Journal of Advances in Microbiology

Volume 22, Issue 12, Page 83-90, 2022; Article no.JAMB.94525
ISSN: 2456-7116

Antifungal Activity of *Curcuma longa* and *Azadirachta indica* Extracts on Dematiaceous Fungi Isolated from Environmental Samples at Uli Community, Anambra State Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2022/v22i12696

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/94525

Original Research Article

Received: 17/10/2022
Accepted: 19/12/2022
Published: 21/12/2022

ABSTRACT

Aim: This research is aimed at evaluating the antifungal activity of *Curcuma longa, and Azadirachta indica* extracts on dematiaceous fungi isolated from environmental samples at Uli community, Anambra State, Nigeria.
Methods: Soil, rotten wood, and water samples were randomly collected from three communities at Uli, Ihiala LGA. Also, leaf of neem and rhizoids of turmeric were purchased from an agro-based farm. The samples were analyzed for the presence of dematiaceous fungi using pour plating technique. The isolates were identified based on their morphology and slide culture technique. The

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plant samples were dried at room temperature, and extraction was done using a Soxhlet extractor and ethanol. The phytochemical composition of the extracts was evaluated qualitatively and quantitatively using spectrophotometer. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using a broth medium and sabourod dextrose agar.
Results: A total number of 10 dematiaceous fungi were isolated from the three communities. The phytochemical analysis of the plants extracts revealed the presence of flavonoids, tannins, cardiac glycosides, saponins, alkaloids, and steroids. The MIC and MFC of the turmeric extract were recorded as 0.50 mg/mL and 12.5 mg/mL, respectively.
Conclusion: The study has shown that *Curcuma longa* ethanolic extract is more potent in tackling subcutaneous mycoses.

Keywords: Antifungal agents; dematiaceous fungi; plants extracts; phytopharmcology.

1. INTRODUCTION

Dematiaceous (dark-pigmented) fungi are a large and heterogenous group of moulds that cause a wide range of diseases such as mycetoma and phaeohyphomycosis. Some of the species that are involved in human diseases are *Alternaria* species*, Curvularia* species, *Exophiala* species and *Madurella* species*.* These organisms are widespread in the environment, being found in soil, wood, and decomposing plant debris. The saprophytic potentials of this group of fungi had been documented by several researchers [1]. They are capable of producing extracellular enzymes which digest their food outside before absorption. Dematiaceous fungi are of clinical importance because of their involvement in mycosis [1]. They majorly cause subcutaneous infections which affect mostly the lower limbs through traumatic inoculation and inhalation of spores [1]. Rural dwellers who engage in some activities such as fetching of firewood, farming and other activities which tend to predispose them to the infectious agents. Most of the fungal infections that affect the lower limbs of rural dwellers had been attributed to dematiaceous fungi. In some cases, prolonged treatment had been documented due to chronic nature of the infection and recurrence had been also observed in some cases [2].

Most are filamentous fungi or moulds, and several yeast species are also important pathogens. Although they represent a very heterogeneous group of fungi, a distinguishing characteristic of these different species is the presence of melanin in their cell walls, which gives their conidia or spores and hyphae a dark colour. Colonies are usually brownish-black in colour [2].

Research had shown that the activities of the dematiaceous fungi can be inhibited by medicinal plants' extracts. Medicinal plants such as *Curcuma longa* and *Azadirachta indica* have phytochemical components that are capable of disrupting the integrity of the cell wall. When the cell wall of fungal species that contain majorly glucan, mannan, and chitin is damaged, growth is impaired [2].

Several researchers, such as [1-2] had worked on the antifungal activity of plant extracts against filamentous fungi but little information had been documented on the antifungal activity of *Curcuma longa* and *A. indica* against dematiaceous fungi isolated from Uli community, Anambra State. Hence, this study aimed to investigate the antifungal activity of *Curcuma longa* and *A.indica* against dematiaceous fungi isolated from the community of Uli, Anambra State. The results from this study will contribute significantly to the prevention of subcutaneous infections.

2. MATERIALS AND METHODS

2.1 Sample Collection

Two hundred and ten (210) different samples (soil, rotten wood, and water) were collected from three communities (Ndikeokwu, Umuchima, and Umuegwungwu) at Uli, Ihiala LGA, Anambra state. Fifty grams (50 g) of each of these samples were collected randomly and put in a sterile cellophane bag. Seventy (70) different soil samples (30 loamy, 20 clay, and 20 sandy soil) were collected using auger and a sterile spoon. Seventy (70) rotten wood samples were also collected from the same community while 60 different water samples were collected from different ponds at the same village using sterile bottles.

2.2 Sample Processing, Isolation and Identification

One gram of each soil sample was weighed out and put into bottles containing 10 mL of distilled

water. The samples and water were homogenized for 10 min and was allowed to settle for 20 min. One mL of the supernatant was used for tenfold serial dilution. One mL of each of the serially diluted sample (10^{-5}) was used to carry out pour plating using SDA medium, which was prepared following manufacturer's instruction. The plates were stored at room temperature (30°C) for 7 days. The isolates were purified by subculturing on SDA and identification was carried out via morphology and microscopy (slide culture technique). The same technique was used to process rotten wood and water sample [3].

2.3 Identification of Isolates

Macroscopically established colonies were evaluated based on characteristics such as texture, elevation, and pigmentation. Also, the color of the reserve side or bottom of the plate was noted. For detailed study of the macro conidia and conidia or arthrospores on the mycelium, slide cultures of the isolates were prepared. In the microscopic evaluation, the appearance observed was matched with those contained in color atlas of pathogenic fungi [1].

2.4 Slide Culture Technique

Slide culture technique was employed so that the structures of the fungal isolates can be visualized using Riddel's method as described by (4). In this technique, potato dextrose agar (PDA) medium was prepared, and an agar block of $4m^2$ area was cut using a sterile glass slide. A Petri dish was covered with a filter paper and sterile glass slides (75 mm× 25 mm× 1 mm) which were kept in an oven at 150°C for 20 mins. The filter papers in the Petri dishes were moistened by pouring a sterile water. The cut agar block was introduced onto the slide and the isolates were aseptically inoculated at the four edges of the agar block. A sterile coverslip was introduced onto the inoculated agar block using a sterile forceps. The plates were kept was at room temperature (30°C) for 4 days as described by [4].

2.5 Lactophenol Cotton Blue Staining

After incubation, a drop of lactophenol cotton blue was dropped on the sterile slide and the coverslip was removed from the agar block and placed on the slide containing lactophenol cotton blue. The edges of the coverslip were covered using nail polish to prevent evaporation of the stain. The preparation was then viewed using a digital microscope of X10 and X40 objective lenses [4].

2.6 Extraction of Plant Materials

The fresh leaf of *Azadirachta indica* (neem leaf) and rhizoid of *Curcuma longa* (turmeric) were dried at room temperature (28°C) for 14 days. The dried leaves were blended 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 (200 mL) of the solvent for extraction (ethanol) 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 boiled at 78 °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 phytochemicals. After extraction process, crude extract was gotten by evaporating the ethanol via open air as described by [5].

2.7 Phytochemical Analysis of the Plant Extracts

The phytochemical components (alkaloids, glycosides, flavonoids, phenolics, tannins, steroids and saponins) of the plant extracts were determined quantitatively using the methods described by [6].

2.7.1 Alkaloids

Five milliliters of the samples were mixed with 96% ethanol and 20% tetraoxosulphate (VI) acid (1:1). One milliliter of the filtrate from the mixture was added to 5 ml of 60 % tertraoxosulphate (VI) acid and allowed to stand for 5 minutes. Then 5

ml of 0.5% formaldehyde was added and allowed to stand for 3 h. The reading was taken at absorbance of 550 nm.

2.7.2 Glycosides

This was carried out using Buljet's reagent. One gram of the fine powder of the sample was soaked in 10 ml of 70 % alcohol for 2 h and then filtered with What man No. 1 filter paper. The extract was then purified using lead acetate solution and disodium hydrogen tetraoxosulphate (VI) solution before the addition of freshly prepared Buljet's reagent. The absorbance was taken at 550 nm.

2.7.3 Flavonoids

Five milliliters of the extract were mixed with 5 ml of dilute hydrochloric acid and boiled for 30 minutes. The boiled extract was allowed to cool and then filtered with Whatman No. 1 filter paper. One milliliter of the filtrate was added to 5 ml of ethyl acetate and 5 ml of 1 % ammonia solution. The absorbance was taken at 420 nm.

2.7.4 Phenolics

Ten milliliters of the sample was boiled with 50 ml acetone for 15 minutes. Five milliliters of the solution was pipetted into 50 ml flask. The 10 ml of distilled water was added. This was followed by addition of 2 M ammonium hydroxide solution and 5 ml of concentrated amyl alcohol solution. The mixture was left for 30 minutes and absorbance was taken at 550 nm.

2.7.5 Tannins

Ten milliliters were pipetted into 50 ml plastic containing 50 ml of distilled water. This was mixed for 1 h on a sterile mechanical shaker. The solution was filtered with Whatman No. 1 filter paper, and 5 ml of the filtrate was mixed with 2 ml of iron (III) chloride solution in 0.1 M hydrochloric acid. The absorbance was taken at 550 nm.

2.7.6 Steroids

The extract was eluted with normal ammonium hydroxide solution. Two milliliters of eluate was mixed with 2 ml of chloroform in a test tube. Three milliliters of ice cold acetic anhydride was added to the mixture and allowed to cool. The absorbance was taken at 420 nm.

2.8 Test for Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentration (MFC)

MIC of the fungal isolates was determined using disk diffusion technique. Different concentrations of the crude ethanolic extracts of turmeric and neem plant were prepared as follows: 50 mg/mL was prepared by dissolving 5 g in 100 mL of distilled water, 25 mg/mL was prepared by dissolving 2.5 g in 100 mL of distilled water, 12.5 mg/mL was prepared by dissolving 1.25 g in 100 mL of distilled water, and 6.25 mg/mL was prepared by dissolving 0.625 g in 100 mL of distilled water. The concentrations of commercial antifungal agents were prepared using the same procedure. SDA was prepared following manufacturer's instruction. The plates were poured and inoculation of the fungal isolates was carried out aseptically. A sterile filter paper was used to prepare a local disk of $4mm^2$ diameter. The disks were impregnated with the concentrations of the ethanolic extracts and commercial antifungal agents. The disks were aseptically placed on the inoculated plates and allowed at 30°C for 3 days. Fungal colonies surrounding the local disks showed growth while clear zone surrounding the local disks showed inhibition, which was measured using a meter rule in millimeter. The lowest concentration of the ethanolic extract and antifungal agents that inhibited the growth of the fungal isolates was considered as the MIC.

Also, the plates that had no growth were subcultured on a freshly prepared SDA . The lowest concentration that had no growth was considered as MFC of the ethanolic extract and the antifungal agents MFC [7].

3. RESULTS

3.1 Morphological and Microscopical Features of the Isolates

The result of morphological and microscopical features of the isolates is presented in Table 1. From the result, the colony of the isolates showed various colors such as black, brown, gray and white. The reverse pigmentation of the isolates was mainly black and dark brown. Meanwhile, the surface texture showed velvety, wooly, and glabrous. Microscopically, hyphae were septate, dark/brown colored and conidia were mainly blastoconidia, chlamyconidia, annelloconidia, and poroconidia. These morphological and microscopical features of the

isolates showed that they are dematiaceous fungi as revealed in clinical mycology atlas.

3.2 Phytochemical Analysis of the Medicinal Plants

The result of phytochemical composition of the extracts is presented in Table 1. From the result, alkaloids, steroids, saponins, cardiac glycosides, terpenoids, flavonoids, tannins, and phenolics were detected. Higher value was recorded by *Azadirachta indica* (3.45 ± 0.03) while *Curcuma longa* recorded 2.82 ± 0.03. These phytochemicals are otherwise known as chemical constituents that are responsible for medicinal potentials of the leaves.

3.3 Antifungal Activity of the Ethanolic Extracts on the Fungal Isolates

The results of antifungal activity of plants' extracts on the fungal isolates are presented in Tables 3 and 4. In Table 3, TM extract exhibited antifungal activity against all the isolates except isolates MCA02 and MCA10. A MIC value of 6.50 mg/mL was recorded against isolates MCA03 and MCA09 while other isolates recorded a MIC value of 25 mg/mL. In other hand, TM extract recorded a MFC value of 12.50 mg/mL against isolates MCA03 and MCA09. Similarly, the result presented in Table 4 showed that NM extract exhibited antifungal activity against all the isolates except isolates MCA03, MCA06, and MCA07. The MIC value of 6.25 mg/mL was recorded against isolate MCA04. Also, MFC value 0f 12.50 mg/mL recorded against isolate MCA04. The conventional antifungal agents (Ketoconazole, Nystatin, and Fluconazole) used as control exhibited antifungal activity against some of the isolates, though KZ recorded the highest activity as shown in the MIC which revealed 6.25 mg/mL against isolates MCA02 and MCA06. Also, NS and FC exhibited similar antifungal activity, though FC inhibited the growth of isolate MCA10 while NS inhibited the growth of isolate MCA09. KZ recorded MFC value of 12.50 mg/mL while NS and FC recorded similar MFC value of 25.00 mg/mL (Table 4). Statistically, there was no significant ($P > 0.05$) difference between the MIC and MFC of the extracts and the conventional antifungal agents.

4. DISCUSSION

Evaluating the distribution of dematiaceous fungi in different soil types, rotten wood, and water provides vital information in clinical mycoses and medical microbiology. Understanding the phytochemical composition of medicinal plants is highly essential in diversification of antifungal agents in order to curtail high alarming rate of antifungal resistance. The morphological and microscopical features of the fungal isolates agree with the isolation carried out by [1], which confirmed their dematiaceous nature. The presence of steroids, flavonoids, cardiac glycosides, phenolics, tannins, alkaloids, and saponins in the extracts agrees with the phytochemical analysis performed by several researchers [7-9].Turmeric extract exhibited higher antifungal activity on most of the fungal isolates with a minimum inhibitory concentration (MIC) value of 6.25 mg/mL. The only resistance was recorded by *Exophiala jeanselmei* and *Scedosporium prolificans.* The ability of *Curcuma longa* extract to inhibit the growth of dematiaceous fungi could be attributed to a high content of flavonoid curcumin. These phytochemicals had been shown to disrupt the ergosterol components in fungal plasma membrane as demonstrated by [10] who exposed dermatophytes such as *Scedosporium apiospermum*, *Trichophyton*, *Fonsecaea pedrosoi*, and *Exophiala jeanselmei* to turmeric extract and obtained MIC values of 7.2 and 7.8 mg/mL. Also, [11] reported the ability of turmeric extract to inhibit ATPase activity against pathogenic fungi. The antifungal activity of neem plant extract could be attributed to its antioxidant property and other bioactive components such as salannin and azadirachtin [12]. The antifungal activities of synthetic antifungal agents against dematiaceous fungal isolates were higher than the plants' extracts, but their activities were not broad enough compared to the plants extracts, which exhibited antifungal activity against most of the isolates. The three commercial antifungal agents used (ketoconazole, fluconazole, and nystatin) exhibited poor antifungal activities on *Cladophosphora carrionii, Exophiala jeanselmei, Ochroconis, Phialemium, Rhinocladiella,* and *Phialophora verrucose.* The low activities of the synthetic antifungal agent against most of the fungal isolates could be attributed to resistance. This finding corresponds to observation made by [3] who recorded poor activities of fluconazole and ketoconazole on dematiaceous fungal species. Also, high level of toxicity of the antifungal agents had been documented, which had limited their optimization in tackling fungal infections [3]). There is a clear distinction between the activities of plants extract and commercial antifungal agents. The natural plants extracts proved to be more potent, and low

Table 1. Morphological and microscopical features of the fungal isolates

Isolate	Colony appearance on PDA	Texture/ and Elevation	Microscopic appearance	Type of Conidia	Reverse pigmentation
MCA01	Olive brown	Velvety, downy, rough surface and smooth edge	Dark septate hyphae	Blastoconiddia with branching chains	Black
MCA02	Dark brown	Mucoid, velvety, flat, rough surface and smooth edge	Brown septate hyphae	Brownish annelloconidia	Dark brown
MCA03	White	glabrous to wooly, flat, rough surface and edge	Dark septate hyphae	Dark blastoconidia	Dark
MCA04	Dark gray	velvety, raised surface, rough surface and edge	Dark septate hyphae	Dark blastoconidia,	Dark
MCA05	Black	velvety, raised, rough surface and edge	Dark septate hyphae	Dark annelloconidia	Black
MCA06	Greyish brown	Raised, smooth edge and surface	Dark septate hyphae	Dark chlamydoconidia	Black
MCA07	Dark gray	Velvety to wooly, raised, smooth edge and surface	Dark septate hyphae	Dark blastoconidia	Black
MCA08	Greyish black	Raised, smooth edge and surface	Hyaline septate hyphae	Brown arthroconidia	Dark brown
MCA09	White	Wooly, flat rough surface and edge	Brown septate hyphae	Brown blastoconidia	Black
MCA10	White	Velvety, rough surface and edge	Dark septate hyphae	Dark annelloconidia	Black

MCA01= Cladophialophora carrionii; MCA02= Exophiala jeanselmei; MCA03= Phialemonium curvatum; MCA04= Cladophialophora abundans; MCA05= Rhinocladiella species; MCA06= Ochroconis mirabilis; MCA07= Phialophora verrucose; MCA08= Scytalidium dimidiatum; MCA09= Botrytis species; MCA10= Scedosporium prolificans

Table 2. Phytochemical constituents of the medicinal plants extract

Parameter	*Curcuma longa* (g/100g)	*Azadirachta indica* (g/100g)
Steroids	0.64 ± 0.00	0.16 ± 0.0
Flavonoids	2.82 ± 0.03	0.49 ± 0.01
Cardiac Glycosides	0.71 ± 0.02	0.22 ± 0.01
Phenolics	1.62 ± 0.01	3.45 ± 0.03
Tannins	0.31 ± 0.11	0.28 ± 0.14
Alkaloids	1.10 ± 0.01	2.19 ± 0.01
Saponins	1.08 ± 0.11	0.88 ± 0.01

Table 3. Antifungal activity of tumeric extract on the isolate

Isolate code	MIC(mg/mL) TM	MIC(mg/mL) KZ	MIC(mg/mL) NS	MIC(mg/mL) FC	MFC(mg/mL) TM	MFC(mg/mL) KZ	MFC(mg/mL) NS	MFC(mg/mL) FC
MCA01	25.00	12.50	25.00	25.00	50.00	25.00	25.00	50.00
MCA02	-	6.25	-	-	-	12.50	-	-
MCA03	6.25	12.50	-	-	12.50	25.00	-	-
MCA04	25.00	-	-	-	25.00	-	-	-
MCA05	25.00	-	25.00	25.00	50.00	-	50.00	25.00
MCA06	25.00	6.25	25.00	25.00	25.00	12.50	25.00	50.00
MCA07	25.00	-	-	-	50.00	-	-	-
MCA08	25.00	-	-	25.00	50.00	-	-	25.00
MCA09	6.25	12.50	25.00	-	12.50	25.00	25.00	-
MCA10	-	12.50	-	25.00	-	50.00	-	25.00

TM= Tumeric extract; KZ= Ketoconazole; NS= Nystatin; FC= Fluconazole;

Table 4. Antifungal activity of neem extract on the isolates

Isolate code	MIC(mg/mL) NM	MIC(mg/mL) KZ	MIC(mg/mL) NS	MIC(mg/mL) FC	MFC(mg/mL) NM	MFC(mg/mL) KZ	MFC(mg/mL) NS	MFC(mg/mL) FC
MCA01	12.50	12.50	25.00	25.00	25.00	25.00	50.00	25.00
MCA02	25.00	6.25	-	-	50.00	12.50	-	-
MCA03	-	12.50	-	-	-	25.00	-	-
MCA04	6.25	-	-	-	12.50	-	-	-
MCA05	12.50	-	25.00	25.00	25.00	-	25.00	50.00
MCA06	-	6.25	25.00	25.00	-	12.50	50.00	25.00
MCA07	-	-	-	-	-	-	-	-
MCA08	25.00	-	-	25.00	25.00	-	-	50.00
MCA09	12.50	12.50	25.00	-	25.00	25.00	25.00	-
MCA10	25.00	12.50	-	25.00	25.00	25.50		25.00

NM= Neem extract; KZ= Ketoconazole; NS= Nystatin; FC= Fluconazole

toxicity had been documented by several researchers [10-12]. Also, their broad spectrum of activity had been documented. Therefore, these plant-based extracts are suitable alternatives which can be optimized in treating subcutaneous infections caused by dematiaceous fungi.

5. CONCLUSION

Dematiaceous or melanized fungi are fungal species that are found in the soil, rotten wood, and water, which produce dark reverse pigmentation on mycological media. Plants' extracts of *turmeric* and *neem* contain phytochemicals which confer antifungal activity to them, as observed through MIC and MFC. Theses plants' extracts especially turmeric had exhibited optimum antifungal activity compared to synthetic antifungal agents. Therefore, they can be optimized as alternative to commercial antifungal agents in tackling cutaneous and subcutaneous fungal infections due to their efficacy, low toxicity, and zero resistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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