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Evaluation of the anti-candidal activity of the ointment formulation containing the admixture of *Allium sativum* (Liliaceae) and *Citrus sinensis* (Rutaceae)

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ABSTRACT

Candidiasis is a highly recurring disease with an history of resistance, thus the need for discovering newer anti-fungal drugs. The aim of this study was to evaluate the anti-candidal activity of the admixture of *Allium sativum* bulb (ASB) and *Citrus sinensis* peel (CSP) extracts and its ointment formulation. The *in vitro* anti-candidal activity of the admixtures and the formulated admixture was evaluated using agar well diffusion method and the minimum inhibitory concentration (MIC) was obtained using solid dilution method. The anti-candidal activity of the admixtures in the ratios 1:1, 1:2 and 2:1 at the concentration of 50 mg/mL gave zones of inhibitions (mm) of 15.50, 21.00 and 16.50 respectively. The zone of inhibition (18.50 mm) of ASB extract at this concentration was smaller than that of the admixture (1:2) while that of the formulated admixture (25.50 mm) was higher than the admixture (1:2). The formulated admixture gave no sensation and was poorly homogenous in appearance. It had a pH of 6.89. Also, the nauseating odour of ASB extract was reduced in the formulation containing the admixture.

Keywords: *Allium sativum, Citrus sinensis,* anti-candidal, ointment.

INTRODUCTION

Medicinal plants have been used for years as alternative treatment for diseases in different areas of the world due to their therapeutic value (Busato *et al.,* 2016). Natural plant products either as pure compounds or as standardized plant extracts have provided unlimited opportunities for new drug leads. This results to a never-ending need to discover new antimicrobial compounds for new and re-emerging infectious diseases (Ekwenye and Edeha, 2010).

Allium sativum L. (garlic) is a member of the Liliaceae- family and has been widely recognized as a valuable spice and popular plant remedy for various ailments and physiological disorders (Derba and Kidanamariam, 2015). Garlic has been widely used as supplements, and it also has antioxidant, blood pressure lowering properties, antiviral, antibacterial and antifungal activities (Konaklieva and Plotkin, 2006; Papu *et al.,* 2014).

Citrus sinensis (orange) is a member of Rutaceae family. It is one of the most important commercial fruit crops grown in all countries of the world (Tao *et al.*, 2008). *C. sinensis* is widely distributed in the tropics. The fruit originated from Southern China, North Eastern India and Southern Asia. It is a perennial tree. The entire part of the tree has useful medicinal properties (Strange *et al*., 1993). The peels of some species of orange have been found to possess antimicrobial properties (Strange *et al*., 1993; Tao *et al.,* 2009).

Candida albicans is the most common human fungal pathogen, causing a variety of skin and soft tissue infections in healthy people. It is also known to cause more virulent invasive and disseminated diseases in patients with compromised immune systems (Noble and Johnson, 2007). The ability of this microorganism to persist in healthy hosts and also cause some diseases in the immuno-compromised host are questions of significant biological interest as well as major clinical and economic importance (Noble and Johnson, 2007). Limited studies have been carried out on the formulation of an effective and pleasant smelling anti-candidal ointment from the extracts of *Allium sativum* bulb (ASB) and *Citrus sinensis* peel (CSP) to the best of the authors' knowledge. This study was carried out to evaluate the anti-candidal activity of the admixture of ASB and CSP extracts and its ointment formulation. The evaluation of the physical properties of the ointment formulation was also carried out and compared with Mycoten® cream (a commercial brand of Clotrimazole 1% $^{w}/_{w}$).

MATERIALS AND METHODS

Collection and Extraction of Plant materials

The *Allium sativum* bulbs and *Citrus sinensis* peelswere obtained from the local market (Mushin) in Lagos, Nigeria. The plant materials were identified and authenticated

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at the Herbarium of the Department of Botany and Microbiology, University of Lagos, Akoka, Lagos, Nigeria. Voucher specimens of *A. sativum* (LUH 5251) and *C. sinensis* (LUH 5250) were deposited for future reference.

The ASB were peeled, rinsed, weighed and blended in the extracting medium (20% ethanol), using an electric blender (Nakai blender 242, Japan), 898 g of the blended ASB was macerated in 3.592 L of 20% ethanol for 48 hours (Daka, 2011; Singla *et al.,* 2011). CSP were dried for about seventeen days under a shady condition in a room with temperature of 26±1 ^{o}C. The dried peels were size reduced using a milling machine (Christy and Norris Ltd., England), one kilogram of the milled powder was then weighed and macerated in 4L of 20% ethanol for 48 hours. The allicin constituent of the *Allium sativum* has been found to be most stable in 20% ethanol when compared with some other solvents (Singla *et al.*,2011).

The macerated ASB and CSP were whirled occasionally in their containers and were kept in a refrigerator (4 ^{o}C). The content in each container was filtered afterwards and kept in the refrigerator till further use. The filtered plant extracts were dried using Telstar Cryodos -80 ^{o}C Freeze dryer (Terrassa, Spain).

Phytochemical Screening

The extracts of the plants were tested for the presence of saponins, alkaloids, reducing sugars, cardiac glycosides, steroidal rings, tannins, anthraquinones and flavonoids using methods employed in earlier studies (Sofowora, 1982; Edrah *et al.*, 2016).

Evaluation of the Anti-Candidal Activity of the Extracts

The anti-candidal activity of the hydro-ethanolic extracts of ASB, CSP and their admixtures in the ratios 1:1, 1:2 and 2:1 were evaluated by using agar well diffusion method (Rojas *et al*., 2003; Balouiri *et al*., 2016). Concentrations (mg/mL) of 50, 100 and 200 were prepared for each extract. The fungal broth cultures were prepared to a density of about 10^{8} sfu/mL. Sabouraud dextrose agar (SDA) was inoculated with *Candida albicans* by adding 1 mL each of the fungus to labelled empty petri dishes and adding 25 mL of autoclaved SDA solution. The mixtures were whirled north- south, east- west and circularly to ensure homogeneity and were made to solidify at 25 ^{o}C. Four wells were made with a cork borer (diameter 10 mm) under aseptic condition on each solidified agar plate. Approximately 0.2 mL of the plant extracts was introduced into each well. The plates were incubated at 37 ^{o}C for 24 hours. Zones of inhibition were measured and expressed in millimeters. The anti-candidal test was carried out in duplicates (Pundir and Jain, 2010).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC of the ASB extract and the most effective admixture ratio (*A. sativum: C. sinensis* admixture (1:2) were determined. To determine the MIC, single strength (0.065% $^{w}/_{v}$) or one and half strength (0.0975% $^{w}/_{v}$) SDA, was mixed with the extracts in different ratios to obtain various concentrations ranging from 0.25 mg/mL to 128.00 mg/mL. The various mixtures of concentrations were transferred into labeled empty petri dishes under aseptic conditions and were made to solidify at room temperature. One drop each of the fungal culture at the density of 10^{6} sfu/mL was dropped at the labeled spots and the plates were incubated at 25 ^{o}C for approximately 72 hours. The MIC was indicated by the first concentration at which no growth was observed (Chhetri *et al*., 2010).

The plates from the MIC determinations that showed no growth were sub-cultured on SDA plates to determine if the inhibition was reversible or permanent. The first concentration at which no growth was observed on the sub-cultured plate was taken as the MFC (Patil and Ravindra, 2009). This shows the least concentration at which the *Candida albicans is* completely dead rather than just being inhibited.

Formulation and evaluation of the ASB: CSP admixture (1:2) 50 mg/mL ointment

The ASB: CSP admixture (1:2) at the concentration of 50 mg/mL was formulated as ointment using a method employed in a previous study (Chhetri *et al*., 2010). This concentration was chosen because it is the concentration of the ASB: CSP admixture that gave the highest zone of inhibition (Table 1).

The ointment was evaluated for homogeneity, spreadability, odour, skin irritation and pH. The evaluation parameters were carried out according to previous studies (Chhetri et al., 2010; Patil and Ravindra, 2009). The evaluation parameters of the ointment formulation were compared with that of Mycoten® (Clotrimazole 1% w/w). The anti-candidal activity of the formulated ointment and Mycoten® (Clotrimazole) was also carried out using the same procedures used for the anti-candidal activity of the plant extracts.

Table 1: Percentage composition of the *ASB: CSP admixture (1:2) 50 mg/mL ointment*

Ingredient	Concentration
Allium sativum	3.33%
Citrus sinensis	6.67%
Glycerine	20.00%
Benzoic acid	0.10%
Petrolatum q.s	100.00%

RESULTS

Phytochemical Screening

The phytochemical screening of the *A. sativum* extract showed the presence of saponins, alkaloids, reducing sugars, cardiac glycosides and steroidal rings while tannins, anthraquinones and flavonoids were absent. The phytochemical screening of the *C. sinensis* showed the presence of saponins, reducing sugars, cardiac glycosides, steroidal rings, tannins, anthraquinones and flavonoids.

Evaluation of the Anti-Candidal Activity of the Extracts

The anti-candidal activity of different concentrations of the standard drug (Clotrimazole) used as positive control are presented in Table 2. The anti-candidal activity of the 5% ethanol (diluent) used as negative control showed no zone of inhibition. The results of the anti-candidal activity of the plant extracts are presented in Table 3.

Table 2: Anti-candidal activity of clotrimazole (positive control)

Concentration (µg/ml)	Diameter of zone of Inhibition (mm)
160	45.00±0.00
80	42.75±1.26
40	40.75±0.50
20	38.75±0.96
10	38.00±1.63

NA: No activity ; Values are mean ± Standard deviation of 2 assays.

Table 3: Anti-candidal activity of the plant extracts

Concentration (mg/mL)	Zone of Inhibition (mm)				
	Allium sativum	*C. sinensis*	Admixture 1:1[a]	Admixture 1:2[b]	Admixture 2:1[c]
50	18.50±1.00	26.00±1.41	15.50±0.71	21.00±1.16	16.50±1.29
100	19.50±1.29	[d]NA	18.00±1.83	16.75±1.50	18.00±1.16
200	22.75±1.50	[d]NA	21.25±0.96	20.50±1.00	20.25±1.50

Values are mean ± Standard deviation of 2 assays.
[a]Admixture 1:1- A. sativum: C. sinensis admixture in the ratio 1: 1; [b]Admixture 1:2- A. sativum: C. sinensis admixture in the ratio 1: 2; cAdmixture 2:1- A. sativum: C. sinensis admixture in the ratio 2: 1; [d]NA: No activity

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC of *A. sativum* extract *was* 32.00 mg/mL, while that of the the ASB: CSP admixture (1:2) was 24.00 mg/mL. The MFC of the ASB: CSP admixture (1:2) was also 24.00 mg/mL while that of *A. sativum* extract was also 32.00 mg/mL.

Formulation and evaluation of the ASB: CSP admixture (1:2) 50 mg/mL ointment

The results of the physical properties of the formulated admixture, in comparison to that of Mycoten® and Petrolatum B. P. are presented in Table 4.

Table 4: Physical Properties of the Formulated Ointment

Formulations	Homogeneity	Spreadability	Odour	Skin irritation test	pH
ASB:CSP[a]	Poor	Good	Slightly pungent	No sensation	6.89
Mycoten®	Good	Good	Neutral	No sensation	6.48
Petrolatum B. P	Good	Good	Neutral	No sensation	5.30

[a]*ASB: CSP is admixture (1:2) formulation at the concentration of 50mg/mL*

DISCUSSION

The result of the phytochemical screening of the *A. sativum* extract is in agreement with earlier studies (Ameh and Nwammoh, 2010; Mikail, 2010). The presence of alkaloids and saponins has been shown to be responsible for its anti-microbial activity (Maatalah *et al.*, 2012).

The phytochemical evaluation of the *C. sinensis* extract also showed similar results to that obtained in a previous work (Osarumwense *et al.,* 2011). The presence of alkaloids, saponins, and flavonoids in plant extracts has been shown to be responsible for its anti-microbial activity (Silva *et al*., 2016).

The standard drug (Clotrimazole) at different concentrations of 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml) gave very wide zones of inhibition of 38.00 mm, 38.75 mm, 40.75 mm, 42.75 mm and 45.00 mm respectively. This served as positive control for this research. Although these values are quite higher than the values obtained from the anti-candidal activities of the extracts as shown in Table 3.

The anti-candidal activity of the 5% ethanol (diluent) implied that the diluent had no anti-candidal activity. The zones of inhibition of *A. sativum* extract as presented in Table 3 at the concentrations (mg/mL) of 50, 100 and 200 respectively revealed that these values are quite different from those obtained in a previous study (Bodhankar and Patil, 2011). This disparity might be attributed to the differences at the time of collection of the *A. sativum* bulb and the mode of extraction employed in the two studies. In the previous study, it was the volatile oil constituent that was used while crude extract was used in this present study. At the concentration of 50 mg/mL, the ASB: CSP admixture (1:2) gave a zone of inhibition of 21.00 mm while *A. sativum* extract alone gave 18.50 mm (Table 3). This showed that the *A. sativum: C. sinensis* admixture (1:2) was more efficacious than the *A. sativum* extract alone at this concentration.

For the anti-candidal activity of the *C. sinensis* extract (Table 3), there was no activity at higher concentrations of 100 and 200 mg/mL. It is possible that the optimum anti-candidal activity of *C. sinensis* could be at 50 mg/mL. In a previous study at a concentration as low as 0.5 mg/mL, *C. sinensis* gave zones of inhibition of between 8 and 12 mm (Jwanny *et al*, 2012). The inhibition of the anti-candidal activity of the extract at higher concentrations might be due to the increase in the antifungal nullifying components in the crude extract as a result of the increase in the concentration of the *C. sinensis* extract.

The MIC of *A. sativum* extract (32.00 mg/mL) value has been found to be higher than the MIC of 14.9 mg/mL after 24 hours and 12.5 mg/mL after 48 hours obtained in a previous study (Iwalokun *et al*., 2014). This difference might be in the species and the different biological conditions employed in the two studies. The MIC of the ASB: CSP admixture (1:2) was 24.00 mg/mL. This showed that the ASB: CSP admixture (1:2) was more potent than the *A. sativum* extract with MIC value of 32.00 mg/mL as MIC is the least concentration at which inhibition of the *C. albicans* would occur.

However, the MIC of *Allium sativum* extract -32.00 mg/mL and ASB: CSP admixture (1:2) -24.00 mg/mL are lower when compared with the MIC of Clotrimazole (16 µg/ml) in an ealier study (Khan and Bagai, 2010). This showed that Clotrimazole is more potent than *Allium sativum* extract and ASB: CSP admixture (1:2).

The values of the MFC was found to be equal to the MIC. This suggests that the evaluation of MIC could be sufficient for measuring fungicidal activity (Natarajan *et al*., 2003).

The results of the physical properties of the formulations as presented in Table 4 showed that ASB: CSP admixture (1:2) formulation showed poor homogeneity unlike petrolatum B.P and Mycoten® which had good homogeneity. The poor homogeneity might be attributed to the high solid content as well as possible variations in the particle sizes and shapes of the extracts used. All the formulations showed good spreadability. The odour of ASB: CSP admixture (1:2) was slightly pungent while that of Mycoten® and

petrolatum B.P were neutral. The odour of the formulated herbal ointment (ASB: CSP admixture (1:2) 50 mg/mL) was considerably reduced in comparison to the very nauseating and pungent odour of ASB extract alone. All of the products gave no sensation. Table 4 also showed that only petrolatum B. P. (pH- 5.3) conformed to the standard range for skin care products considering that the pH of the skin is between 4-5.5 (Prakash *et al.,* 2017). However, the pH values of the herbal formulation (6.89) and Mycoten® (6.48) were not outrageously high. This has been shown in the index for sensation as there was no sensation experienced by using the two formulations. The pH can however be reduced by adding a buffer to the formulation (Zbacnik *et al.,* 2017) as extreme pH may cause skin irritability especially under extreme storage conditions.

The anti-candidal activity of the formulated admixture showed that at a concentration of 50mg/ml, the herbal ointment formulated gave a zone of inhibition of 25.50 mm, while Mycoten® at a concentration of 1mg/ml gave a zone of inhibition of 30.00 mm. It is worthy to note that the concentration of the formulated admixture (50 mg/mL) compared to that of Mycoten® (1 mg/mL) that achieved relatively similar anti-candidal activity is very high. This might be attributed to the fact that Clotrimazole, the active ingredient in Mycoten® had been isolated and purified unlike the admixture that contains both bioactive and inactive substances for anti-candidal activity. This shows that there is a need for isolation and purification of the bio-active substances that are responsible for the anti-candidal activity of the formulated admixture in order to reduce the concentration of the solid content of the formulation and hence improving homogeneity and possibly the efficacy.

The zone of inhibition of the formulated ointment (25.50 mm) was significantly higher than the zone of inhibition of the pure admixture (21.00 mm) as shown in Table 3, this increase in zone size could be as a result of the impact of the antifungal activity of petrolatum. Petrolatum contains aromatic hydrocarbon -benzene which has been found to show anti-candidal activity (Uyanik *et al.,* 2009).

CONCLUSION

This study showed that the admixture of *A. sativum* extract and *C. sinensis* extract (1:2) and its ointment formulation have anti-candidal effect. Also, the pungent and nauseating odour of ASB extract was reduced in the formulation containing the admixture. Thus, this formulation can be recommended for various candidal infections like vaginal infection, thrush and diaper rash.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' DECLARATION

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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