples (seed and pericarp together) showed inhibitory effect. This suggests that there may be synergy from both the bio-active components from the seed and pericarp and not necessarily the seed nor pericarp alone. There are not many findings yet on this as no experiment have specifically reported the effect of these extracts against fluconazole-resistant *Candida albicans* isolates.

The minimum inhibitory concentration (MIC) results as shown in Tables 1 and 2 suggest that among the three selected *Capsicum annuum* samples, *Capsicum annuum* variety atarugu gave the least MIC value ranging from 6.25 mg/ml to 25 mg/ml (both hot water and ethanol extract) and demonstrated a relatively higher antifungal effect against fluconazole- resistant *Candida albicans* isolates compared to other pepper extracts. This is also in tandem with the findings of Anikwe *et al.,* who reported that the ethanol extracts of *Capsicum annuum* varieties tested were active against all the clinical fungal isolates. Amongst the pepper extracts, *Capsicum annuum* var. atarugu also gave the lowest MIC values. This supports the report that samples collected from same geographical location show similar antimicrobial activity (Kahni *et al*., 2011). *Capsicum annuum* variety Nsukka yellow pepper and *Capsicum annuum* variety shombo displayed relatively equal activity against the tested isolates. These findings suggest that these flavouring plant materials may serve as potential sources of drugs to combat resistant microbial strains.

Conclusion

Although this is a preliminary study, our study has established that ethanol extracts of these local spices displayed relatively higher inhibitory activity on the test organisms. Moreover, the biochemical components from the seed or pericarp alone could not produce any inhibition on the tested isolates. Inhibition was observed using extracts from whole *Capsicum annum* fruit. This study further justifies the use of *Capsicum annuum*, *Zingiber officinale* and *Allium sativum* as therapeutic agents.

Conflict of Interest

The authors have no conflict of interest to declare.

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