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Antimicrobial efficacy of Cymbopogon citratus (Lemon grass) on toenails dystrophy amongst females attending pedicure outlets in Makurdi metropolis

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Abstract:

This research project was aimed at determining the antimicrobial efficacy of Cymbopogon citratus (lemon grass) on toenails dystrophy amongst females attending pedicure beauty salons in Makurdi metropolis. Dermatophytes fungi, Trichophytonspp. and Microsporium spp. were isolated as major cause of toenails dystrophy with a total prevalence of. 13 (33.33%) and 17 (43.59%), respectively. Non dermatophyte fungi, Aspergillus spp. has showed a prevalence of 9 (23.08%) which confirmed that the causes of most toenails' dystrophy arise from dermatophyte fungi. Several bacterial species such as Bacillus spp., Streptococcus spp., Staphylococcus spp., Klebsiella spp., Salmonella spp., Escherichia coli and Shigella spp. were isolated. The high total prevalence of Staphylococcus spp., 37 (31.36%) confirms the implications of the bacterial ability to colonize the keratinous portions of the toenails. Phytochemical screening of the extracts confirmed the presence of Flavonoids, Saponins, Alkaloids, Phenols, and Tannins in the methanol, aqueous and ethyl acetate extracts. Quinone was present in methanol and aqueous extracts but absent in the ethyl acetate extract. Susceptibility tests were done by agar well diffusion method to determine the mean zone of inhibition for all the extracts. The average mean zone of inhibition was 13.00±1.4 and 13.50±0.71 for Microsporium spp. and Aspergillus spp., respectively while Trichophyton spp. was resistant to all the extracts. Bacillus spp. was susceptible to ethyl acetate extract but resistant to methanol and aqueous extracts. Streptococcus spp. was susceptible to methanol and ethyl acetate extracts but resistant to aqueous extract. Staphylococcus spp. was susceptible to ethyl acetate and aqueous extracts but resistant to methanol extract. Klebsiella spp. was resistant to all the extracts. Salmonella spp. was susceptible to methanol and aqueuos extracts but resistant to ethyl acetate extract. Escherichia coli was susceptible to methanol and ethyl acetate extracts but resistant to aqueous extract. Shigella spp. was susceptible to all the extracts. This research study concluded that ethyl acetate extract proved to be the most potent of all the extracts; it is recommended that the extracts of Cymbopogon citratus could be explored as an alternative to conventional antibiotics.

Key words: *Toenails dystrophy, Onychomycosis, Cymbopogon citratus, Dermatophytes, Antimicrobial resistance, Public health*

1. Introduction

The human being is an intelligent animal that places considerable values on their appearance. They invest heavily on cosmetics that enhance not just the appearance of the skin but the nails too. This is common amongst female who continually apply colour enhancers on their nails to avoid cosmetic sabotage by external factors. Certain factors can damage the tissues and affect appearances of the nail resulting in dystrophy (Cleveland clinic, 2022).

Nail dystrophy is a general term that describes malformed fingernails and toenails; in this condition the nails are often deformed, ridged, pitted or discoloured (Health Jade, 2019). The deformities arising from dystrophy can be due to different causes ranging from nail traumatism to microbial infections. About 50% of nail dystrophy are results from fungal infections (Adigun, 2022). The remainder results from various causes, including trauma, congenital abnormalities, psoriasis, lichen, benign tumors, and occasional cancer (Adigun, 2022). Toenails dystrophy caused by fungi is known as onychomycosis (Health Jade, 2019). Onychomychosis also called tinea unguium is a fungal infection of the nail

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plate, nail bed, or both and the nails typically are deformed and discoloured white or yellow (Adigun, 2022). About 60 to 80% are caused by resistant dermatophytes (e.g*Trichiphyton rubrum*). Many of the remaining cases are caused by resistant non-dermatophyte molds (e.g. *Aspergillus*, *Scopulariopsis*, *Fusarium*) (Adigun, 2022).

Antimicrobial resistance (AMR) is associated with most ailments in developing countries and this is due to poor environmental sanitations (CDC, 2007). It is important to seek alternatives to failing antibiotics. Hence, plants are exploited to utilize their therapeutic properties (DeFilipps and Krupnick, 2018). Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which have been found to have antimicrobial properties (Chrubasik *et al*., 2005). Medicinal plants have therefore been described as the ones in which one or more of their organs contain substances that can be used for therapeutic purposes (Rios and Recio, 2005). These may be in the form of vegetable drugs which may either be organized (material which possesses cellular structure e.g. leaf, bark, petal, flower, stem, root, etc.) or unorganized drugs (acellular structural medicinal agents such as gums, balsams and latex) (Ewansiha et. al., 2012). For the past two decades, there has been an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents (Bonjar and Ferrokhi, 2004). According to the Center for Disease Control indication, about 33% of drugs produced in the developed countries are derived from plants.

This study investigated the antimicrobial efficacy *of Cymbopogon citrates* on toenails dystrophy amongst females attending pedicure outlets in Makurdi metropolis. The phytochemical screening of the extracts was done and their antimicrobial potential was ascertained.

2. MATERIALS AND METHODS

2.1 Area of Study

This research work was carried out in the city of Makurdi in Benue state, Nigeria. Makurdi is the capital territory of Benue state which lies on the south bank of the Benue River. Makurdi is in the north-central region of the country and it lies between latitude 7°44'N and longitude 8°54'E Makurdi.

2.2 Sampling Area

The samples were collected from different pedicure outlets in the following locations; High level, Wadata, North Bank, and Wurukum.

2.3 Sample Collection

A total of thirty (30) toenail samples were collected randomly from the outlets available in each of the aforementioned locations. The samples were stored in sterile airtight universal bottles. Fresh leaves *Cymbopogon citratus* (lemon grass) were collected from Achusa area of Makurdi. They were identified and authenticated at the Botany department of Joseph Sarwuan Tarka University Makurdi. The plant leaves were washed in running water to remove adhesive contaminants. The plant leaves were dried under shade at room temperature for 5-7 days and ground to obtain the dried powdered plant, which were stored in an airtight universal container. Care was taken to avoid contamination of the samples and they were transported to microbiology laboratory, Joseph Sarwuan Tarka University Makurdi.

2.4 Preparation of Samples

The scrapped particles of the nails were differentiated and labeled accordingly. These samples were suspended in 5ml sterilized distilled water in a test tube and allowed to stand for an hour to obtain a broth.

2.5 Procedure for Extraction

A mass of 50g dried powdered *Cymbopogon citrates* leaves was gathered, each was suspended in 700ml distilled water, 700ml methanol and 700ml ethyl acetate. The suspensions were allowed to stand for 24hrs and filtered using a filter paper to remove the solutes. The methanol and ethyl acetate extracts were exposed for evaporation. The residues were collected as a gel semi-solid greenish substance and stored in well corked sterile universal containers. The aqueous extract was heated in a water bath till evaporation was complete. The residue was collected and stored in a well corked sterile universal container.

2.6 Preparation of Media

The media used for this research were plate count agar (PCA), nutrient agar (NA) and potato dextrose agar (PDA). They were prepared according to the manufacturer's instructions. Plate count agar (PCA) is a non-selective bacteriological substrate used for the determination of the total number of live, aerobic bacteria in a sample. Nutrient agar is a general purpose media and was used to subculture bacteria. Nutrient agar was also used to perform antimicrobial assay of the crude extracts on bacterial isolates. Potato dextrose agar was used to isolate the fungi species and their susceptibility testing.

2.7 Sample Inoculation

Sample Inoculation for Bacterial Isolates

The method employed for bacterial inoculation was pour plating. 1ml of thebroth was poured on plate count agar plates and incubated at 37°C. The plates were observed for the growth of bacterial colonies after 24hrs. The number of viable colonies were noted and counted.

Sample Inoculation for Fungal Isolates

The method employed for fungal inoculation was pour plating. 1ml of the broth was pouredon potato dextrose agar plates and incubated at temperature of 25°C. The growth of fungal colonies/hyphae was observed on the plates after 48-72hours. The number of viable colonies were noted and counted.

2.8 Identification of Bacterial Isolates

Differentiation of Bacteria Colonies

The colonies of bacteria observed on the plate count agar media were sub-cultured individually to a fresh nutrient agar plates to obtain a pure culture by pour plating. The plates were incubated at 37°C for 24hrs. The colonies were observed and studied for their growth pattern.

Gram Staining/Microscopy

Gram staining method was used to differentiate the gram negative and gram positive bacteria. It was also used to magnify and study the morphology, shapes, spores and also gram staining reaction of each isolate.

2.9 Biochemical Test

The following biochemical characterisation of the isolates was done to further identify the organisms; Catalase test, SIM (Sulphide, Indole and Motility) test, Citrate test and Urease test.

2.10 Identification of Fungal Isolates

Cultural Characteristics

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The colonies and hyphae of fungi observed on the potato dextrose agar media were sub-cultured into a fresh potato dextrose agar (PDA) plate to obtain a pure culture by pour plating and incubated at 25^{0}C for 48 to 72 hours. The colonies and hyphae of fungi on the incubated medium were observed macroscopically to note the colour and texture of the colonies.

Fungal Staining/microscopy

A drop of lactophenol cotton blue stain was placed on a clean glass slide. A fragment of the fungal colony was placed in the drop of the stain using inoculating needle and teased gently. A cover slip was applied and the preparation was viewed under the microscope using oil immersion technique for the presence of fruiting body structure and mycelia.

2.11 Phytochemical Screening of Extracts

Phytochemical analysis was performed to screen the extracts for the presence of the following phytochemical properties: Tannins, Flavonoids, Phenols, Alkaloids, Saponins and Quinones. All procedures were as described by (Ewansiha *et al.,* 2012 and Umar, 2012).

Test for Tannins

Zero point five milliliters (0.5mls) of each extract was added to 10.0mls of distilled water and mixed with few drops of Ferric Chloride ($FeCl_3$) solution.

Test for Flavonoids

One milliliters (l.0ml) of each extract was dissolved in sodium hydroxide (NaOH) solution.

Test for Alkaloids

Mayer's test: (Ewansiha *et al.,* 2012)

1 ml of conc. HCl to was added to 1ml of extract followed by few drops of Mayer's reagent.

Test for Phenols

Ferric chloride test: (Ewansiha *et al.,* 2012).

1ml of 5 % ferric chloride solution was added to 1ml of extract.

Test for Saponins

Foam test: (Ewansiha *et al.,* 2012).

1ml of distilled water was added to 1ml of the extract and shake vigorously.

Test for Quinones

Add 0.5 ml of con HCl to 1ml of extract.

2.12 Preparation of Extracts

One gram (1g) of methanol, ethyl acetate and aqueous extracts were weighed and dissolved in 5ml each of methanol. This gives 200mg/ml concentration of the extract.

2.13 Antibacterial Assay of Crude Extracts

The isolates were dispensed in 5ml sterilized distilled water. The agar well diffusion method was employed as described by Ewansiha (2012), Umar *et al*. (2016), and Unachukwu (2017). One ml each of the prepared isolates was dispensed in Petri dishes for pour plating. Nutrient agar media were used for pour plating of all the isolates. The media were allowed to solidify and labelled accordingly; sterile cup borer (5mm) was used to bore four holes in the culture media. In a drop-wise manner, two drops of the prepared methanol, ethyl acetate and aqueous extracts were added into each well. Two drops of acetic acidwas added accordingly into the fourth well in all the media. Acetic acid is an inorganic acid that poses antibacterial effects. The cultures

were allowed to stand for 30 minutes before they were transferred into the incubator. The cultures were incubated for 24-48 hours at 37°C before final readings were taken. Zones of inhibition were measured to the nearest millimeter.

2.14 Antifungal Assay of Crude Extracts

The agar well diffusion method was employed as described by Ewansiha (2012), Umar *et al*. (2016), and Unachukwu (2017). The test organisms were inoculated into test tubes containing 5ml sterilized distilled water and tween 80 and were allowed to stand for an hour. One ml each of the isolates was inoculated into a potato dextrose agar through pour plating method and the media were labelled accordingly. A sterile cork borer (5mm) was used to bore four holes in each plate. In a drop-manner, two drops of methanol, ethyl acetate and aqueous extract were added into each of the well. Two drops of fluconazole were added to the fourth well which serves a control and the cultures were allowed to stand for 30 minutes before incubation at room temperature for 72 hours. After 72 hours of active growth, the zones of inhibition were measured with the aid of a meter rule considering the diameter of the cork borer.

2.15 Statistical Analysis

Data were entered and analysed using Statistical Package for Social Science (SPSS) version 20 software. Results were presented through tables. Statistical significance of means was measured by using the ANOVA. A ($P<0.05$) was considered as statistically significance. All results were expressed as the mean ± standard deviation.

3. Results

Thirty samples of female dystrophic nails were collected randomly across different outlets within Makurdi metropolis. The following bacteria were isolated; *Bacillus spp., Streptococcus spp., Staphylococcus spp., Klebsiella spp., Salmonella spp., Escherichia coli* and *Shigella spp*. The fungal isolates were *Trichophyton spp., Microsporium spp.* and *Aspergillus spp*.

The antimicrobial susceptibility of the isolates to methanol, ethyl acetate, and aqueous extracts were determined using acetic acid and fluconazole as standard for bacterial and fungal isolates, respectively. The phytochemical analysis of *Cymbopogon citratus* extracts confirms the presence of Flavonoids, Saponins, Alkaloids, Phenols and Tannins small in all the extracts. Quinone was present in all the extracts except ethyl acetate extract.

Table 1 data showed the mean values and standard deviation values of Total Viable Count (TVC) of bacterial and fungal isolates. The fungal total viable count of female aged 18 to 34 showed 21.70 ± 8.17^{b}, female aged 35 to 64 showed 23.10 ± 9.49^{b}, and female aged 65 and above showed 49.80±15.19. The bacterial total viable count of female aged 18 to 34 showed 53.60±16.02, female aged 35 to 64 showed 62.80±30.96 and female aged 65 and above showed 56.80±22.12.

Tables 2 and 3 data showed the percentage occurrence of bacterial and fungal isolates, respectively. The total prevalence of bacterial isolates were *Bacillus spp*. (47(39.83%)), *Streptococcus spp*. (13(11.02%)), *Staphylococcus spp*. (37(31.36%)), *Klebsiella spp*. (8(6.78%)), *Salmonella spp*. (3(2.54%)), *Escherichia coli* (5(4.24%)), and *Shigella spp*. (5(4.24%)). The total prevalence of fungal isolates were *Trichophyton spp*. (13(33.33%)), *Microsporium spp*. (17(43.59%)) and *Aspergillus spp*. (9(23.08%)).

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Table 1: Total Viable Count of Bacterial and Fungal Isolates

Female age groups (years)	Bacterial Isolates (CFU/ml)	Fungal Isolates (CFU/ml)
18 – 34	53.60±16.02	21.70±8.17[b]
35 – 64	62.80±30.96	23.10±9.49[b]
65 and above	56.80±22.12	49.80±15.19[a]
p-value	0.685	0.000

Bacterial isolates: df= 9, p>0.005, Fungal isolates: df= 9, p<0.005

Key: CFU/ml = colony forming units per milliliter.

Results are mean±standard deviation of duplicate values.

Note: mean on the same row with different superscript differ significantly.

Table 2: Percentage Prevalence of Bacterial Isolates (%)

Isolates	*Bacillus spp.*	*Streptococcus spp.*	*Staphylococcus spp.*	*Klebsiella spp.*	*Salmonella spp.*	*Escherichia coli*	*Shigella spp.*	Total
A	17(14.24)	5(4.24)	15(12.71)	0(0.00)	0(0.00)	3(2.54)	0(0.00)	40(33.90)
B	10(8.48)	3(2.54)	7(5.93)	2(1.69)	0(0.00)	1(0.85)	0(0.00)	23(19.50)
C	10(8.48)	5(4.24)	6(5.08)	5(4.24)	2(1.69)	1(0.85)	2(1.69)	31(26.27)
D	10(8.48)	0(0.00)	9(7.63)	1(0.85)	1(0.85)	0(0.00)	3(2.54)	24(20.34)
Total	47(39.83)	13(11.02)	37(31.36)	8(6.78)	3(2.54)	5(4.24)	5(4.24)	118(100.00)

Key: % = percent, A = Sample A sub-cultured from each plate, B = Sample B sub-cultured from each plate, C = Sample C sub-cultured from each plate, and D = Sample D sub-cultured from each plate.

Table 3: Percentage Prevalence of Fungal Isolates across the Samples

Isolates	*Aspergillus spp.*	*Trichophyton spp.*	*Microsporium spp.*	Total
A	5(12.82)	4(10.26)	2(5.13)	11(28.21)
B	3(7.69)	8(20.51)	5(12.82)	16(41.03)
C	1(2.56)	1(2.56)	10(25.64)	12(30.77)
Total	9(23.08)	13(33.33)	17(43.59)	39(100.00)

Key: % = percent, A = Sample A sub-cultured from each plate, B = Sample B sub-cultured from each plate, C = Sample C sub-cultured from each plate, and D = Sample D sub-cultured from each plate.

Tables 4 and 5 showed the antimicrobial susceptibility of the bacterial and fungal isolates to the extracts. The Clinical Laboratory Standards Institute (C.LS.I) criterion for antimicrobial susceptibility testing was employed taking note of the mean zone of inhibitions of the extracts on the isolates. The antimicrobial susceptibility of the bacterial isolates showed *Bacillus spp.* Was 12.00±0.00 for ethyl acetate, 0.00±0.00 for both methanol and aqueous extracts and 66.50±2.12 for acetic acid. *Streptococcus spp.* was 10.00±0.00 for methanol, 12.00±0.00 for ethyl acetate, 0.00±0.00 for aqueous and 66.50±2.12 for acetic acid.*Staphylococcus spp.* Was 0.00±0.00 for methanol, 12.00±1.41 for ethyl acetate, 10.00±0.00 for aqueous and for acetic acid. *Klebsiella spp.* Was 0.00±0.00 for all the extracts and 53.50±4.95 for acetic acid. *Salmonella spp.* was 12.50±0.71 for methanol, 0.00±0.00 for ethyl acetate, 14.50±0.71 for aqueous and 65.00±7.07 for acetic acid. *Escherichia coli* was 10.50±0.71 for methanol, 16.00±0.00 for ethyl acetate, 0.00±0.00 for aqueous and 62.50±2.83 for acetic acid. *Shigella spp.* was 13.00±1.41 for methanol, 10.00±0.00 for both ethyl acetate and aqueous extracts, and 62.50±0.71 for acetic acid. The antimicrobial susceptibility of the fungal isolates showed *Trichophyton spp.* was 0.00±0.00 for all the extracts and 20.00±0.00 for fluconazole. *Microsporium spp.* was 0.00±0.00 for methanol and aqueous extracts, 13.00±1.41 for ethyl acetate and 29.50±4.95 for fluconazole, *Aspergillus spp.* was 0.00±0.00 for methanol and aqueous extracts, 13.50±0.71 for ethyl acetate and 16.50±0.71 for fluconazole.

Table 4: Zone of Inhibition of Extracts and Acetic Acid on Bacterial Isolates (mm)

Extracts	*Bacillus spp.*	*Streptococcus spp.*	*Staphylococcus spp.*	*Klebsiella spp.*	*Salmonella spp.*	*E. coli*	*Shigella spp.*
Methanol	0.00±0.00	10.00±0.00	0.00±0.00	0.00±0.00	12.50±0.71	10.50±0.71	13.00±1.41
Ethyl acetate	12.00±0.00	12.00±0.00	12.00±1.41	0.00±0.000	0.00±0.00	16.00±0.00	10.00±0.00
Aqueous	0.00±0.00	0.00±0.00	10.00±0.00	0.00±0.00	14.50±0.71	0.00±0.00	10.00±0.00
Acetic acid	66.50±2.12	66.50±2.12	75.00±7.07	53.50±4.95	65.00±7.07	62.50±2.83	62.50±0.71
p-value	0.00	0.000	0.000	0.000	0.000	0.000	0.000

df = 1, $p < 0.005$

Key: mm = millimeters.

Results are mean ± standard deviation of the duplicate values.

Note: mean on the same column with different superscript differ significantly.

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Table 5: Zone of Inhibition of Extracts and Fluconazole on the Fungal Isolates (mm)

Extracts	*Trichophyton spp.*	*Microsporium spp.*	*Aspergillus spp.*
Methanol	0.00±0.00	0.00±0.00	0.00±0.00
Ethyl acetate	0.00±0.00	13.00±1.41	13.50±0.71
Aqueous	0.00±0.00	0.00±0.00	0.00±0.00
Fluconazole	20.00±0.00	29.50±4.95	16.50±0.71
p-value	0.000	0.001	0.000

df = 1, $p<0.005$

Key: mm = millimeters.

Results are mean ± standard deviation of the duplicate values.

Note: mean on the same column with different superscript differ significantly.

4. DISCUSSION

The dermatophytes (*Trichophyton spp.* and *Microsporium spp.*) and non-dermatophytes (*Aspergillus* spp.) isolated in this study possesses the capacity to adhere to nail regions of the body as indicated in a study by Adigun (2022) and Hainsworth *et. al.* (2015). As observed in this project study, adults above 65years showed higher total viable counts of (49.80 ± 15.19^{a}) for fungal isolates as compared to 21.70 ± 8.17^{b} and 23.10 ± 9.49^{b} in the other age groups as agreed by a study of Adigun (2022) confirming that older adults are prone to fungal infections compared to younger adults. The following bacteria isolated as follows; *Bacillus spp., Streptococcus spp., Staphylococcus spp., Klebsiella spp., Salmonella spp., Escherichia coli* and *Shigella spp.* have been previously isolated from nails (Risan, 2017). The bacterial counts of 53.60±16.02, 62.80±30.96 and 56.80±22.12 between the various age groups (18 to 34, 35 to 64 and 65 and above, respectively) with a p-value of 0.685 (p-value > 0.05) showed no significant difference.

The predominance of dermatophytes in this study was evident in the total prevalence of *Trichophyton spp.* (13(33.33%)) and *Microsporium spp.* (17(43.59%)) as compared to non-dermatophyte, *Aspergillus spp.* (9(23.08%)).

The antimicrobial potential of the extracts was noticed around the zones of inhibition on the agar plate. The susceptibility of the extracts against the bacterial isolates was due to the essential oil and phytochemical properties of *Cymbopogon citratus*. This project study observed varying degree of susceptibility of the extracts which agrees with a study by Ewasinha *et. al.* (2012) and Singh (2011) on various strains of bacterial isolates. This project study observed that the extracts were sparingly effective against the fungal isolate which agrees with a study by Shah (2011).

This study acknowledges the differences in the invitro analyses of zones of inhibition amongst bacterial and fungal isolates which can be different in applications. Hence, the C.L.S.I standards used cannot compare the already existing antibiotics standards to the extracts used. Fluconazole was used as a standard which showed a clear zone of inhibition on the fungal isolates. Acetic acid was used as a control since it possesses antibacterial properties.

5. CONCLUSION

This research has concluded, based on its findings, that dermatophyte such as *Trichophyton spp* and *Microsporium spp.* and non-dermatophyte, *Aspergillus spp.* can colonize the toenails. The prevalence of the dermatophytes was evidently higher as seen in the percentage prevalence of *Trichophyton*

spp. and *Microsporium spp*. compared to the percentage prevalence of *Aspergillus spp*. The bacterial species associated with nails such as *Staphylococcus spp*. were isolated and other bacterial species which were also isolated as indicated by Risan (2017) under the nail beds such as *Klebsiella spp*., *Streptococcus spp*., *Shigella spp*., *Escherichia coli, Bacillus spp*., and *Salmonella spp*. were isolated.

The phytochemical substituents such as Flavonoids, Tannins, Saponins, Phenols and Alkaloids small were all present in methanol, ethyl acetate and aqueous extracts hence, providing the plants with some degree of antimicrobial properties. Also, quinones presence in the methanol and aqueous extracts contributed to this property. The invitro differences of zones of inhibition recorded indicates the antimicrobial potential of *Cymbopogon citratus*.

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