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Evaluation of the Antifungal Activity of Aqueous and Alcoholic Extracts of Six Spices

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Abstract

Background: Nigeria is plagued with a variety of socioeconomic problems mainly poverty, poor access to quality healthcare and poor hygienic conditions resulting in the myriad of fungal infections that are frequently encountered in clinical practice. Method: The antifungal activity of aqueous, methanolic, propanolic and benzyl alcohol extracts of *Capsicum annuum* fruits and seeds, *Capsicum chinense* fruits and seeds, *Aframomum melegueta* pods and seeds, *Allium sativum* bulbs, *Allium cepa* bulbs and *Zingiber officinale* rhizomes on *Candida albicans* (yeast), *Aspergillus niger* (mould) and *Trichophyton rubrum* (dermatophyte) were evaluated by the agar well diffusion method. The aqueous and alcoholic filtered plant extracts were obtained by maceration, and also methanolic extracts were also obtained by Soxhlet extraction. The results were obtained by measuring the inhibition zone diameter in millimeters and were presented by subtracting the activity of the control. Results: Aqueous and methanolic extracts of *Allium sativum* gave the highest inhibition of the growth of *Candida albicans* (22 mm), followed closely by its propanolic extract with inhibition zone diameter of 15 mm, and also, propanolic extracts of *Aframomum melegueta* and *Allium cepa* gave inhibition zone diameters of 12 mm each. Soxhlet methanolic extract of *Allium sativum* had the highest inhibition of the growth of *Aspergillus niger* with an inhibition zone diameter of 25 mm, followed closely by *Zingiber officinale* Soxhlet methanolic extract with an inhibition zone diameter of 22 mm, also, the propanolic extract of *Allium sativum* gave an inhibition zone diameter of 21 mm, whereas Soxhlet methanolic extracts of *Aframomum melegueta* and *Allium cepa* gave an inhibition zone diameter of 19 mm each. The highest activity against *Trichophyton rubrum* was obtained with the Soxhlet methanolic extract of *Allium sativum* (39 mm), followed closely by its propanolic extract with an inhibition zone diameter of 27 mm. An inhibition zone diameter of 22 mm was recorded with the benzyl alcohol extract of *Allium cepa*, 22 mm with the Soxhlet methanolic extract of *Aframomum melegueta* and 19mm with the aqueous extract of *Capsicum chinense* seeds. Conclusion: The *in-vitro* inhibitory effects of these spice extracts indicated that the test spices could serve as potential candidates for developing new systemic and topical

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antifungal drugs against the wide range of pathogenic fungal strains, and they could also serve as natural prophylaxis against the fungal infections.

Keywords

Spices, Antifungal Activity, Agar Well Diffusion

1. Introduction

People all over the world are still affected by quite a large number of microbial infections with fungi causing a good number of them. It has since been discovered that active medicinal substances are present in plants and this has encouraged the inclusion of herbal remedies in the delivery of health care [1] [2].

Spices are aromatic or pungent plant parts used for enhancing the taste of foods. Although spices are commonly used to improve the taste of foods, they have also been exploited for their medicinal as well as their antimicrobial activities [3] [4].

Capsicum annuum (red pepper) is a fruit spice of the Solanaceae family, rich in proteins, lipids, vitamins, carbohydrates, and health phytochemicals such as carotenoids, flavonoids and capsaicinoids known to prevent diseases such as asthma, coughs, sore throats etc. [5] [6]. *Capsicum chinense* (Cameroon pepper) is also from the family Solanaceae. It is very popular in the Nigerian market and is used by so many households and individuals.

Aframomum melegueta (Alligator pepper) is from the family Zingiberaceae together with *Zingiber officinale* (ginger). Alligator pepper seeds have been shown to possess phytoconstituents that have potent antimicrobial effect [7]. Alligator pepper have been found to contain the phytoconstituents; gingerol, methyl-6-gingero, shogaol and paradol that contribute to its antimicrobial properties [8]. Ginger has strong aromatic and medicinal properties [9]. It has been utilized for the treatment of infectious diseases in many countries [10] [11]. The pungent taste of ginger has been attributed to gingerols, shagaols, and zingerone.

Allium cepa (onions) is from the family Amaryllidaceae and contains water, sugar, protein, fat and fibre [12]. Flavonoids and organosulphur compounds are the two major classes of phytochemicals present in onions believed to provide its beneficial health effects [13]. *Allium sativum* (garlic) also from the Amaryllidaceae family has been used both for its culinary and medicinal purposes [14]. The sulphur containing compounds give them the characteristic flavor and exhibit potent antifungal properties [15] together with the non-sulphur compounds [16]-[18].

Fungal pathogens of humans are grouped as: Yeasts (*Candida albicans*), Moulds (*Aspergillus niger*), and Dermatophytes (*Trichophyton rubrum*). *C. albicans* is responsible for a wide range of superficial and systemic infections. 75% of women are affected with genital Candidiasis in their lifetime while in men, alcoholics and diabetics are prone to genital Candidiasis [19] [20]. *Aspergillus niger* is ubiquitous in nature, exposure is common but disease is rare. Aspergillosis occurs in immunocompromised individuals. *A. niger* has been implicated in otomycosis in healthy persons. Massive inhalation of *A. niger* spores can cause allergic reactions such as asthma and pneumonitis. Dermatophytes are fungi that are capable of colonizing the skin, nails, or hair. *Trichophyton rubrum* is an anthropophilic saprotroph, it colonizes the upper layers of dead skin and it is the most common cause of *Tinea pedis*, *Tinea unguuim*, *Tinea manuum*, *Tinea cruris*, and *Tinea corporis*. Infection can be avoided by lifestyle and hygiene modification [19].

The aim of this study is to investigate the inhibitory effects of six commonly used spices on the growth of three pathogenic fungi to determine whether they can serve as natural prophylactics and the possibility of developing systemic and topical antifungal agents with them using the agar well diffusion technique.

2. Methods

2.1. Test Micro-Organisms

Three microorganisms were tested in this study: *Candida albicans*, *Trichophyton rubrum*, *Aspergillus niger*. They were characterized strains obtained from the Laboratory of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria.

2.2. Plant Materials

In this study, six commonly used spices were selected to analyze their antifungal activity based on previous literature and their popularity in the Nigerian markets. The fresh bulbs of *A. cepa* and *A. sativum*, fresh rhizomes of *Z. officinale*, fresh fruits of *C. annuum*, dried fruits of *C. chinense* and pods and seeds of *A. melegueta* were purchased from Eke Awka and Ose market in Onitsha, Anambra State in the month of April. They were identified and authenticated by Mrs Oduche Anthonia in the Department of Pharmacognosy and Traditional medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka.

2.3. Extraction of Crude Drugs

The fresh fruits of *C. annuum* were dried in a hot air oven at 60˚C for 8 hours. Seeds from some of the dried fruits of *C. annuum* and *C. chinense* were separated, along with the seeds of *A. melegueta* and were powdered separately. The dried materials including the whole fruits of *C. annuum* and *C. chinense* and pods of *A. melegueta* and the separated seeds were reduced to fine powder with a mechanical grinder. The bulbs of *A. cepa* and *A. sativum*, and the *Z. officinale* rhizome were washed thoroughly using tap water, a clean kitchen knife was used to manually peel the outer coverings, and then the fleshy parts were washed and rinsed with distilled water again. A sterile ceramic mortar and pestle was used to crush the parts into smaller marshy parts. Four solvents were used for the preparation of the extracts namely: cold water, methanol, propanol and benzyl alcohol.

The aqueous extracts were prepared by weighing out 2 g of the powdered seeds and whole fruits of *C. annuum*, *C. chinense* and *A. melegueta*, and 5 g of the crushed bulbs of *A. cepa* and *A. sativum* and rhizome of *Z. officinale*, and macerating in 40 ml of cold water in a glass jar. The combination was allowed to stand for 24 hours at room temperature (32˚C ± 2˚C) with occasional agitation. The alcoholic extracts; methanol 99.5%, propanol 99.5% and benzyl alcohol 99% were obtained by weighing out same fraction (2 g) of the powdered whole fruits and separated seeds of *C. annum*, *C. chinense* and *A. melegueta*, and 5 g of the crushed bulbs of *A. cepa* and *A. sativum* and rhizome of *Z. officinale* and macerating in 40 ml of the 99.5% methanol, 99.5% propanol and 99% benzyl alcohol in a covered glass jar. The combination was allowed to stand for 24 hours with occasional agitation at room temperature (32˚C ± 2˚C). The extracts were then filtered using a Whatman no. 1 filter paper into plastic sample bottles and stored in a refrigerator at 2˚C - 8˚C. There was altogether nine aqueous extracts, nine methanolic, nine propanolic and nine benzyl alcohol extracts.

The Soxhlet extractor was also used to afford nine different methanolic extracts of the whole fruits and separated seeds of *C.annum*, *A. melegueta* and *C. chinense*, and the bulbs of *A. cepa* and *A. sativum* and rhizome of *Z. officinale*. The extracts were then stored in a refrigerator at 2˚C - 8˚C. Altogether, 45 extracts was tested.

2.4. Identification of Organisms

Three test tubes of 5 ml sterile Sabouraud dextrose broth was prepared and labeled appropriately and loopfuls of organisms were collected from the stock cultures of *C. albicans*, *A. niger* and *T. rubrum* and placed in the individual test tubes. The organisms were identified using morphological and cultural characteristics.

2.5. Fungal Identification

The morphological and cultural characteristics of the fungi isolates were used to identify them. The method that was used is the direct observation of plates. Colony morphology includes the type of pigment (if present), size of colony, texture (opaque, translucent, or transparent), adherence to agar and undulating/round/dentate edge. The plates were observed daily for the rate of growth of each of the isolates. The colour and morphology of the colonies were noted. The base of the plate, odour, were noted.

Standard suspensions containing 10^5 cfu/ml of the test organism was made by transferring colonies from the subculture into 5 mls of sterile water and then adjusting and comparing with McFarland's 0.5 standard.

2.6. Antifungal Activity of the Extracts

The antifungal activity of these crude extracts was determined against a yeast (C. *albicans*), mold (*A. niger*) and dermatophyte (T. *rubrum*). Agar well diffusion technique was used to determine the antifungal activities of the extracts. 25 mls of molten sterilized Sabouraud's dextrose agar, fortified with 0.05 mg/ml of chloramphenicol in

a McCartney bottle was seeded with 0.2 mls of 0.5 McFarland standard suspension of each fungal isolate and poured into appropriately labeled sterile Petri dishes aseptically. The Petri dishes were rotated gently to achieve a uniform distribution of the fungal isolate and was then allowed to set. A standard sterile cork borer of 8 mm diameter was used to cut uniform wells on the surface of the solidified seeded agar. The wells were then filled with 0.1 ml of each extract with the aid of a sterile syringe and needle. One of the wells in each SDA agar plate was filled with 0.1 mls of sterile water, 99.5% methanol, 99.5% propanol or 99% benzyl alcohol as a control. The plates were then incubated at room temperature (32˚C ± 2˚C) on the laboratory bench for 24 hours (*C. albicans*), 48 hours (*A. niger*), and 72 hours (*T. rubrum*) respectively and observed for zones of inhibition. A zone of clearance round each well signifies inhibition and the diameter of the zones were measured in millimeter (mm) and presented by subtracting the activity of the control. This was performed in triplicates for each fungal isolate and extracts and the average of the three readings was taken.

3. Results and Discussions

The fungal isolates were identified by their morphological characteristics (**Table 1**).

The spice extracts differed significantly in their potential to inhibit the growth of *C. albicans*, *A. niger* and *T. rubrum.* Even some of the aqueous extracts had inhibition of the growth of the fungal isolates. The fruits of *C. annuum* and *C. chinense* had ten (10) seeds each. This seeds were separated, milled and tested alone for their inhibitory effects on the test fungi. The seeds of *A. melegueta* were also purchased from the market, different from the pods, milled, and also tested for antifungal activity.

The highest activity on *C. albicans* was obtained with the methanolic and propanolic extracts, moderate activity was obtained with the aqueous extracts and the lowest activity was with the benzyl alcohol and Soxhlet methanolic extraction extracts. There was no growth on the C. *albicans* agar plates filled with the propanol and benzyl alcohol extracts for three and four days respectively. The table below shows the IZD of tested spice extracts on *Candida albicans*.

Soxhlet methanol extraction gave the best activity on *A. niger*, propanol gave moderate activity while aqueous and benzyl alcohol extracts gave the lowest activity. There was no growth on the *A. niger* agar plates filled with the propanol and benzyl alcohol extracts for four days. The table below shows the inhibition zone diameter of the tested spice extracts on *Aspergillus niger.*

Methanolic extracts gave the highest activity against *T. rubrum*; aqueous extracts gave intermediate activity, while the lowest activity was obtained with the propanolic extracts. There was no growth on the *T. rubrum* agar plates filled with the propanol and benzyl alcohol extracts for seven days. The table below shows the inhibition zone diameter of the tested spice extracts against *T. rubrum*.

Significant antifungal activities were evident with extracts of selected spices from members of Solanaceae, Zingiberaceae and Amaryllidaceae families. The result obtained from this study indicated that aqueous or methanolic, propanolic or even benzyl alcohol extracts of dried fruits of *C. annuum*, its seeds, *C. chinense*, its seeds, *A. melegueta* pods and seeds, *A. cepa* and *A. sativum* bulbs and *Z. officinale* rhizomes inhibited the growth of *C. albicans*, *A. niger* and *T. rubrum.*

From the Solanaceae family, *C. annuum* and *C. chinense* dried fruits and seeds were studied. Only the aqueous extracts of the seeds of *C. annuum* and *C. chinense* inhibited the growth of *C. albicans*. Only the aqueous extract of *C. annuum* seeds inhibited the growth of *A. niger* whereas the aqueous extract of *C. annum fruit* and *C. chinense* seeds inhibited the growth of *T. rubrum*. This indicates that these peppers have active constituents that inhibit the growth of these fungi. Benzyl alcohol extracts of *C. annuum* seeds and fruits had no activity on *C. albicans* indicating that the active constituents might be insoluble in the alcohol. Methanolic and

Table 1. Colony morphology of fungal isolates.

Isolate Identification Code	Cultural Characteristics	Organism
A	White to cream coloured opaque colonies, with fast rate of growth, produced in less than 24 hours.	*Candida albicans*
B	Fast growing, white, yellow, yellow-brown to black opaque colonies, arranged in whorls.	*Aspergillus niger*
C	The colonies are opaque, flat to slightly raised, white to cream with a pale brown reverse pigment and a slow rate of growth.	*Trichophyton rubrum*

benzyl alcohol extracts of *C. annuum* and *C. chinense* fruits and seeds had no activity on *A. niger* but the propanol extracts did indicating that their active constituents for antifungal activity are best extracted with propanol. The aqueous and propanol extracts of *C. annuum* seeds had the same inhibition zone diameter (15 mm) on *A. niger*. The aqueous and methanolic extracts of *C. chinense* seeds inhibited the growth of *T. rubrum* with IZD of 19 mm and 16 mm respectively. The aqueous extract had more activity than its methanol and benzyl alcohol extracts on *T. rubrum*. The propanol extract had no activity.

In the Zingiberaceae family, *A. melegueta* seeds and pods, and fresh rhizomes of *Z. officinale* was studied. Their aqueous extracts had no activity on *C. albicans* (**Table 2**) and *A. niger* (**Table 3**) The aqueous extract of *A. melegueta* pods had activity (18 mm) on *T. rubrum*. The methanolic extract of *A. melegueta* pods and seeds and ginger had activity on *A. niger* and *T. rubrum*. *A. melegueta* had more activity on the three test fungi than *Z. officinale*. In a study conducted by Odetunde *et al*., 2015, *A. melegueta* leaves aqueous and methanolic extracts inhibited the growth of *C. albicans* and *A. niger* with 1ZD up to 40 mm in both cases indicating that *A. melegueta* possess phytochemicals with potent antifungal activity [21]. The antifungal activities of *A. melegueta* have been attributed to phenolic compounds such as gingerol, shagaol, and paradol present in the plant parts [22]. The phytochemical screening of A. melegueta extracts indicated that it contains high doses of tannins, saponins, glycosides and polyphenols than many other weed plants [23].

Only the methanolic extract of ginger had activity on *T. rubrum*. Also, only the methanolic and benzyl alcohol extract of ginger had activity on *A. niger*. An inhibition zone diameter of 11 mm was obtained with ethanolic extracts of ginger against *C. albicans* in a study conducted by Atai *et al*., 2009. Similarly, an IZD of 16 mm was

Table 2. Inhibition zone diameter of tested spice extracts on *Candida albicans*.

Spices	Aqueous Extracts (mm)	Methanolic Extracts (mm)	Soxhlet Methanolic Extracts (mm)	Propanol Extracts (mm)	Benzyl Alcohol Extracts (mm)
C. annuum	-	8 mm	-	-	-
C. annuum seeds	5 mm	5 mm	-	3 mm	
C. chinense	-	8 mm	-	3 mm	7 mm
C. chinense seeds	6 mm	6 mm	7 mm	4 mm	6 mm
A.melegueta	-	-	10 mm	12 mm	1 mm
A.melegueta seeds	-	8 mm		2 mm	
A .cepa	-	10 mm		12 mm	
Z. officinale	-	9 mm	1 mm	5 mm	
A. Sativum	22 mm	22 mm	17 mm	15 mm	4 mm

Table 3. Inhibition zone diameter of tested spice extracts on *Aspergillus niger*.

Spices	Aqueous Extracts (mm)	Methanolic Extracts (mm)	Soxhlet Methanolic Extracts (mm)	Propanol Extracts (mm)	Benzyl Alcohol Extracts (mm)
C. annuum	-	-	-	10 mm	-
C. annuum seeds	15 mm	-	-	15 mm	-
C. chinense	-	-	14 mm	12 mm	-
C. chinense seeds	-	-	17 mm	6 mm	-
A.melegueta	-	15 mm	19 mm	13 mm	-
A. melegueta seeds	-	10 mm	12 mm	7 mm	-
A. cepa	-	11 mm	19 mm	-	11 mm
Z. officinale	-	5 mm	22 mm	-	7 mm
A. sativum	20 mm	14 mm	25 mm	21 mm	7 mm

obtained with ethanolic extracts of ginger against *C. albicans* in a study conducted by Supreetha *et al*., 2011 [24]. This indicates that active constituents responsible for its activity against *C. albicans* are more soluble in ethanol as methanolic extracts used in this study only gave IZD of 9 mm against *C .albicans*. This study also suggests that the different antifungal agents present in ginger are insoluble in water as none of the aqueous extracts of ginger inhibited the growth of the three test fungi. Gingerols were identified as the major active component in the fresh ginger rhizome [10] indicating that it potential as an antifungal agent. Thus, methanol is a good solvent for obtaining *A. melegueta* extracts whereas ethanol is better for *Z. officinale* extracts.

A. sativum and *A. cepa* are members of the Amaryllidaceae family studied. The aqueous and methanolic extracts of garlic had the same activity (22 mm) on *C. albicans*. Similarly, in a study conducted by Iwalokun *et al*., 2004 [17], Suleiman and Abdallah, 2014 [25], an inhibition zone diameter of 27 mm and 16 mm respectively were obtained with aqueous extracts of garlic against *C. albicans*. The difference in activity obtained from the aqueous extracts of garlic in the different studies can be attributed to the geographical location, age of plant, time of harvesting, freshness of plant materials, and even the time of harvesting of plant materials. Also, the aqueous extract of garlic had activity on *A. niger* and *T. rubrum*. The aqueous extract of onion had activity only against *T. rubrum* which is in line with the study conducted by Bakht *et al*., 2013 [26] and Irkin and Korukluoglu, 2007 [27]. Aqueous extract of garlic gave an inhibition zone diameter of 20 mm against A. niger which is in line with that obtained by Avasthi *et al*., 2010 [28] (34 mm), Irkin and Korukluoglu, 2007 [25] (16 mm) and Suleiman and Abdallah, 2014 [27] (14 mm). Again, the difference in the inhibition zone diameter can be attributed to the geographical location, age of plant, time of harvesting, freshness of plant materials, physical factors (temperature, light, and water), contamination by field microbes and even the time of harvesting of plant materials. Thus, the aqueous extract of garlic had activity on the three tested fungi. The propanolic extract of onion had more activity on *C. albicans* than its methanol extract whereas the methanolic extract of garlic had more activity on *C. albicans* than its propanolic extract as shown in **Table 3**. This indicates that the active components inhibiting the growth of *C. albicans* are more soluble in propanol (onion) and methanol (garlic) in each case. The propanolic extract of garlic had more activity on *A. niger* than its benzyl alcohol, methanol and aqueous extract as shown in **Table 4**. Only the propanolic extract of garlic had activity on *T. rubrum*, the propanolic extract of the other test spices didn't. The benzyl alcohol extract of onion gave an inhibition zone diameter of 22 mm against *T. rubrum* which is higher than that obtained with its aqueous and methanolic extracts. *A. sativum* had more activity than *A. cepa*. Flavonoids and organosulphur compounds present in onion has been found to be responsible for its antifungal properties [13]. Ajoene present in garlic has been noted to have a potent effect on yeasts, molds and dermato-phytes [16]. Some studies revealed that garlic is as effective as fluconazole at inhibiting *C. albicans* [17]. When crushed, garlic yields allicin, an antifungal compound and other sulphur containing compounds.

The activity of these spice extracts is comparable to the activity of some synthetic antifungal agents' invitro. In a study conducted by Atai *et al*., 2009 [29], Nystatin was used as a positive control. Whereas ginger gave an IZD of 11 mm against *C. albicans*, nystatin produced an IZD of 12 mm. Similarly, fluconazole produced an IZD of

Table 4. Inhibition zone diameter of tested spice extracts on *Trichophyton rubrum.*

Spices	Aqueous Extracts (mm)	Methanolic Extracts (mm)	Soxhlet Methanolic Extracts (mm)	Propanol Extracts (mm)	Benzyl Alcohol Extracts (mm)
C. annuum	10 mm	4 mm	-	-	-
C. annuum seeds	-	-	-	-	-
C. chinense	19 mm	-	-	-	-
C. chinense seeds	18 mm	16 mm	17 mm	-	10 mm
A. melegueta	-	15 mm	22 mm	-	12 mm
A. melegueta seeds		17 mm	18 mm	-	-
A. cepa	12 mm	10 mm	-	-	22 mm
Z. officinale	-	9 mm	17 mm	-	-
A. sativum	21 mm	19 mm	39 mm	27 mm	-

30 mm when used as positive control whereas aqueous garlic extracts produced an IZD of 27 mm against *C. albicans* in a study conducted by Iwalokun *et al*., 2004 [17].

Soxhlet extraction is a better extraction method than maceration but its high cost could be the reason while it is not usually employed. Macerated methanol extracts of *C. chinense* seeds, *A. melegueta* pods, its seeds, *Z. officinale* and *A. sativum* gave inhibition diameters of 16 mm, 15 mm, 17 mm, 9 mm, and 19 mm respectively against *T. rubrum* whereas their Soxhlet methanol extracts gave IZD of 17 mm, 22 mm, 18 mm, 17 mm, and 39 mm respectively. Also, macerated methanol extracts of *C. chinense*, its seeds, *A. melegueta* pods and seeds, *A. cepa*, *Z. officinale* and *A. sativum* gave inhibition zone diameters of 0 mm, 0 mm, 15 mm, 10 mm, 11 mm, 5 mm, and 14 mm respectively against *A. niger* whereas their Soxhlet methanol extracts gave IZD of 14 mm, 17 mm, 19 mm, 12 mm, 19 mm, 22 mm, and 25 mm respectively as shown in **Table 4**.

The zones of inhibition obtained from extracts of these spices shows that they have great potential as remedies for diseases caused by the test fungi and can even serve as natural prophylactics.

4. Conclusion

The study demonstrated that the six spices used had activity on the yeast (*Candida albicans*), the mould (*Aspergillus niger*) and the dermatophyte (*Trichophyton rubrum*). *Allium sativum* had the greatest activity on all the test organisms signifying that it had greater promise as an antifungal agent. Active constituents with potent activity against tested fungi were also present in the separated seeds of *C. annuum*, *C. chinense* and *A. melegueta.* This encouraged the use of these spices for controlling fungal infections and as natural prophylactics. They offered the advantage of being used indefinitely in large amounts.

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