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In-vitro Antifungal Evaluation of *Cinnamomum zeylanicum* and *Leptadenia hastata* on *Malassezia* species

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Abstract
In-vitro antifungal evaluation of two plant extracts (*Cinnamomum zeylanicum* and *Leptadenia hastata*) was carried on *Malassezia* spp. using agar well-diffusion method. The ethanolic extraction of the bark of cinnamon spp. yielded more extract than leaves of *L. hastata* with a percentage yield of 6.20 and 4.28, respectively. At the concentration of 20 mg/ml, Cinnamon had the greatest activity with a zone of inhibition measuring 28 mm followed by *L. hastata* with 24 mm at the same concentration. Concentrations of 10 mg/ml, 5 mg/ml and 2.5 mg/ml also show antifungal effects on the test organism. Cinnamon had an MIC value of 5 mg/ml while that of *L. hasta* was 20 mg/ml with an MFC of 10 mg/ml and 20 mg/ml, respectively. The phytochemical screening of the extracts show the presence of Saponins, tannins, glycosides, alkaloids, phenols and flavonoids, which might be responsible for the antifungal effects of the extracts on *Malassezia* spp. The findings of this work suggests that infections by *Malassezia* spp. can be treated using Cinnamon spp. or *Leptadenia hastata*.
Key words: *Malassezia* spp., Cinnamon spp., *Leptadenia hastata*, Zone of inhibition.

INTRODUCTION

Global priority is currently placed on combating malaria and HIV/AIDS (Ahumuza and Kirimuhuzya, 2011). In developing countries, rapid urbanization increases overcrowding in slum areas, exacerbating the problem of skin diseases, which are highly contagious with one-third or more of the population affected. Families often sacrifice part of their overstretched household budgets trying to treat these infections. Thus, controlling skin diseases through simple but effective public health measures is necessary and realistic for alleviating a common and solvable source of ill health (Hay *et al*., 2006). There is an evolving awareness that traditional medicine has a central role to play in combating new and re-emerging diseases with significant effect on human health (Burford *et al*., 2000).

Skin infections are very common in childhood worldwide and between 49 - 80.4% of African school children are affected (Dagnew and Erwin, 1991). It has been observed to be common in adolescent and young adult males in hot climates (Kevin, 2008). The prevalence of fungal skin infection is about 20% among the American populations at any given time while in Ethiopia, 49.2% and Taiwan, 28.2%, (Frank, 2008). Skin infections have been reported to be a major problem in Tanzania where about 34.7% of the rural populations have skin diseases (Mollel, 1994; Satimia *et al*., 1998). In Nigeria, the prevalence rate is about 40.4% among pupils in primary schools (Oyedeji *et al*., 2006). Clinical investigations have shown that, dermatophytosis of *Tinea versicolor* and *Tinea corporis* are responsible for more than 15% of all skin infections in Nigeria (Ogunbiyi *et al*., 2005). An increase in the prevalence of *T. versicolor*, among others, was once recorded in the University College Hospital (UCH), Ibadan, between 1994 and 1998 (Ogunbiyi *et al*., 2005). Poor socio-economic status was identified as the major cause of skin infections in the developing countries (Odueko *et al*., 2001). Resistance to antifungal agents has important implications for morbidity, mortality and health care in the community (Tasleem, 2011).

Pityriasis versicolor,(PV), (*Tinea versicolor*) is caused by *Malassezia* species(Gupta and Foley, 2015). This condition is one of the most common superficial fungal infections worldwide, particularly in tropical climates(Gupta and Foley, 2015). PV is difficult to cure and the chances for relapse or recurrent infections are high due to the presence of Malassezia in the normal skin microbiota.

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality especially in developing countries with lower health status indices (Isaka *et al*., 2017).Due to the fact that *Malassezia* is a eukaryote

containing egesterol, treatments against it using synthetic antifungals may affect the patient because ergesterol is a homologue to cholesterol found in humans. The objective of this research was to study the efficacy of *Cinnamomum zeylanicum* and *Leptadenia hastate in vitro* against *Malassezia* species.

MATERIALS AND METHODS

Samples Collection and Processing

Clinical specimens of *Malassezia* spp. isolated from skin of patients with *Pityriasis versicolor* were collected from the Microbiology laboratory of Aminu Kano teaching hospital, Kano- Nigeria.

Fresh leaves of *Leptadenia hastata* were collected from Kalori village, Dutse Jigawa State, and Cinnamon Sticks were obtained from "yankaba" market in Kano. The plant specimens were identified by a botanist in the Federal University Dutse, Biology laboratory. The leaves of *L. hastata* and cinnamon sticks were air dried at room temperature (28ºC) for 7 days. The dried plant materials were ground into powder using mortar and pestle. The powdered samples were stored in an air-tight plastic container (Bello *et al.*, 2011).

Identification of isolate

Macroscopic examination

The isolates were sub cultured onto Saboraud's dextrose agar plates containing 0.05% chloramphenicol with 1ml of olive oil incorporated into it. The plates were incubated at 32^0C for 5 days (Khosravi *et al.*, 2009). Creamy mucoid colonies were observed which a characteristic morphology of yeasts is.

Microscopic examination

The microscopic morphology of the yeast cells was studied by making gram stained smears of the colonies from Saboraud's dextrose agar after five days of incubation at 32°C.Characteristic "spaghetti" and "meat balls" were observed which typical microscopic features of Malassezia were (Karhoot*et al.*, 2012).

Biochemical test

Catalase test was carried out by applying a drop of hydrogen peroxide (3% solution) onto a portion of colony on a glass slide. The production of gas bubbles indicated a positive reaction (Kordbacheh *et al.*, 2004), this is a feature of all *Malassezia* species except *M. restricta*.

Maintenance of Isolates

Stock cultures of the identified isolates of *Malassezia* were stored in Sabouraud's Dextrose Agar (SDA) slants at 4ºC until needed (Anejionu *et al.*, 2012).

Extraction of Plant Materials

Twenty five grams (25g) of the powdered samples of *cinnamomum zeylanicum* and *L. hastata* were soaked in 250ml each of absolute ethanol and allowed to stand for 24 hours. The mixture was stirred occasionally for 3 days. The sample was filtered using Whatman No.1 filter paper and the filtrate was transferred into a rotary evaporator to recover the crude extract and ethanol separately. The crude extract was then transferred into an evaporating dish and placed in a water bath set at 78.37°C to allow the excess ethanol escape (Handa, 2008). The recovered crude extracts were stored at 4°C for future use.

Phytochemical Analysis

The extracts were qualitatively analyzed for the presence of Tannins, Alkaloids, flavonoids, Saponins, phenols and Glycosides (Talkudar *et al.*, 2010; Waterman, 1993).

Antifungal activity of *Cinnamomum zeylanicum* and *Leptadenia hastata*

Media Preparation

Saboraud's dextrose agar was prepared according to manufacturer's instructions and autoclaved at 121°C for 15 min.

Inoculum Preparation/ Standardization

A suspension of the test fungi (*Malassezia* spp.) was made by emulsifying loop full of colony into a test tube containing normal saline. Inoculum density of the yeast suspension was adjusted to that of 0.5 McFarland standard (Amit *et al.*, 2011).

An Initial concentration of 20mg/ml was prepared by dissolving 0.2g each of the crude extracts of *Cinnamomum zeylanicum* and *L. hastata* in 10ml of distilled water, concentrations of 10mg/ml and 5mg/ml were also prepared by aliquoting 1ml from the stock solution into 9ml of distilled water. Standardized inocula of *Malassezia* were inoculated on the surface of SDA plates by surface spreading using a sterile cotton swab and evenly spread over the entire surface of the agar plate. Wells of 6 mm diameter and 5 mm depth were made on the solid agar using a sterile cork borer. Ethanolic extract of the concentration 20mg/ml, 10mg/ml and 5mg/ml were dispensed into respective wells and fluconazole (40 mg/ml) was used as a positive control. Physiological saline was used as negative control. The plates were incubated for 5days at 32°C after which the zones of inhibition were measured.

Minimum Inhibitory Concentration (MIC)

The MIC of *C. zeylanicum* and *L. hastata* was determined using a modified method of (Isaka *et al.*, 2017) where fresh Saboraud's dextrose broth was dispensed into a series of five labeled test tubes, out of which the first test tube contained 10ml of the broth whereas the other four contained 5ml of it, respectively (Audu et al. 2005).

These were sterilized at 121◦C for 15 minutes and allowed to cool. Two-fold serial dilutions of the extract in the broth were made to obtain the concentrations of 20.0mg/ml, 10.0mg/ml, 5.0mg/ml, 2.5mg/ml and 1.2mg/ml respectively. The highest concentration was obtained by dissolving 200 mg of the extract in 10ml of the SDA (Braca et al. 2005). Having obtained the different concentrations of the extract in the broth, 0.1 ml of the standard inoculum was inoculated into the test tubes containing different concentrations of the extracts. Thereafter, the test tubes were incubated at 32◦C for 5days. The lowest concentration of the extract in the broth, which inhibited the growth of *Malassezia* spp., was recorded as the Minimum Inhibitory Concentration (MIC).

Minimum Fungicidal Concentration (MFC)

Minimum fungicidal concentration (MFC) of the extracts was also carried out using a modified method described by (Isaka *et al.*, 2017).Saboraud's dextrose agar was prepared according to the manufacturer's instructions (65g in 1litre), sterilized at 121^{0}C for 15 minutes, cooled at 40^{0}C and poured into sterile Petri dishes to solidify. Tubes from the MIC test that showed no growth (turbidity) were used to inoculate the agar plates and incubated at 32^{0}C for 5 days after which they were observed. The MFC was the plate with lowest concentration without colony growth (Audu et al. 2005).

RESULTS

The physical properties of *Cinnamon* spp. and *L. hastata* are shown in Table 1. Percentage yields of 6.20% and 4.28%, respectively from *Cinnamon* and *L. hastata* were obtained. *Cinnamon* bark had a crispy texture with a "red-brick" color, while *L. hastata* leaves had a sticky texture with a dark green color.

Table 1: Physical properties of *Cinnamomum zeylanicum* and *L. hastata*.

Properties	**Cinnamon(Bark extract)**	***L. hastata* (Leaf extract)**
Percentage yield (%)	6.20	4.28
Color	Red brick	Dark green
Texture	Crispy	Sticky

Table 2 shows the phytochemical components of *Cinnamomum zeylanicum and L. hastata*. It is clear that Saponins, Alkaloids and glycosides were present in both extracts. In addition, Flavonoids and Tannins were also present in cinnamon extracts while they were absent in *L. hastata*. However, *L. hastata* extract tested positive for phenols which were absent in cinnamon extracts.

Table 2: Phytochemical components of *Cinnamomum zeylanicum and L. hastata*.

	Phytochemicals					
Plant part(s)	**Saponins**	**Flavonoids**	**Tannins**	**Alkaloids**	**Phenols**	**Glycosides**
Cinnamon (Bark)	+	+	+	+	-	+
L. hastata (Leaf)	+	-	-	+	+	+

Key: + = positive - = negative

Table 3 shows the antifungal activity of *Cinnamomum zeylanicum and L. Hastata* against *Malassezia* spp. *Cinnamon* had the highest activity with a zone diameter of 28 mm, 24 mm, and 12 mm at concentrations of 20 mg/ml, 10 mg/ml and 5 mg/ml respectively. *L. hastata* had a zone diameter of 24 mm, 18 mm, and 11 mm at the same concentrations. This implies that *Cinnamon* has a greater antifungal effect on *Malassezia* spp. than *L. hastata*.

Table 3: Antifungal Assay

	Zones of inhibition (mm) of the extracts concentration (mg/ml) against *Malassezia* spp.			**Negative control**	**Positive control**
	20	**10**	**5**		
Cinnamon	28	24	12	00	32
L. hastata	24	18	11	00	32

Key: mg/ml = Milligrams per milliliter, mm= Millimeters

Table 4 shows the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Cinnamomum zeylanicum* and *L. hastata* against *Malassezia* spp. Five (5) mg/ml was the least concentration of *Cinnamomum zeylanicum* that inhibited the growth of *Malassezia* spp. while 10 mg/ml was the least concentration of *L. hastata* that inhibited the growth of *Malassezia* spp.Also, 10 mg/ml of *Cinnamomum zeylanicum* completely killed *Malassezia* spp. while 20 mg/ml of *L. hastata* killed it.

Table 4: Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of *Cinnamomum zeylanicum* and *L. hastata*.

Extracts	20mg/ml	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml
Cinnamon	-	MFC	MIC	+	+
L. hastata	MFC	MIC	+	+	+

Key: -=No growth observed, MIC=Minimum inhibitory Concentration, +=Growth observed, MFC=Minimum Fungicidal Concentration

Discussion

The infection by different species of *Malassezia* is quite common in tropical countries like Nigeria and like most fungi and other microbes, *Malassezia* has developed resistance to most of the antifungal drugs available. Also, *Malassezia* being a eukaryote, the treatments against it may adversely affect the patient which makes the discovery of new synthetic antifungals difficult hence, the current study evaluated the susceptibility of *Malassezia* yeast to *Cinnamomum zeylanicum* and *Leptadenia hastata*. *Cinnamomum zeylanicum* and *Leptadenia hastate* were found to contain some secondary metabolites which contributed to their antifungal effect (Table 2). According to sofowora (1993), the presence of secondary metabolites in plants produce some biological activity in man and animals which is responsible for their use as medicinal agents. Results of the phytochemical screening of *Cinnamomum zeylanicum* were in line with the findings of (Preeti Gauniyal *et al.,* 2014). The zones of inhibition obtained from the antifungal assay at varying concentrations are very encouraging. It shows that *Malassezia* spp. are susceptible to the crude extracts of *C. zeylanicum* and *L. hastata*. *C. zeylanicum* had a greater antifungal effect on *Malassezia* spp. than *L. hastata* which might be due to the fact that *L. hastata* is a common and ancient plant found mostly in Northern Nigeria where it is being used as a source of food and traditional medicine, this frequent use could be the reason behind its lower antifungal activity as compared with *C. zeylanicum* which is not common. However, both *C. zeylanicum* and *L. hastata* showed lower activity than the standard antifungal drug fluconazole (Table 3). The antifungal assay results show a similar pattern with the findings of (Pooja *et al.*, 2013), where cinnamon oil showed greater antifungal activity when compared with Kapur Tulis oil, *Eucalyuptus* oil, Cajeput oil, Tea tree oil and Karanj oil against *Malassezia furfur*. Another reason that might be responsible for the greater activity of cinnamon is that *C. zeylanicum* was found to contain Saponins which are not present in *L. hastata* (Table 2). This compounds have the ability to form pores in membranes. Furthermore, fungi as eukaryotes contain ergesterol which is a homologue to cholesterol and according to (Francis *et al.*, 2002), ginsenosides including Saponins interact with membrane cholesterol and displace it from the immediate environment of ATPases leading to an increase in membrane fluidity and conformational changes that ATPases undergo during their transport cycle.

Results of the minimum inhibitory concentration and minimum fungicidal concentration of *C. zeylanicum* and *L. hastata* concretizes the fact that these plants have a great antifungal effect on *Malassezia* spp. This is evident as low concentrations of 5mg/ml and 10mg/ml of *C. zeylanicum* and *L. hastata* inhibited the growth of the fungus respectively (Table 4). Similarly, 10mg/ml of *C. zeylanicum* killed the fungus however, a higher concentration of *L. hastata* was needed to obtain a fungicidal effect (Table 4).

CONCLUSION

Cinnamon bark and *L. hastata* leaves can be used as alternative treatments to Tinea versicolor infection caused by *Malassezia* spp.

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