Microbial Quality and Antimicrobial Potential of some Herbal Remedies Marketed in Owerri-West Nigeria

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Abstract

BACKGROUND

Herbal remedies are complex mixtures of plant extracts and natural products. Their adoption for therapeutic purposes dates back to ancient times. Despite the acceptance of these herbal remedies, there is still concern about their purity and safety. The objective was to determine the quality of the herbal remedies by their microbial load and organisms present in the samples. The study also determined the therapeutic potential of selected pathogenic organisms from clinical sources.

MATERIALS AND METHODS

Microbial enumeration and identification of bacteria and fungi from 20 common herbal remedies were performed using standard microbiological protocols. Further identification was done using molecular typing. Antibiotic susceptibility tests, minimum inhibitory and bactericidal concentration of the remedies was also determined on *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus* were done using agar well diffusion method.

RESULTS

Bacteria counts in the remedies varied from $1.0x10^4$ – $1.4x10^5$ while fungi counts ranged from $2.0x10^3$ – $1.6x10^5$. *S. aureus, P. aeruginosa, E. coli* and *Bacillus spp* were the bacterial species isolated from the remedies. *Bacillus* was the highest in the occurrence of (53%), while the least was *E. coli* (7%). Seven species of fungi namely *Penicillium citrinum (MN960659)* 21.1%, *Pichia cecembensis (MN960658)* 21.1%, *Aspergillus niger* 26.3% *(MN960657), Saccharomyces spp* (5.3%), *Mucor spp* (5.3 %), *Fusarium spp* (7. 3%) and *Rhizopus spp* (13.2 %), were identified. The inhibitory activity of herbal remedies on pathogenic organisms ranged from 8mm -18mm. Minimum inhibitory concentration (MIC) ranged from 62.5 – 125mg/ml while Minimum bactericidal concentration (MBC) ranged from 125mg/ml – 250mg/ml. The lowest activity was seen with *Candida albicans* and *S. aureus* while the highest activity was seen with *P. aeruginosa,* while *Salmonella typhi* was resistant.

CONCLUSIONS

Producers of herbal remedies should observe standard protocols and regulatory agencies should monitor and enforce microbial quality of products continually.

Keywords: *Antimicrobial, Contaminant, Herbal Remedies, Microorganisms, Quality*

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Introduction

For decades herbal formulations from plants have been used for medical treatments in most developing countries. It was estimated that herbal medicine is used as a therapeutic intervention for 80 per cent of ailments.[1] These herbal remedies are used for the first point of call treatment due to costs, availability, fewer side effect and the belief that they can treat all diseases.[1, 2] In most developing countries including Nigeria herbal remedies have been in use for centuries. These remedies are available in shops and supermarkets and have been used for the prevention and treatment of some diseases. Most times the remedies are not regulated and the components can vary from one location to another.[2, 3]

The advantage of herbal remedies is their availability and antioxidant properties have made them highly sought after. [4] It is generally believed that herbal remedies are effective, safe and without side effects, which has resulted in the use of these products. Some reports claim that these herbal remedies are effective against cancer, diabetes, asthma and end-stage kidney disease, drug-resistant malaria and AIDS.[3, 5]

A study reviewed different articles on natural products that are effective in different types of cancers. These herbs from studies show that they have fewer side effects and are less destructive to cells that are close to cancer cells. There are no reliable studies on the effective use of herbal remedies for the treatment of cancers.[3] This has contributed to the continuous rise in the number of people using them.[5, 6] The use of herbal remedies in Nigeria gained ascendency with improved branding of products, National Agency for food, drug administration and control (NAFDAC) certification of some products and their several media and trade campaigns. [7] Despite the general acceptance of traditional remedies in Nigeria, there is concern about the purity and safety of these products.[8, 9] Traditional remedies are sometimes contaminated with microorganisms that can affect the property of the remedy either physically, chemically or organoleptic property.[4] When these remedies are released into the market without being sterile and not supervised, they can affect the therapeutic ability of the herbal remedies making them non-effective. [7, 8]

Previous studies have shown the contamination of herbal remedies with microorganisms. Modern medicine depends on evidence-based pharmaceutical drugs. This practice uses many plants derived compounds, with applications of modern standards to ensure high-quality clinical standards of purity, effectiveness and dosage often lacking for most herbal formulations in use.[1, 2] Several microorganisms have been detected in herbal products such as *E. coli, Klebsiella, Pseudomonas, Proteus, Salmonella typhi*, *Shigella spp.* and *Staphylococcus aureus*. Moulds isolated from herbal products from previous studies include *Aspergillus flavus, A. parasiticus, Trichosporium spp Mucor spp, Candida spp Penicillium* sp.[7, 10-14] The contamination of herbal remedies with microorganisms above the permissible limit can be of negative effect on the elderly and immunocompromised patients, thus the need for more stringent rules and policies guiding the production of theses remedies to prevent complications.[15]

Materials and Methods

Study area and sample source

The research was carried out in Owerri West Local Government Area (L.G.A), Imo State, Nigeria**.** Twenty (20) herbal remedies were randomly purchased from herbal stores, hawkers and motor parks in Owerri West L.G.A of Imo state. The remedies were marketed for the treatment of typhoid fever, stomach aches, skin infections, headaches, toothaches, piles, sexually transmitted diseases and diabetes. They were used either in liquid or powdered form to be reconstituted into a suspension as directed.

Study design

The study was designed to determine the microbial quality and efficacy of herbal remedies marketed in Owerri -West. The samples selected were those that have National Agency for Food and Drug Administration (NAFDAC) number. The NAFDAC number certifies that the herbal remedy is certified for use.

Microbiological analysis of the herbal remedies

The bacteria count of the selected herbal remedies was determined by inoculating the herbal formulations onto sterile nutrient agar and incubating at 37^{o} C for 48 hrs according to the methods of previous methods. Fungi were isolated from the herbal remedies by inoculating them onto Sabouraud Dextrose Agar (SDA) plates in duplicates and incubated at 25^{o}C for 5 days. [16, 17]

Characterization and identification of isolates

Bacterial and fungal isolates were characterized and identified based on colonial, microscopic, biochemical, Germ tube test and molecular methods. [18]

DNA extraction and purification of bacteria from herbal remedies

Bacteria isolates from the remedies were identified and confirmed by previous studies [19, 20] In brief, five millilitres of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^{0}C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{o}C for further analysis. Extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. Bacterial DNA was amplified using 16S rRNA.

Molecular identification of fungi from herbal remedies

The genomic DNA of the fungal isolates was extracted using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. Their regions of the rRNA genes of the isolates were amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 DreamTaq Master mix supplied by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as templates.

The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV trans illuminator. [21]

Sequencing and phylogenetic analysis

This was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul; the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x Big Dye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min". Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. The obtained ITS and 16SrRNA sequence from the isolate produced an exact match during the megaBlast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database.[22-25]

Ethical approval

Ethical approval was obtained from the Ethical Committee of Federal Polytechnic Nekede Medical Center with the number FPN/MC/85B/1/41

Isolation and identification of clinical isolates

Clinical isolates were obtained from urine, wound and stool of patients suffering from urinary tract infection, wound infection and gastroenteritis. The samples were collected from Jahmuel Diagnostic and Scientific, Umuofocha Nekede Laboratory between the months of March to October 2019. Urine samples were cultured on CLED agar, and wound on MacConkey agar, while stool samples were cultured on Salmonella-Shigella agar and MacConkey agar. The clinical isolates were identified by standard microbiological methods which included *Pseudomonas* spp. and *Staphylococcus aureus* were isolated from wounds, *Salmonella typhi* from stools while *Candida albican* and *E. coli* were isolated from urine.

Detection of inhibitory activity of herbal remedies on clinical isolates

The inhibitory activity of concentrated herbal formulations was determined. The chemotherapeutic agents used in the positive control were ciprofloxacin 10 µg/ml (Nichola laboratories limited, England) and Nestation. Fungal. Prepared plates of Muller Hilton agar were seeded with the bacteria test organisms while SDA was seeded with fungi organisms. A sterile cork borer was used to make holes in the plates (each with a diameter of 0.5cm). These holes were filled with 250 mg/ml of the extracts and incubated at 37^0C for 24 hrs. Zones of inhibition were measured with a micrometre screw gauge and the diameter was recorded. [26]

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Different concentrations of 250mg/ml, 125 mg/ml, 62.5 mg/ml, and 31.25 mg/ml were obtained using two-fold serial dilutions. The initial concentration was obtained by diluting 1ml of the TMF in 4ml of nutrient broth. Having obtained the different dilutions and concentrations, three were inoculated into the dilutions and incubated at 37^0C for 24 hrs. The lowest concentration of the TMF, which inhibited the growth of the test organism, was recorded as MIC. [26] Tubes showing no visible growth from the MIC test were sub-cultured into nutrient agar and incubated at 37^0C for 24 hrs. The lowest concentration of the herbal remedy yielding no growth was recorded as the MBC.

Results

Of the twenty brands of Herbal formulations used in this study, seventeen were used to cure more than one ailment and three had no indications for intended usage. All the

herbal remedies were made in Nigeria. Nineteen of the herbal remedies were in suspension form while one was in liquid form; all had their batch number written on them. Four (4) of the same had no manufacturing date, while the rest had. Five had an expiry date, while fifteen 15 had no expiry date written on them. Only two did not have a NAFDAC number but the rest had. The content of sixteen was written while four had no content indicated. Results for bacterial and fungal counts are presented

In Table 1 Seven samples (A, B, C, E, F, G and M) had no bacteria counts in them (Figure 1). The lowest count of $1.0x10^4$ was recorded in sample D while the highest count of $3.2x10^5$ was recorded in sample O. There were no fungi count in sample A and M. Sample 'R' recorded the highest fungi count of $1.6x10^5$ while sample 'B' and 'G' had the least fungi load of $1.0x10^3$ respectively. Following morphological and biochemical characteristics of the bacterial isolates from the herbal remedies *S. aureus*, *P. aeruginosa*, *E. coli* and *Bacillus* spp were identified. *Bacillus had the highest* occurrence (53%), while the least was *E. coli* which occurred just once at **7**% as presented in Figure 1. Identified fungal isolates include *Penicillium citrinum, Aspergillus niger, and Rhizopus spp. Pichia cecembensis, fusarium spp, mucor spp.* and *Saccharomyces spp. A. niger* had the highest occurrence of eight 8(21.1%) each while Mucor spp. and *Saccharomyces spp* had the least 2(5.3%) each as presented in Figure 2.

The phylogenetic tree of the identified bacteria from the herbal remedies showed that the phylogenetic placement revealed a close relatedness of *Pseudomonas aeruginosa* and *Escherichia coli* while the phylogenetic placement within the *Aspergillus, Penicillium, and Pichia* revealed a close relationship to *Aspergillus niger, Penicillium citrinum* and *Pichia cecembensis* (Figures 3 and 4).

Table1:
Total Bacteria Count and Fugal Count of the Herbal Remedies

Sample Code	**Bacteria Count 0**	**Fungal Count 0**	**Identified Fungal Isolates**
A			
B	0	$1.0X10^3$	*Rhizopus spp, Pichia cecembensis, Penicillium citrinum*
C	0	$1.2X10^3$	*Pichia cecembensis* and *Aspergillus niger*
D	$1.0X10^4$	$1.4X10^3$	*Mucor spp* and *Aspergillus niger*
E	0	$1.3X10^3$	*Fussarium spp and Aspergillus niger*
F	0	$1.0X10^3$	*Pennicillum citrium, Apergillus niger* and *Rhizopus spp*
G	0	$1.0X10^3$	*Pennicillum citrium* and *Mucor spp*
H	$23.0X10^4$	$30.0X10^3$	*Pichia cecembensis spp,*
I	$14.0X10^4$	$126.0X10^3$	*Pennicillum citrium, Apergillus niger* and *Rhizopus spp.*
J	$18.0X10^4$	$15.0x10^3$	*Penicillium citrinum*
K	$23.0X10^4$	$160.0X10^3$	*Aspergillus niger*
L	$15.0X10^4$	$31.0X10^3$	*Pichia cecembensis, Pennicillum citrium* and *Sacharomyces spp*
M	0	0	
N	$27.0X10^4$	$24.0X10^3$	*Aspergillus niger, Fussarium spp,* and *Pennicillum citrium*
O	$32.0X10^4$	$19.0X10^3$	*Aspergillus niger* and *Pichia cecembensis*
P	$25.0X10^4$	$28.0X10^3$	*Pichia cecembensis* and *Fussarium spp*
Q	$17.0X10^4$	$11.0X10^3$	*Aspergillus niger* and *Pichia cecembensis*
R	$14.0X10^4$	$15.0x10^3$	*Aspergillus niger, Rhizopus spp* and *Pichia cecembensis*
S	$24.0X10^4$	$48.0x10^3$	*Rhizopus spp*
T	$28.0X10^4$	$29.0x10^3$	*Pichia cecembensis*

Inhibitory activity (Figure 5) of herbal remedies on clinical isolates showed that sample M (18mm) was most effective on *P. aeruginosa*, 17mm (*E. col)* and 15mm (*S. aureus*). Sample D had an intermittent activity of 15mm *S. aureus* and *P. aeruginosa*. Sample B also had a similar inhibitory activity with reduced inhibition on *E. coli* and *S. aureus* (9mm and 8mm) recorded on the sample while no activity was recorded on samples 'D' and 'E' against *E. coli*. Samples (C and D) had bactericidal activity on *P. aeruginosa*, but no activity was recorded on samples F, G and M. Samples A, B and E had bacteriostatic activity on the organism. On *C. albican* samples A and B had bactericidal activity, while there was no activity on the sample.

Discussion

The study was conducted to investigate the microbial quality and antimicrobial activity of some herbal remedies sold in Owerri West LGA of Imo State.

Twenty (20) were used in the study. Of the 20 herbal remedies used, 17 (85%) had their indications written on them while 3 (15%) did not. Again, 5 (25%) of the herbal remedies have their expiry date while 15 (75%) did not have their expiry date written on them. Medicinal products should have their indications written on them for proper guidance, use and cue to the expected duration of potency. In Nigeria, any medicinal product in use that doesn't have a NAFDAC number is deemed not suited for use by the Nigerian populace. These findings are in agreement with other studies[10], that revealed that the majority of herbal medicinal products sold in South-Western Nigeria had no manufacturing and expiry dates stated, they were not registered by NAFDAC and did not have their content stated but had their therapeutic claims indicated on the container.

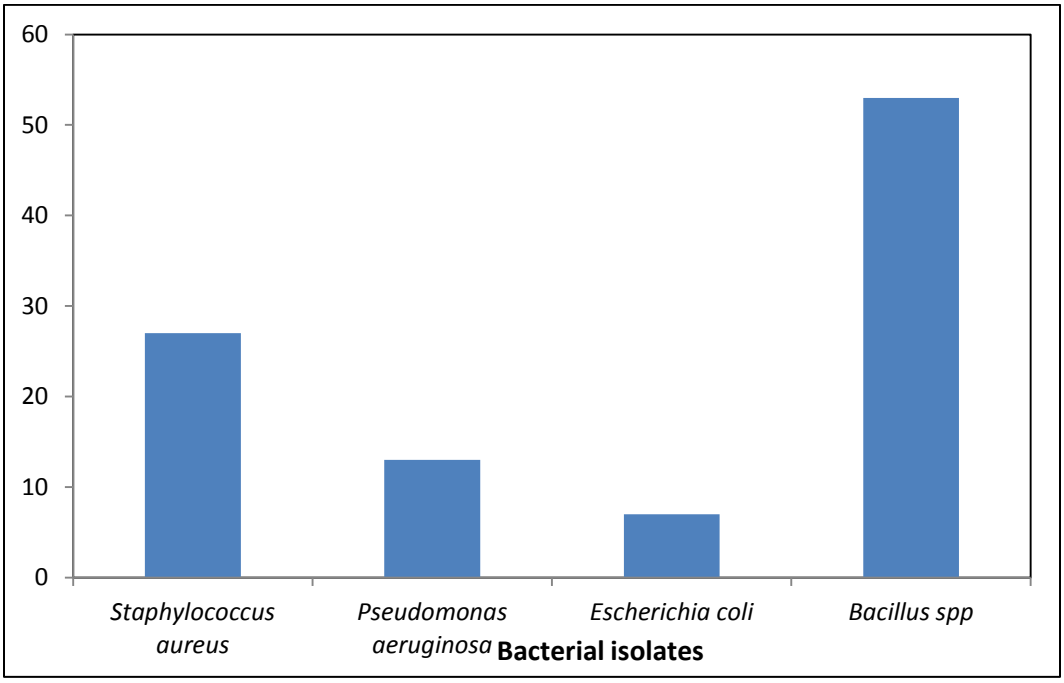

Figure 1:
Percentage occurrence of bacteria isolates from herbal remedies

Contrary to our findings, a previous study reported that all of the herbal medications sampled had expiry and manufacturing dates stated. [27] The present study showed that the herbal remedies analyzed were contaminated. Sixty (60%) of the samples had microbial loads beyond the officially permissible limits. A high level of fungal and bacterial counts was observed in some of the samples. The total aerobic count was compared with the WHO standard. [28]The specification of WHO for total aerobic count is $\leq 10^7$ cfu/g for the plant material used as tea or infusion and $\leq 10^5$ cfu/g for internal use. WHO specifications for yeast and mold are $\leq 10^4$ cfu/g for plant use as tea or infusion and $\leq 10^3$ cfu/g for internal use.

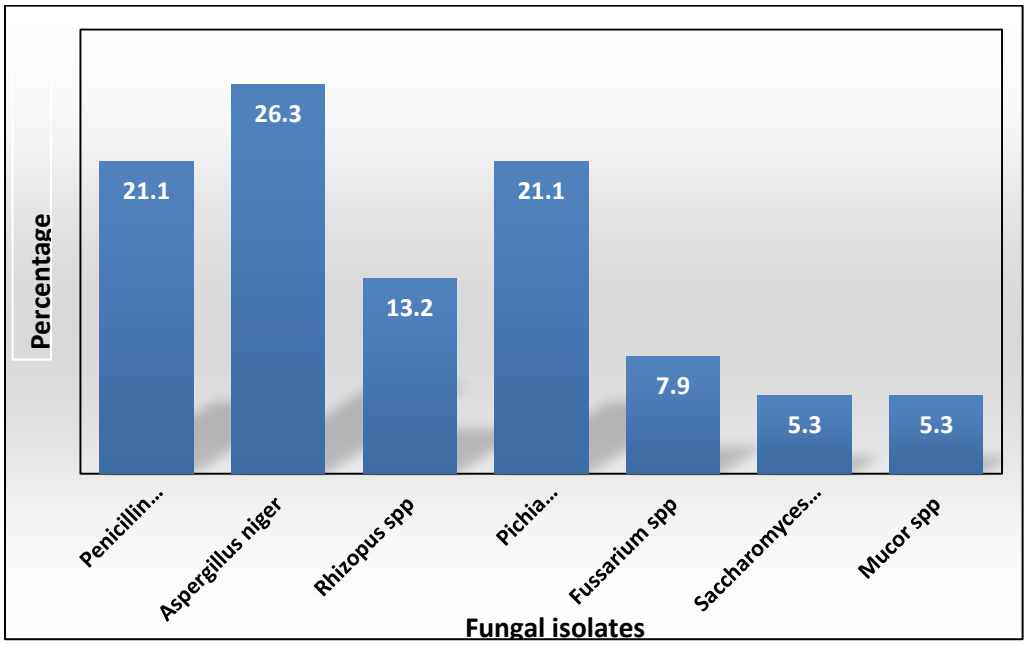

Figure 2:
Percentage Occurrence of Fungi Isolates from Herbal Remedies

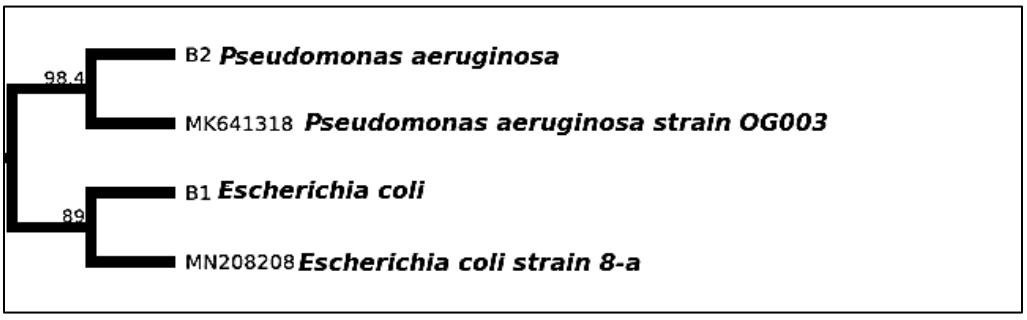

Figure 3:
Phylogenetic Tree Showing the Evolutionary Distance between the Bacteria Isolates

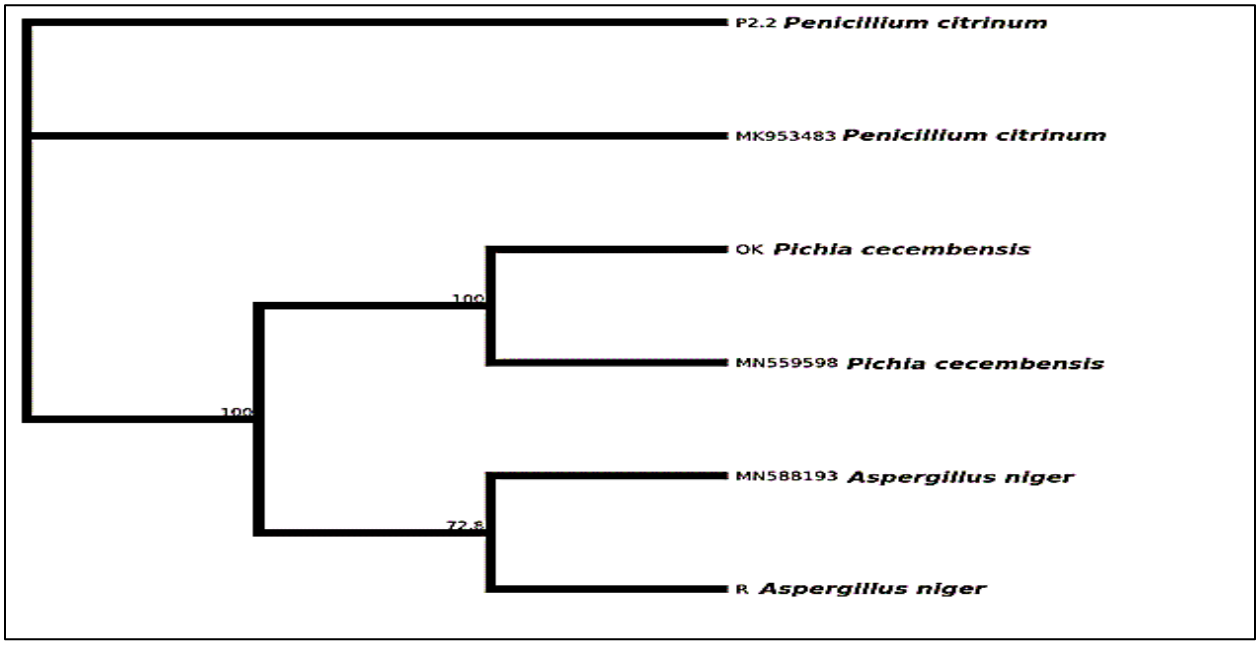

Figure 4:
Phylogenetic tree showing the evolutionary distance between the fungal isolates

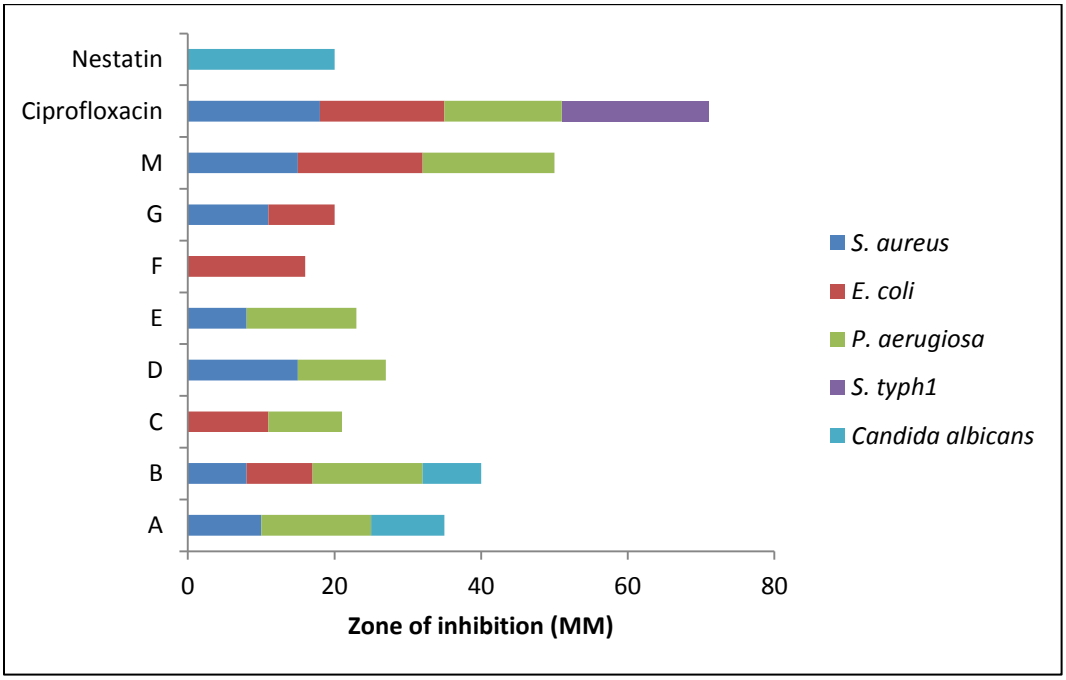

Figure 5:
Effect of herbal remedies on clinical isolates
CLSI Standard = Clinical laboratory standard institute. R =Resistant ≤ 12; I = Intermediate 12-17; S=Susceptible ≥17

Of the 19 oral suspensions and one liquid sample, 12 had total aerobic bacterial load of 1.4 X 10^5 – 2.8 X 10^5 cfu/ml and total fungi load of 1.5 X 10^4 – 1.6 X 10^5 cfu/ml. According to [30], the main microbial contaminants of herbal remedies are aerobic mesospheric enterobacteria, yeast and mold. Bacteria identified from the herbal remedies in this present work were *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli* and *Bacillus spp.*, with *Bacillus spp.* the most prevalent. Similar microorganisms were isolated from the herbal remedies in previous studies. [4, 10, 13, 30, 31] Some of the isolates found in the herbal remedies are normal flora of the soil, water and vegetation. [32] Microbiological qualities of herbal medicaments are influenced by the atmosphere, harvesting, poor drying, processing, storage and improper handling. Some of these bacteria are part of the microbiota of the human intestine and other animals. They are used as indicator organisms and as an index of possible contamination by human faecal matter. *Staphylococcus aureus* may cause infections such as skin infection, food poisoning, septicemia, toxic shock syndrome and arthritis. *Bacillus cereus* produces heat-stable spores and causes food intoxication when ingested. Sources of contaminants in herbal remedies occur during extraction and preparation. The microbiota of the final preparation may represent contaminants from the raw materials, equipment, atmosphere, water and personnel. Plants materials are usually contaminated through the methods used in their processing such that the microbial contaminants are a reflection of the environmental/processing conditions, and they are mostly aerobic mesospheric enterobacteria, mould and yeast. Although to consider a herbal medication objectionable, the magnitude of contaminants should be considered although *Enterobacteria* including *E. coli* should not be present in any amount. [34, 35] This group of coliform indicates undesirable hygiene conditions.

Microorganisms such as *E.coli* and *Pseudomonas* reported in this study are known to cause UTI while *Staphylococcus aureus* and *Bacillus* spp. are commonly isolated from air. These organisms can constitute serious health hazards, especially in immune-comprised patients. Most *Pseudomonas species* are implicated in UTI, respiratory system infection, dermatitis, soft tissue infection, bacteremia, bone and joint infection, gastroenteritis and systemic infections.

Table 2:
Minimum Inhibitory and Minimum Bactericidal Concentrations and of the Herbal Remedies on test Organism

Herbal Remedies	***S. aureus***		***E. coli***		***P. aeruginosa***		***Candida abican***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
A	125	0	0	0	125	250	125	0
B	125	250	125	0	125	250	125	0
C	0	0	250	0	125	0	0	0
D	125	250	0	0	125	0	0	0
E	250	0	0	0	125	125	0	0
F	0	0	62.5	125	0	0	0	0
G	125	0	125	0	0	0	0	0
M	125	250	62.5	125	62.5	62.5	0	0

These bacteria can cause serious health challenges when ingested. Some of the fungal species isolated such as *Aspergillus* and *Penicillium* may cause health problems to consumers. *Aspergillus* produced potent mycotoxins that have been implicated in carcinogenicity, dermatitis nephrotoxicity and hepatoxicity. [22]

Some of the sampled TMF (A, B, C, D, E, F, G and M) showed acceptable microbiological quality and thus were used for susceptibility tests on clinical isolates. Sample B and E showed the lowest inhibitory activity, while sample M had the highest inhibitory activity. The herbal remedies showed pronounced activity on *E.coli* and *P.aeruginosa. Salmonella typhi* was resistant to all the samples. This could be because the organism acquired resistance easily. The effect of samples B, C and G were bactericidal on *S. aureus* while samples F and M were bacteriostatic. No effect was recorded on samples D and E against *S.aureus*. In the case of *P. aeruginosa*, the effect of samples C and D was bactericidal, samples A, B and E were bacteriostatic while no effect was recorded on samples G and M of the same organisms. Samples A and B had a bactericidal effect on *C. albican* while samples C, D, F, G, and M had no activity. The result of this present work conforms with studies from other states[35] that reported herbal preparations showed good susceptibility to tested organisms even in the least concentrations ranging from 5µl/ml to 200µl/ml which is contrary to the present findings. The least concentration of 31.5mg/ml in this study was not able to inhibit the test organisms as against the 5µl/ml in theirs. The alternative medicine practice in Nigeria is guided by a dubious claim of "professional secrecy" which does not permit scientific verifications of content and claims, and this has extended to neglect in checking for purity and safety of herbal remedies. The business is being promoted by charlatans who lay claims to some ancestral lineage of the secret knowledge of the spiritual language of plants for the cure of everything that ails man[35, 36]. Antimicrobial activities demonstrated by these herbal remedies in the present study justify some of the claims of the producers. The present study thus concludes that herbal remedies H, I, J, K, L, N, O, P, Q, R, S and T did not show acceptable microbial quality. Unfortunately, these were among the products that are very popular and expensive in Owerri west L.G.A of Imo State. Despite the claims by peddlers of herbal remedies, the microbial quality should routinely be checked, to ensure the safety of the products.

Conclusion and recommendations

The present study showed that some of the herbal remedies did not show acceptable microbial qualities, unfortunately, they are been consumed by the masses who believed that they are cheaper and more effective than conventional drugs. However, some were of good quality and should be enhanced for therapeutic purposes. To guarantee the safety and quality of herbal remedies marketed in Nigeria the products should undergo microbial quality checks and safety regularly. Policies regarding herbal remedies should be strictly adhered to. There should be constant training of producers of herbal remedies and public enlightenment on consumers.

Limitations of the study

Based on the availability of funds, the study was restricted to Owerri -West of the Imo States and few samples. Some of the samples were not stated specifically what they were used for. In future studies, these herbal remedies should be compared with those marketed in pharmaceuticals.

Competing Interests

The authors declare that this manuscript was approved by all authors in its form and that no competing interest exists.

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Contribution of authors

Onyewenjo Chiegeiro and Onyewenjo Simson Chukwuemeka sourced the literature and did the lab work. Agbagwa Obakpororo Ejiro and Frank-Peterside Nnenna designed and supervised the study. Agbagwa Obakpororo Ejiro prepared the final manuscript

References

1. **Onyambu MO, Chepkwony HK, Thoithi GN, Ouya GO and Osanjo GO**. Microbial quality of unregulated herbal medicinal products in Kenya. Afr. J. Pharmacol. *Therapeut*. 2013; 2: 70-7
2. **Ali N, Hashim NH, Saad B, Safan K, Nakajima M and Yoshizawa T.** Evaluation of a method to determine the natural occurrence of aflatoxins in commercial traditional herbal medicines from Malaysia and Indonesia. *Food Chem. Toxicol*. 2005; 43: 1763-1772.
3. **Pallavi, K.** Natural and Herbal Remedies for Cancer Treatment. *Inventi Impact: Planta Activa.* 2016. 140 - 147.
4. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *J. Amer. Med. Assoc*. 1998; 280: 1569–1575.
5. **Itharat A, Houghton PJ, Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. J. *Amer. Med. Assoc*. 1998; 280: 1569–1575.
6. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *J. Amer. Med. Assoc*. 1998; 280: 1569–1575.
7. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *J. Amer. Med. Assoc*. 1998; 280: 1569–1575.
8. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C**. (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *J. Amer. Med. Assoc*. 1998; 280: 1569–1575.
9. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *J. Amer. Med. Assoc.* 1998; 280: 1569–1575.
10. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *J. Amer. Med. Assoc*. 1998; 280: 1569–1575

4. **Eisenberg D.M, Davis R.B, Ettner S.L, Appel S, Wilkey S, Van Rompay M, Kessler R.C.** (1998) Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national

survey. *J. Amer. Med. Assoc*. 1998; 280: 1569–1575

5. **Braide W, Sokari TG, Nwaoguike RN, Okorondu SI**. Microbes from soil associated with Metamorphosing Moth Larvae, Current Trends in Microbiology, 2008;**4**:11 – 14.
6. **Gyasi RM, Darko ET, Mensah CM**. Use of Traditional Medicine by HIV/AIDS Patients in Kumasi Metropolis, Ghana: A Cross-sectional Survey. *Am. Int. J. Contemp. Res* 2013; 3(4): 117.
7. **Roozbeh J, Hashempur MH, Heydari M**. Use of Herbal Remedies among patients undergoing hemodialysis. *Iranian Journal of Kidney Diseases*. 2013;7 (6): 492-495
8. **Oluwatoyin AI, Adebayo L.** Assessment of the microbial quality of some oral liquid herbal medicines marked in Ile-Ife, South-Western Nigerian. *African Journal of Microbial research*, 2016;10 (38): 1618-1624.
9. **World Health Organization (WHO).** WHO guidelines on good manufacturing practices (GMP) for herbal medicines. 2007; Geneva, *World Health Organization*.
10. **Temu-Justin, M, Lyamuya EF, Makwaya CK.** Source of microbial contamination of natural therapeutically used locally prepared herbal medicines sold on the open market in Dares Salaam, Tanzania. *African Journal of Health Sciences*. 2011; 18: 1-2.
11. **Halt M**. Moulds and mycotoxins in herb tea and medicinal plants. *European Journal of Epidemiology*, 1998;14: 269-274.
12. **Bugno A, Almodovar AAB, Pereira TC, Pinto TJA, Sabino M**. Occurrence of toxigenic fungi in herbal drugs. *Brazilian Journal of Microbiology*, 2006; 37: 47-51.
13. **Okunlola A, Babatunde AA, Oluwatoyin AO.** Evaluation of Pharmaceuticals and Microbial Quality of some Herbal Medicinal Product in South Western Nigeria. *Tropical Journal of Pharmaceutical Research*, 2007**;** 61 (1): 661-670.
14. **Abba D, Inabo HI, Yakubu SE, Olonitola OS**. Contamination of herbal medicinal products marketed in Kaduna Metropolis with selected pathogenic bacteria. *African Journal of Traditional, Complementary and Alternative Medicines* 2009; 6: 70-77.
15. **Amaike S, Keller NP.** *Aspergillus flavus*. *Annual Review of Phytopathology*, 2011; 49: 107-133.
16. **de Sousa Lima CM, Fujishima MAT, de Paula Lima B. *et al.*** Microbial Contamination in herbal medicines: a serious health hazard to elderly consumers. *BMC Complement Med Ther* 2020; 17.
17. **Pelczar MJ (Jr), Chan ECS, Krieg NR**. *Microbiology: Concepts and Applications*, 1st ed. McGraw- Hill Inc., New York, USA. 2018; 80- 100, 158- 161,370.
18. **Cheesbrough M.** *District Laboratory Practice in Tropical Countries, Part 2*, Cambridge University Press, UK. 2000; 157-234.
19. **Willey JM, Sherwood IM, Woolverton CJ**. *Isolation of Pure Bacterial Culture from Specimen:* Prescott's Microbiology, 10th Edition: Boston WCB McGraw's Hill Companies: 2017;714 – 796.
20. **Thatcher S.** DNA/RNA preparation for molecular detection. *Clinical Chemistry* 2015; 61: 89-99
21. **Aldemir D, Jannink JL, Sidro-Sanchez J.** Locally epistatic models for genomic- wide prediction and association by importance sampling. *Genetic Selection Evolution*. 2017; 49(1): 74.
22. **Jin J, Lee YK, Wickes BL**. Simple chemical extraction method for DNA isolation from *Aspergillus* species. *Journal of Clinical Microbiology* 2004;42: 4293-4296

23. **Erickson K**. The jukes – cantor model of molecular evolution, *Primus*, 2010;20(5): 438 – 445.
24. **Kumar S, Stechar G, Tamura K.** Molecular Evolutionary Genetics Analysis version 7.0 for Bigger Datasets, *molecular biology and evolution*. Academic Oup.com 2016; 33 (7): 1870 – 1874.
25. **Tamura K, Peterson D, Peterson N**. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular biology and evolution* 2011; 28 (10): 2731 – 2739.
26. **Huey RB, Gerland J. Turelli M.** Revisiting a key innovation in Evolutionary Biology: Felsenstein's phylogenies and the comparative method. *Am Nat* 2019;193 (6): 755 – 772.
27. **Akujobi CO, Ogbulie JN, Uchegbu UN**. Antibacterial activities and preliminary phytochemical screenings of Veronica amygdalina and Citrus aurantifoloia. *Nigeria journal of microbiology*. 2006; 20(1): 649-654
28. **Odonkor TS, Osei J, Amin A, Laar C**. Microbial quality of some herbal products sold in Accra, Ghana. *Elixir pharmacy*. **40**: 2011; 5499-5503
29. **World Health Organization**. Quality control methods for medicinal plant materials. Geneva, 1998 World Health Organization.
30. **Kneifel W, Czech E, Kopp B**. Microbial contamination of medicinal plants- A review. *Planta Medica*, 2002; 68**:** 5-15.
31. **Noor R, Huda N, Rahman F, Bashar T, Munshi SK.** Microbial contamination in herbal medicines available in Bangladesh. *Bangladesh Medical Research council Bull* 2013; 39: 124-129.
32. **Yesuf A, wondimeneh Y, Gebrecher-kos T, Moges F.** Occurrence of potential bacteria pathogens and their antimicrobial susceptibility patterns isolated from herbal medicinal. 2016;
33. **Nester E, W Roberts, CE, Pearsall NN, Anderson DG, Nester MT**. *Microbiology. A Human Perspective*, 7th Edition WBC/McGraws- Hills Science/Engin New York, USA, 2011; 434 -435.
34. **Kosalec, I, Cvek J, Tomic S**. Contaminants of medicinal herbs and herbal products. *Archives of Industrial Hygiene and Toxicology***,** 2009***;*** 60: 485-501.
35. **Czech E, Kneifel W, Kopp B.** Microbiological status of commercially available medicinal herbal drugs- A screening study. *Planta Medica* 2001; 67: 263- 269.
36. **Foysal H, Saha O, Mukharjee Sk, Khan HI, Amin RM, Chowdhury, HMM.** Evaluation of antimicrobial activity and determination of suitable method for antibiogram of some commercial homoeopathic drugs available in Bangladesh **2016; 5(11):240-249.**
37. **Esimone CO, Chad KF, Ikedigide SC**. Microbiological Quality of herbal Preparation marketed in South East Nigeria. *Journal of Natural remedies* 2002**;** (1): 42-48.