Isolation and Identification of Microorganisms from Herbal Mixtures Sold Within, Abuja, Nigeria

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Abstract: The safety, efficacy and quality of herbal mixtures has become a concern to health authorities and health professionals, especially, with rise in microbial resistance to drugs and the demand herbal mixtures as alternative remedies. Thus, the need to isolate and identify microorganisms from some liquid herbal mixtures sold within Abuja, Nigeria. A total of ten samples of herbal mixture were selected at random, from herbal shops in Wuse market in sterile bottles and were analysed according to microbiological standard in Microbiology laboratory, Nile University of Nigeria, Abuja. Samples were inoculated onto Nutrient agar, MacConkey agar and Potato Dextrose Agar, and incubated at 37^{o}C for 24 hrs. Potatoe dextrose agar slants were also inoculated for the isolation of fungi. Viable bacterial counts of the samples were also performed using nutrient agar, molecular identification of sample was also carried out using DNA sequencing and blasting. The organisms isolated were identified using biochemical tests, and the following organisms were identified *Aspergillus flavus*, *Aspergillus* spp., *E. coli*, *Fusarium* spp., *Salmonella* spp., *Staphylococcus aureus*, *Proteus* spp., *Pseudomonas* spp., *Klebsiella* spp. and *Mucor fragilis.* However, the National center for biotechnology database, showed that the nucleotide query sequence of the 16S RNA gene was 99.47% similar to *Pseudomonas geniculate* (Gram positive), uncultured bacterium and *Stenotrophomonas maltophilia* (Gram negative). The herbal medicine were highly contaminated and most of the organisms, isolated could be as a result of unhygienic handling of raw materials used during the preparation of this herbal mixtures.
Key word: 16S RNA, Agar, Gram positive, Gram negative, nucleotide query sequence

INTRODUCTION

Phytomedicine, also known as herbal medicine, is a fast-growing field with a long tradition that makes use of natural plant products to treat diseases (Fazly Bazzaz *et al*., 2021). The stems, barks, leaves and roots of the plants are essentials components used by herbalists to prepare complex mixtures of organic chemicals. The chemical compositions of herbal mixtures differ depending on the genus, species and the raw or processed part of the specific plant (Fazly Bazzaz *et al*., 2021). The methods of usage of these botanical plant-derived products to cure disease vary with societies around the globe (Urumarudappa *et al.,* 2019).

The World Health Organization (2020) has defined herbal medicine as medicine based on the all-inclusive utilization of theories, beliefs, skills, knowledge of specific societies around the world, in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness. Over the past years, herbal medicines have been promoted as natural and safe, therefore, the preferred choice, used to treat various types of aliments, including diarrhoea, urinary tract infection, typhoid fever, and skin disease (Fazly Bazzaz *et al*., 2021).

The phytomedicinal plants contribute significantly to both traditional and modern medicine. These plants have been in use as traditional home remedies, as they form an important component of the indigenous medicinal systems from various countries around the world (Urumarudappa *et al*., 2019). According to WHO (2020), 80% of the world's population solely depend on these traditional herbal mixtures, made out of medicinal plants. In Africa, people make use of herbal medicines to fight ailments, including headaches, which is the most frequent health condition in both children and adults (Frimpong *et al*., 2021).

During the high times of the COVID-19 pandemic, several studies suggested the use

of herbal medicines as a preventive and curative option against the coronavirus. The findings of these studies were not clinically proven by the world health organization (Khadka *et al.,* 2021). But populous nations such as China and India have integrated such approaches in combination with modern western medicines to boost the immunity power of COVID-19 patients (Khadka *et al*., 2021).

However, the WHO (2020) stated that despite the plethora of studies on medicinal plant research in developing countries from Africa and Asia, there is still little supportive scientific data about folk medicines (Okaiyeto and Oguntibeju, 2021). On the other hand, among certain populations around the world, there has been some widespread belief that herbal products are harmless to human health as they are derived from natural plants (Sánchez *et al*., 2020). In Nigeria, herbal medicines are commonly used to treat so many illnesses and health conditions (Oreagba *et al*., 2011).

However, herbal medicines are prone to microbial contamination, especially bacterial and fungal. Because, most of the time, these products are unscientifically harvested, processed, transported and stored in non-hygienic circumstances with congenital climatic conditions renders the raw medicinal plant's material prone to infestation and exposed them to many microbial contaminations (Kayombo *et al.,* 2013).

The conventional procedures used to manufacture a safe, effective and quality medicine include microbial free, specific dosages etc. Herbal mixtures used as traditional medicines are indistinct heterogeneous liquid compounds. In addition to that, consumers of these mixtures may not be aware of the conditions in which they have been processed or stored. Thus, whether, the environment was microbial free or not, is uncertain as the processing, storage and distribution chain may not have been processed according to microbiological standards, therefore prone to microbial contamination.

Microbial contamination remains till this day one of the biggest issues related to the consumption of herbal mixtures as traditional medicines. Hence, it is crucial to determine the microbial profiling of herbal mixtures sold in the Wuse market, Abuja; in order to assess their safety, efficacy and quality for human consumption.

MATERIALS AND METHODS

Study Area: The herbal mixtures used in this study were purchased from vendors within Abuja metropolis. Abuja is the capital city of the Federal Republic of Nigeria. The Federal Capital Territory commonly called FCT is located between latitude 8.25 and 9.20 north of the equator with longitude 6.45 and 7.39 east of the Greenwich Meridian. Abuja is situated in the center of Nigeria with a landmass of approximately 7,315 square kilometer and latest known current metro area population of about 3,652,000, a 5.43% increase in 2022. The laboratory work was performed in the Microbiology laboratory, Faculty of Natural and Applied Sciences, Nile University of Nigeria, Abuja.

Sample Collection: A total number of ten different herbal mixtures were purchased from different part of Abuja metropolis. The samples were stored in the refrigerator at 4ºC before the analysis. They were analysed in Microbiology laboratory, Nile University of Nigeria, Abuja.

Preparation of Media: Growth media used for the work were Nutrient Agar (NA), MacConkey Agar (MCA), and Potato Dextrose Agar (PDA) for the isolation of bacteria and fungi respectively. In addition, peptone water was also made. The culture media were prepared in accordance with the manufacturer's instructions, and poured into sterile petri plates.

Inoculation of the Media for Microbial Isolation:

The membrane filtration method was used to filter each herbal mixture, as described by Mulamattathil *et al.* (2014). The filtered herbal products were thoroughly mixed and inoculated into the prepared culture plates, inoculating wire loop (De Sousa-lima *et al*.,

2020). The inoculation was done in the presence of the Bunsen flame, for aseptic environment. However, 2 ml of each mixture sample were added to 5 ml of peptone water, then incubated at 37ºC overnight. The peptone water culture was later sub-cultured onto the NA and PDA media plates. Then the plates were incubated at 37ºC for 24 h and at 25ºC for 7 days for bacteria and fungi culture, respectively. The growth pattern, pigmentation and size of the obtained microbial colonies were recorded at the end of the incubation, which were later used for the morphological identification of the susceptible organisms (De Sousa-lima *et al*., 2020).

Morphological Identification of Bacterial and Fungal Colonies: This was carried out using light microscope, following standard laboratory techniques as described by Garcha *et al.* (2016). Thus, visible colonies from the sub-cultured plates were smeared on microscope slide then viewed. The bacterial and fungal isolates were morphological identified with regards to the growth pattern, shape, pigmentation, elevation, margin, arrangement of cells, size of colonies and opacity.

Colony Count: This was done using the colony counter machine (SC6+). However, the number of colonies on each plate was counted and recorded. The countable plate was between 23 and 100 colonies.

Lactophenol Cotton Blue (LPCB) Stain of Fungal Colonies: The LPCB was made of phenol which acts as a disinfectant by killing undesired microorganisms; lactic acid which preserves the structures of the fungal cells; and the cotton blue which gives the blue colour to the chitin, hyphae and sporangia.

Biochemical Test for Identification of Bacterial Colonies: The biochemical tests including Gram staining, methyl-red, indole test, coagulase test, catalase, citrate, and Voges Proskauer test were performed to confirm the identified microorganisms (Kumadoh *et al.*, 2022).

Molecular Confirmation of Isolates: Molecular diagnosis is playing an increasingly important role in the rapid detection and identification of pathogenic organisms in clinical samples. The genetic variation of ribosomal genes in bacteria offers an alternative to culturing for the detection and identification of these organisms.

Amplification of Bacterial DNA by PCR: The DNA fragment is terminally labeled with fluorescence-labeled nucleotides by PC by adding 18 µL of nuclease free water to the negative control and 16uL of nuclease free water to all the samples in a hot start premix. Afterwards 2 µL of primer (1uL forward primer and l µL reverse primer) was added to all the samples and including the negative control and 2 µL of DNA extract was added only to all the samples and then run in a PCR machine (Bioneer, 2018).

Gel Electrophoresis of Bacterial DNA: The amplified DNA products extracted from water samples were analyzed with electrophoresis on 1.2% agarose w/ gels stained with Ethidium bromide stain. Five microliters (5 µL) of PCR product were loaded on to agarose gel. A 100bp DNA ladder was used as a marker for PR product difference of electric potential of 110 V was applied to each gel and thereafter visualized by UV illumination. The PCR products positive for each of the 4 genes were further run through gel extraction kit to obtain the elute of the positive bands. These elutes were sent to a DNA laboratory for sequencing (Pei, 2012).

Sequencing of the Bacterial DNA: Sequencing as a process involves the determination of a nucleotide sequence of a particular DNA fragment. This process used four types of fluorescence-labeled nucleotides, and they are dideoxynucleotides (daNTPs). ddNTPs lack a 3' OH group to which the phosphate group of the incoming nucleotide is attached. Therefore, ddNIP was added to the growing chain, there was no further addition of nucleotides at the 3' end of the chain. That means the addition of a ddNTP into the growing chain terminated the chain growth. Since ddNTPs were added to the PC mixture in low concentrations, each growing chain was terminated at

different level. The emitting fluorescence was detected to determine the nucleotide sequence of the DNA fragment at the end of the PCR (Chauhan, 2021).

Statistical Analysis: The generated data were analysed using Microsoft Excel 2010, version software will be at 95% confidence interval and $\alpha = 0.05$ ($p \leq 0.05$) for performing statistical analyses (De Sousa-lima et al., 2020). Also, descriptive statistics was used to describe variable's mean and standard deviation and generated DNA sequences were analysed in the NCBI system, following a modified method of Bhutia *et al*. (2021). Thus, the obtained DNA contigs were subjected to BLAST (Basic Local Alignment Search Tool) (Bhutia *et al.,* 2021) for nucleotide similarity search.

RESULTS

The microbes from each sample were morphologically characterized on the different growth media agar plates. Bacterial species were grown and isolated on the NA. The appearance, form, size, elevation and pigmentation of bacterial colonies were recorded. And based on these morphological traits, we recorded circular and irregular; large and punctiform; raised, dome-shaped, flat, columella and curve-tapered; entire across all plates; mucoid and soupy; off-white, red-pink, glistening, yellow and colourless as colonies form, size, elevation, margin, surface appearance and pigmentation respectively (Table 1). However, the probable bacterial isolated could be *Escherichia coli*, *Salmonella* spp, *Klebsiella* spp, *Proteus* spp, *Staphylococcus aureus* and *Pseudomonas* spp. PDA was used for the growth and isolation of fungal species. The morphological presentation of microorganism on PDA plates are optical characteristic filiform, echinulate, beaded,effuse, arborescent and rhizoid; flat, raised, curved, tapered and columella; regular and irregular; grey-green, blackish, yellow-green, reddish-yellow, white-grey; and large as the colonies surface appearance, margin, elevation, size, form and pigmentation respectively. However, the probable fungal species could be Aspergillus spp, *Aspergillus flavus*, *Fusarium* spp, and *Mucor fragilis*.

Table 1: Sample location and ID

SAMPLE ID	LOCATION
A	NAFDAC
B	NAFDAC
C	NAFDAC
D	NAFDAC
E	NAFDAC
F	Wuse market
G	Jabi market
H	Utako market
I	Mabushi market
J	Wuye market

Table 2: Colonies Morphology Isolated on Nutrient Agar

Isolate	Form	Elevation	Surface	Size	Pigmentation
A	Circular	Raised	Mucoid	Punctiform	Off-white/milkish
B	Circular	Raised	Mucoid	Large	Red-pink/milkish
C	Circular	Dome-shaped	Mucoid	Large	Glistening/milkish
F	Irregular	Flat	Mucoid	Large	Colorless
G	Circular	Raised	Soupy	Large	Yellow-whitish/milkish
J	Circular	Raised	Soupy	Punctiform	White/milkish

Table 3: Colonies Morphology Isolated on Potatoes Dextrose Agar

Isolate	Form	Elevation	Surface	Size	Pigmentation
D	Circular	Raised	Arborescent	Large	Grey-green/black-blue
E	Irregular	Raised	Filiform	Large	Yellow-green/blackish
H	Circular	Curved/tapered	Effuse	Large	Reddish/yellowish
I	Circular	Columella	Beaded	Large	White-grey

Table 4: Probable Fungal Species Isolated on Potatoes Dextrose Agar

Isolate	Pigmentation	Probable organism
D	Grey-green/black-blue	*Aspergillus* spp
E	Yellow-green/blackish	*Aspergillus flavus*
H	Reddish/yellowish	Fusarium spp
I	White-grey	*Mucor fragilis*

Table 5: Colony count result MacConkey Agar

SAMPLE ID	FIRST COUNT	SECOND COUNT	MEAN	STDEV
A	9	8.95	8.975	0.035355
B	6.4	6.8	6.6	0.282843
C	4.5	5.12	4.81	0.438406
F	3.7	3.7	3.7	0
G	6.2	6.2	6.2	0

Key: STDEV = Standard Deviation

Table 6: Biochemical test results

Samples Isolates	Gram Stain	Cat	Cog	Ind	VP	MR	Cit	Probable organism
A	-Ve (rods)	+	NA	+	_	+	_	*E. coli*
B	-Ve (rods)	+	NA	_	_	+	+	*Salmonella* spp
C	-Ve (rods)	+	NA	_	+	_	+	*Klebsiella* spp
F	-Ve (rods)	+	NA	+	_	+	_	*Proteus* spp
G	-Ve (rods)	+	+	_	+	+	+	*S. aureus*
G	+Ve (rods)	+	_	+	_	_	+	*Pseudomonas* spp

Table 7: Sequences producing significant alignments with the query sequence

Organism	Max Score	Query Cover	E-value	% Similarity	Accession Number
Pseudomonas geniculata	1358	50%	0.0	99.47%	MK342208.1
Uncultured bacterium	1358	50%	0.0	99.47%	MG994994.1
Stenotrophomonas maltophilia	1358	50%	0.0	99.47%	MK823946.1

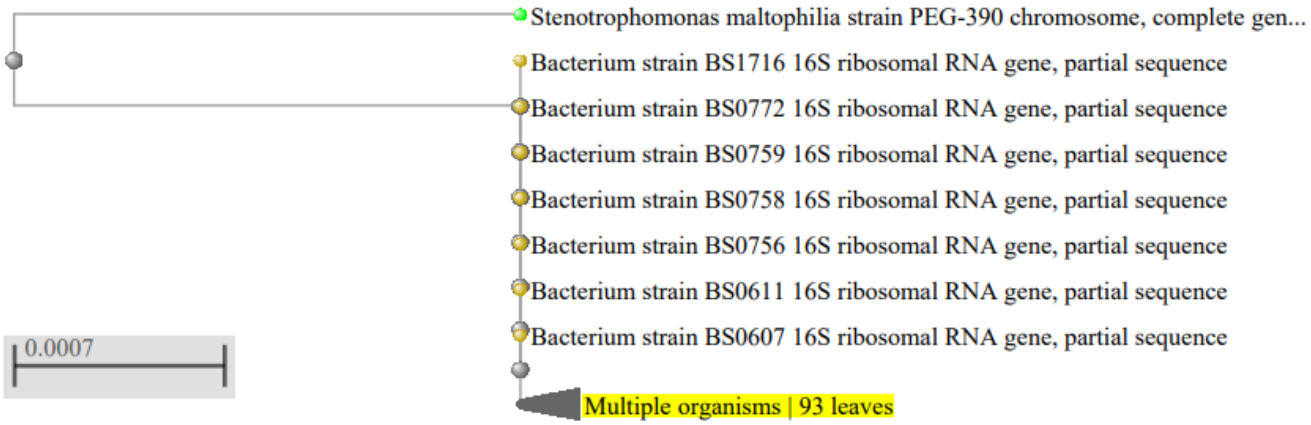

Figure 1: An illustration of the neighbor-joining phylogenetic tree of identified isolates

The colonies count carried out for samples with bacterial cells morphologies was recorded and tabular in the table. At 95% significance interval, the ANOVA single factor test yielded a p-value of 0.88, greater than alpha (0.05). However, fungal growth was observed from samples D, E, H and I.

After applying the LPCB on the fungal colonies, the microscopic view showed spreading hyphae. Sample D had dichotomous branching, septate hyphae, vesicle and conidia bearing conidiophores. And E displayed sparsely septated hyphae sporangiophores. Also, these fungalisolates exhibit dematiaceous sporangia. The sample H exhibited dematiaceous sporangia, pseudo-septate, with pale brown appearance.

Gram staining shows that 67% of the bacterial isolates were Gram negatives. Most observed shapes are bacilli and cocci which are either arranged in chain, pairs or single.Other biochemical tests such as catalase (Cat), coagulase (Cog), indole (Ind), methyl red test (MR), Voges Proskauer test (VP), Citrate utilization test (Cit), were all tests carried out. However, NA stands for Not Applicable, while -Ve and +Ve denote Gram-negative and Gram-positive respectively.

Based on the NCBI database, the nucleotide query sequences of the 16s RNA gene showed an average similarity of 99.47% to *Pseudomonas geniculate* species, uncultured bacterium and *Stenotrophomonas maltophilia*.

DISCUSSION

The findings of this study showed that majority of the herb mixtures are contaminated with microbial pathogens including bacterial and fungal species. Based on the morphological and biochemical characteristics observed from the microbial culture in this study, the possible microbial contaminants are *Aspergillus flavus, Aspergillus* spp., *E. coli, Fusarium* spp., *Salmonella* spp., *Staphylococcus aureus, Proteus* spp., *Pseudomonas* spp., *Klebsiella* spp., and *Mucor fragilis.* However, the National center for biotechnology database, showed that the nucleotide query sequence of the 16s RNA genewas 99.47% similar to *Pseudomonas geniculate* species (Gram positive), uncultured bacterium and *Stenotrophomonas maltophilia* (Gram negative). The high p-value of 0.88 >α (0.05), is an indication that there is a significant difference between the colony count for bacterial species present in the different herbal mixture, could be possibly due to the difference of dose or concentration, our findings agree with the report of Lawrence (2016).

Thus, the Gram-negative bacteria had the highest colony count of about 9.0 x 10^7CFU/plate, compared to Gram-positive organisms with an average colony count of 3.7 x 10^7CFU/plate. This result is similar to the findings of Walther *et al.* (2016) who observed faecal coliforms including *Klebsiella* spp and *Enterobacter* spp. being the predominant contaminants of traditional liquid herbal medicinal products sold in Abuja. However, no pathogenic bacteria such as Salmonella spp and Shigella spp were isolated in their study; which isn't the case in our present study. While fungal contaminants isolated include *Aspergillus* spp, *Aspergillus flavus, Fusarium* spp, and *Mucor fragilis.* The fungal contaminants found in the herbal mixtures in the current study primarily could be attributed to the fungal spores found in the air and soil. And such contaminated herbal products can cause fungal infections and other health complications due to mycotoxins accumulation from toxin-producing fungi such as *Aspergillus* spp.; especially *A. parasiticus* and *A. flavus* (Keter *et al.*, 2017). Cengiz *et al.* (2020), stated that the accepted range for countable coliform bacteria and fungal colonies on a standard nutrient agar plate in the food industry is between 25-250 CFU/plate. The acceptable range for *Aspergillus niger* is between 8-80 CFU/plate.

It can be argued that contaminations of botanical preparations chiefly happen during

a slow drying process because of inadequate drying or during postharvest storage if relative humidity is high or if temperatures are favorable. However, the contamination of these herbal products by the pathogenic microbes can be associated with many factors including soil contamination and environmental pollution and soil (Turkson *et al*., 2020).

CONCLUSION

The herbal preparations were contaminated with 40% and 50% fungal and bacterial pathogens, respectively. While the remaining 10% had no microorganism. The NCBI database, showed that the nucleotide query sequence of the 16s RNA gene was 99.47% similar to *Pseudomonas geniculate* species (Gram positive), uncultured bacterium and *Stenotrophomonas maltophilia* species (Gram negative). Of great concern was the isolation of *Aspergillus flavus,* a highly pathogenic spore forming fungus and others *Aspergillus* spp.

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