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Analysis of in vitro activity of *Cymbopogon citraus* (lemon grass) and *Ocimum gratissimum* (scent leaf) essential oils on dematiaceous fungi isolated from Uli community

Abstract

The increase in subcutaneous infection has prompted the need to diversify antifungal agents. This study investigated an *in vitro* activity of *Cymbopogon citraus* and *Ocimum gratissimum* essential oils on dematiaceous fungi isolated from Uli community, Anambra state. Two hundred and ten soil samples were randomly collected from three sampled communities. Also, two different plants' leaves (*Cymbopogon citratus* and *Ocimum gratissimum*) were purchased from the communities. The soil samples were analyzed for the presence of dematiaceous fungi using standard microbiological technique, and characterization of the isolates was carried out using cultural, microscopy, and mycology atlas. The essential oils were extracted using Soxhlet extractor and normal hexane. An *in vitro* antifungal activity of the essential oils on dematiaceous fungi was evaluated using macro tube dilution method, and Minimum Inhibitory Concentration (MIC), and Minimum Fungicidal Concentration (MFC) were determined using standard microbiological technique. The morphological features of the fungal isolates showed dark reverse pigmentation, which portrayed dematiaceous fungi. A total number of ten dematiaceous fungi were isolated from the three communities, of which Umuaku recorded the highest occurrence (50%), while the least was Aluoha (20%). Statistically, there was no significant (P>0.05) difference in the occurrence of the fungal isolates in the sampled communities. The *in vitro* antifungal activity of the essential oils showed that *Cymbopogon citratus* was higher than *Ocimum gratissimum* as indicated in MFC values of 0.10mg/mL and 0.25mg/mL, respectively. Therefore, these essentials oils could serve as novel antifungal agents in curbing subcutaneous mycoses.

Keywords: Dematiaceous fungi, essential oil, antifungal agents, Subcutaneous infection

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Introduction

Globally, there had been increase rate of infectious diseases which have exerted enormous negative impact on the health of individuals, especially lowincome earners.[1] Pharmaceutical industries have also intensified effort to sourcing possible remedy to the ugly menace. Unfortunately, the high rate of microbial resistance had limited the use of synthetic antifungal agents.[1] Most fungal pathogens had undergone genetic mutation, which eventually alters their genetic components, thereby conferring them resistance to most available antifungal agents.[1] The threat posed by excessive microbial resistance had also led researchers to sourcing for antifungal agents that are naturally based.[2]

Medicinal plants had played a vital role in traditional medicine for decades in tackling different ailments both infectious and non-infectious diseases.[3] These medicinal plants had been proved to be effective due to their bioactive compounds.[3] *Cymbopogon citratus* commonly known as lemon grass had been shown to exhibit optimum activities against different microbes such as fungi, bacteria, and viruses.[1] The lemon grass is generally known for its lemon fragrance, and its antifungal potential had been attributed to citral, one of the phytochemical components in the lemon grass as documented by Ewansiha *et al*.[4] Similarly, *Ocimum gratissimum* locally known as scent leaf is one of the local leaves that had been extensively optimized in tackling diseases traditionally, and high potency had been reported.[1,4] The leaf is considered as miracle plant in some localities due to its healing property such as blood glucose regulation and antifungal potentials as documented by Pandey.[3]

Several researchers had shown that essential oils extracted from *Cymbopogon citratus* and *Ocimum gratissimum* are capable of inhibiting the growth of dematiaceous fungi. Dematiaceous fungi are group of fungi that are darkly pigmented due to the presence of melanin in their cell wall.[5] The fungi are responsible for subcutaneous mycoses that affect mostly farmers in rural areas.[5] Essential oils that are lipid loving is easily absorbed in the skin surfaces and frequent application could result in sensitive skin. When a skin surface has been sensitized, there is an increase in reaction with any substance that enters the skin and glowing body surfaces set in, though irritation and redness had been reported at high concentration.[3] These essential oils are capable of interfering with the fungal cell wall, thereby distorting cell wall components, especially β-glucan.[5,6] This interaction has been described as antimicrobial due to the presence of chemical compounds such as glycosides that impede fungal proliferation.[3]

Numerous researchers had provided adequate information on *in vitro* activity of plants extract against filamentous fungi such as Mousavi and Raftos,[2] Yanar et al.[7] Nyamath and Karthikeyan,[8] and Boukhtem *et al*.,[1] but little study had been documented on the *in vitro* activity of *Cymbopogon citraus* and *Ocimum gratissimum* essential oils on dematiaceous fungi. Hence, the aim of this study is to evaluate an *in vitro* activity of *Cymbopogon citratus* (lemon grass) and *Ocimum gratissimum* (scent leaf) essential oils on dematiaceous fungi isolated

from Uli community, Anambra state. The result obtained in this study would contribute immensely in combating subcutaneous mycoses.

Materials and methods

Study area: The study was conducted at Aluoha, Umuaku, Umuoma, Uli, Ihiala Local Government Area, Anambra State. Uli is a village located between latitudes 5.47°N and 5.783°N and longitude 6.52°E and 6.87°E on the South eastern part of Nigeria. Uli extends westward to the confluence of the rivers of Atammiri and Eyinja, and across Usham lake down to the lower Niger region. Uli has rainforest vegetation with two seasonal climatic conditions: rainy season and dry season, which is characterized by the harmattan between December and February. Uli is characterized by double maxima of rainfall with a light drop in either July or August known as dry spell or August break. The annual total rainfall is about 1,600 mm with a relative humidity of 80 % at dawn.

Sample collection

Collection of soil samples

Two hundred and ten (210) soil samples were collected from three communities at Ihiala Local Government Area, Anambra State namely: Aluoha, Umuaku, and Umuoma. Seventy soil samples were randomly collected from different soil types (loamy, clay and sandy soil) in the three communities (Aluoha, Umuaku, and Umuoma) using a soil auger, which was sterilized using 70 % ethanol. The soil samples were collected at the depth of 10 cm and were transferred into a sterile polyethene bag. The samples were transported to the Department of Microbiology Laboratory, Chukwuemeka Odumegwu Ojukwu University (COOU), for immediate analysis. The samples were processed within 2 h of collection.[5]

Collection of plant materials

Similarly, fresh leaves of *Ocimum gratissimum* (scent leaf) and *Cymbopogon citratus* (lemon grass**)** were purchased from the communities. The leaves were put in a polyethene bag and were conveyed to the Biological Science laboratory, COOU for authentication by Dr. Anthony.[9]

Sterilization of glass wares: The glass wares were sterilized using electric oven. The glass wares were washed with detergent and rinsed with clean water. These were dried and placed inverted inside of the oven and set the thermostat at 150°C for 1h.

Preparation of media for isolation: The media used for this study were potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) which were accurately weighed according to the manufacture's instruction, and sterilized by autoclaving at 121°C, 15psi for 15 minutes.[5]

Isolation and purification of the isolates: A stock solution of the soil samples was prepared by weighing one gram using a weighing balance **(**DC-300), a little normal saline was added to the soil samples in a test tube, the mixture was shook and normal saline was added to make up 10 mL. The stock sample was serially diluted tenfold in which 9.0 mL of normal saline was dispensed into test tubes (pyrex) and 1.0 mL of the stock solution was dispensed into the first test tube, and was stirred after which 1.0 mL was taken from the tube using another pipette, and was transferred into the second test tube. The process continued until the last tube after which 1.0mL was discarded. Inoculation was carried out by aseptically inoculating 0.1 mL of the diluted sample (10^{-5}) on Potato Dextrose Agar (PDA) using spread plate method. The plates were incubated in inverted position at 30±2°C for 7 days. After incubation, the grown colonies were sub-cultured by streaking a single colony aseptically on sterile poured plate containing SDA (60 mm OD × 55 mm ID × 13mm high). All the plates in triplicates were incubated inverted at 30±2°C for 7 days.[5]

Identification of the isolates

The sub- cultured isolates were identified using their colonial and morphological descriptions. The colonial description was carried out to determine the appearances of the isolates on agar media plates, edges, texture, surface, and reverse pigmentation.[5]

Slide culture technique

Slide culture technique was carried to visualize the structural endowment of the dematiaceous fungi as elucidated by Umedum and Iheukwumere.[10] Preparation of sabouraud dextrose agar (SDA) was carried, followed by an agar block which was cut at $4m^2$ area using a sterile glass slide. A Petri dish containing filter paper, glass slides (75mm×25mm×1mm) and coverslip was put in an oven at 200°C for 30mins. After heating, the filter papers in the Petri dishes were soaked with sterile water. The agar block was placed at the center of two slides that formed angle 90°. The colony of the fungi was inoculated at the margins of the agar, and a sterile coverslip was placed on the inoculated agar. The preparation was incubated at 30±2°C for 4 days as illustrated by Umedum and Iheukwumere.[10]

Lactophenol cotton blue staining

After incubation, the coverslip was removed from the agar block using a sterile forceps and was placed on a clean slide containing a drop of lactophenol cotton blue. The margin of the coverslip was sealed using candle wax to conserve the stain. The slide was then viewed using X10 and X40 objective lenses of digital microscope.[10]

Extraction of essential oil

The fresh leaves of *Ocimum gratissimum* (scent leaf) were dried at 30±2°C for 14days. The dried leaves were pulverized using a blender to obtain powdered form of the leaves. Twenty grams of the powdered leaves were weighed using weighing balance. The weighed quantity was packed into a semipermeable known as thimble. Two hundred milliliters (200mL) of the solvent for extraction (n-hexane) was measured using a measuring cylinder and was poured into a flat-bottomed flask. The Soxhlet extractor was then set up by placing the flat-bottomed flask on a heating mantle set at 100°C, extraction chamber containing the powdered leaves was inserted on the flask firmly, facilitated by Vaseline to enable lubrication and ultimate pressure to be actualized. Liebig's condenser was then used to cover the extracting chamber with Vaseline application. The inlet and outlet openings on the condenser were connected to a cooled water tank, for cooling of the condenser during heating process. The set up was clamped using a retort stand for firmness. The power source was turned on at 100°C, the solvent started boiling at 68 °C and vapors entered extracting compact on the extracting chamber, which were further conveyed to the condenser. The condenser converted the vapors to liquid, which entered into the extracting chamber and soaked the powdered leaves on the chamber, extracting its essential oil and phytochemicals. The essential oils then accumulated and were deposited into the flat-bottomed flask through the small channel (siphon) and the process was continued for 16 h. After extraction process, the essential oil was concentrated by evaporating the n-hexane, which was achieved by exposing the solution in the atmosphere. The essential oil was then obtained in liquid form and different concentrations were prepared from a stock solution prior to analysis as described by Okonkwo and Ohaeri.[9]

In vitro antifungal activity of essential oils

Preparation of 0.5 macFarland standard

MacFarland standard solution was prepared using 1% $BaCl_2$ H_2O and 1% concentrated H_2SO_4. One gram of $BaCl_2$ was weighed and dissolved in 100 mL of distilled water [1% $BaCl_2$. H_2O (w/v)]. Also, 1mL of H_2SO_4 was measured using a sterile syringe and put in 99 mL of distilled water (1% Conc H_2SO_4 v/v). Then, 0.05mL of 1% $BaCl_2$. H_2O was pipetted into 9.95 mL of 1% Conc. H_2SO_4 in a conical flask (pyrex), and a turbid solution was formed due to the formation of $BaSO_4$. The turbid solution is known as 0.5 MacFarland standard, which is equivalent to $1.5X10^8$ cells/mL.[11]

Inoculum preparation

Dematiaceous fungal isolates were subcultured on SDA in a Petri dish to obtain a pure culture. A normal saline (0.85%) was prepared by weighing 0.85 g of NaCl in 100 mL of distilled water and the mixture was autoclaved at 121°C 15 Psi for 15min. The fungal isolates in Petri dishes were flooded with 0.85% normal saline and sterile forceps was used to scrub the fungal colonies together with the normal saline into a conical flask (pyrex). The colonies were teased using teasing needle, and the mixture was filtered using Watman No1 filter paper. The filtrate was transferred into a test tube and the prepared normal saline was used to dilute the fungal filtrate to match the turbidity of 0.5 MacFarland standard which is equivalent to $1.5X10^8$ cells/mL.[11]

Macro tube dilution technique

A broth medium (peptone water that contains peptic digest of animal tissue (10g/L) and NaCl (5g/L) was prepared by dissolving 7.50g in 500mL of distilled water, the mixture was autoclaved at 121°C for 15min. Four different concentrations (0.50mg/mL, 0.25mg/mL, 0.10mg/mL, 0.05mg/mL) of the two plants' essential oils viz. *Ocimum gratissimum* (scent leaf), and *Cymbopogon citratus* (lemon grass**)** were prepared using a stock solution of 1.00mg/mL. The stock solution was prepared by dissolving 100mg of the essential oils in 100mL of Dimethyl sulfoxide (DSMO). The concentrations were prepared as follows: 0.5mg/mL was prepared by pipetting 50 mL of the stock solution into 50mL of DMSO, 0.25mg/mL was prepared by pipetting 25mL of the stock solution into 75 mL of DSMO, 0.1mg/mL was prepared by pipetting 10 mL of the stock solution into 90mL of DSMO, and 0.05 mg/L was prepared by pipetting 5mL of the stock of the stock solution into 95mL of DSMO. Similar concentrations were prepared using ketoconazole tab as a positive control antifungal agent, though distilled water was used as diluent, while negative control tube was also prepared for essential oils and antifungal agent, which was not inoculated with the test fungal isolates. The tubes were thoroughly homogenized, followed by inoculation of 0.05 mL (using 1 mL string) of the 10 fungal isolates that had been standardized to 0.5 MacFarland standard ($1.5X10^8$ cells/mL). The inoculated tubes were incubated at 30±2°C for 96 h. After incubation, the tubes were observed for growth and inhibition, considering the turbidity of the control tubes.[12]

Determination of minimum inhibitory concentration (MIC)

The MIC of the plants' essential oils was determined by preparing different concentrations (0.50mg/mL, 0.25mg/mL, 0.10mg/mL, 0.05mg/mL) of the essential oils and ketoconazole using a stock solution of 1.00mg/mL. The stock solution was prepared by dissolving 100 mg of the essential oils in 100 mL of Dimethyl sulfoxide (DSMO). The tubes were thoroughly homogenized, followed by inoculation of 0.05mL (using 1mL string) of the 10 fungal isolates that had been standardized to 0.5 MacFarland standard ($1.5X10^8$cells/mL). The inoculated tubes were incubated at 30±2°C for 96 h. After incubation, the tubes were observed for growth and inhibition, considering the turbidity of the control. T ubes that had lowest concentrations, and inhibited the growth of the test fungal species were recorded as MIC as described by Balouiri *et al*.[11]

Determination of minimum fungicidal concentration (MFC)

Considering the tubes that had no visible fungal growth on the MIC, MFC was evaluated by inoculating the negative tubes on freshly prepared media (peptone water) and incubation was done at 30±2°C for 96h as described by Balouiri *et al*.[11] The tubes that had the lowest concentration but yielded no growth were recorded as the MFC.[11,13]

Statistical analysis

MIC and MFC of the essential oils and ketoconazole, and their therapeutic potentials were compared using ANOVA. P values lower than 0.05 ($P<0.05$) were considered to reflect significant differences.

Results

The *in vitro* antifungal activity of the essential oils showing Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) is presented in Tables 1&2. From the result, Lemon Grass Essential Oil (LGEO) recorded the lowest MIC value of 0.05 mg/mL against isolates DMEO1, DME02, DME03, DME05, DME06, DME07, and DME10, followed by Scent Leaf Essential Oil (SLEO) which recorded the same MIC value (0.05 mg/mL) against 5 isolates (DME01, DME03, DME05, DME07, and DME09). Ketoconazole (KCZ) recorded similar MIC value of 0.05 mg/mL with SLEO against 5 isolates (DME01, DME03, DME06, DME09, and DME10. Similarly, in Table 2, LGEO recorded the lowest MFC value of 0.10 mg/mL against 3 isolates (DME02, DME07, and DME09) while SLEO recorded MFC value of 0.25 mg/mL against 2 isolates (DME03 and DME07). Meanwhile, KCZ recorded MFC value of 0.10 mg/mL against 4 isolates (DME01, DME02, DME09, and DME10). This indicates that KCZ had the lowest MFC value against 4 isolates, followed by LGEO, while the least was SLEO.

Table 1 *In vitro* antifungal activity of the essential oils showing MIC

Isolate Code	MIC of the Essential Oils (mg/mL)		
	LGEO	SLEO	KCZ
DME01	0.05	0.05	0.05
DME02	0.05	0.10	0.25
DME03	0.05	0.05	0.05
DME04	0.10	0.10	0.10
DME05	0.05	0.05	0.25
DME06	0.05	0.10	0.05
DME07	0.05	0.05	0.25
DME08	0.10	0.10	0.10
DME09	0.25	0.05	0.05
DME10	0.05	0.25	0.05

DME, Dematiaceous fungal isolate; MIC, Minimum inhibitory concentration; LGEO, Lemon grass essential oil; SLEO, Scent leaf essential oil; KCZ, Ketoconazole.

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Table 2 *In vitro* antifungal activity of the essential oils showing MFC

Isolate Code	MFC of the Essential Oils (mg/mL)		
	LGEO	SLEO	KCZ
DME01	0.50	0.50	0.10
DME02	0.10	0.25	0.10
DME03	0.25	0.25	0.25
DME04	0.25	0.25	0.25
DME05	0.50	0.25	0.50
DME06	0.25	0.50	0.25
DME07	0.10	0.25	0.50
DME08	0.25	0.25	0.25
DME09	0.10	0.50	0.10
DME10	0.25	0.25	0.10

DME01, *Exophiala jeanselmei;* DME02, *Scedosporium apiospermum;* DME03, *Cladophialophora carrionii ;* DME04, *Cladophialophora abundans;* DME05, *Phialophora verrucose;* DME06, *Ochroconis mirabilis;* DME07, *Scedosporium prolificans;* DME08, *Scopulariopsis brumpti;* DME09, *Scopulariopsis brevicaulis ;* DME10, *Rhinocladiella species*. MIC, Minimum inhibitory concentration; LGEO, Lemon grass essential oil; SLEO, Scent leaf essential oil; BLEO, Bitter leaf essential oil; KCZ, Ketoconazole.

Discussion

The high prevalence rate of fungal infection had led to diversification of sources of antifungal agents globally. Also, conventional antifungal agents have not yielded the desired goal due to high microbial resistance and side effects. The ability of the essentials oils to inhibit fungal growth could be attributed to phytochemical components found in the plants such as flavonoids, tannins, and terpenoids. These phytochemicals had been shown to be capable of disrupting the integrity of plasma membrane by destroying ergosterol. Research had revealed that the phytochemicals are capable of inhibiting the synthesis of β-glucan, which is the major component of fungal cell wall. This finding supports the observation made by several researchers.[3,6,12,14] The low MIC and MFC values of the ketoconazole and *Cymbopogon citratus* indicates high efficacy, but high toxicity and side effects had been associated with ketoconazole administration as documented by several researchers,[15,16] which makes it less desirable for tackling subcutaneous infection. Statistically, there was no significant (P>0.05) difference observed in the MIC and MFC of the essential oils and conventional antifungal agent (Ketoconazole) which indicates that the essential oils extracted from the medicinal plants are potent antifungal agents.

Conclusion

This study has revealed that essential oils extracted from *Cymbopogon citratus* and *Ocimum gratissimum* possess antifungal activity due to their ability to inhibit the growth of dematiaceous fungi, which are agents of subcutaneous infections. Also, *Cymbopogon citratus* exhibited higher antifungal activity than *Ocimum gratissimum,* though not significant (P>0.05) statistically. These essential oils may be optimized for tackling subcutaneous infection, though an *in vivo* study is required to investigate their antifungal potentials on living organisms. However, the ketoconazole cream optimized in this study requires further investigation *in vivo* to ascertain the reaction with cutaneous and subcutaneous tissues.

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Conflict of interest

The authors hereby declare that there was no conflict of interest throughout the period of this study.

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