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Candida albicans in brain, photo by Microbe World

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Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Short Communication: Assessment of tetracycline residue in local milk consumed in Yola, Adamawa State, Nigeria

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Manuscript received: 14 March 2023. Revision accepted: 14 July 2023.

Abstract. *Bello MI, Lamiya A, Hayatu IA, Zailani HA, Ja'afar JN, Raji ML, Abdullahi M. 2023. Short Communication: Assessment of tetracycline residue in local milk consumed in Yola, Adamawa State, Nigeria. Asian J Trop Biotechnol 20: 45-49.* Antimicrobial residues in food beyond the tolerance limit for human consumption will harm health, such as toxicity, allergy, teeth discoloration, teratogenicity, and, most importantly, antimicrobial resistance. Knowing the prevailing tetracycline usage pattern in cattle husbandry and the lack of information on tetracycline residue in the locally consumed milk in Yola Adamawa State, Nigeria, therefore, this study was conducted to assess the tetracycline residue in the region. One hundred and twenty-two local milk samples were collected through simple random sampling in the study area from local retailers. First, the samples were screened for general antimicrobial residue using the microbiological method. Antimicrobial-positive samples were then subjected to HPLC analysis to identify and quantify possible tetracycline residue. The results showed that 23% (28/122) were positive for antimicrobial residue, of which 68% (19/28) were identified as tetracycline. The overall tetracycline residue prevalence was 15.6% (19/122) with a mean concentration of 432.64 µg/L with the lowest and highest concentration of 52.91 µg/L and 1,597.29 µg/L, respectively. The proportion of tetracycline positive to negative samples differed significantly ($P<0.05$), occurring with probabilities of 0.16 and 0.84, respectively. This study revealed tetracycline at a low prevalence, but in most cases, 89.47% (17/19) at dangerous levels. Thus, there is a need to review the present antimicrobial regulatory mechanisms.

Keywords: Antibiotic, health hazard, prevalence, residue, tetracycline

INTRODUCTION

Milk is a nutritious food of animal origin that provides almost all the nutritional requirements of humans and animals (Kurjogi et al. 2019). It has been reported that Nigeria is the largest milk producer in West Africa and can potentially be the largest milk producer in Africa (Olatoye et al. 2016). In Nigeria, as in other African countries, most cattle husbandry is handled by the local trans-nomadic or semi-nomadic herdsmen with poor access to professional veterinary services (Olatoye et al. 2016). Therefore, promoting the indiscriminate use of antibiotics for therapeutic and prophylactic purposes (Olatoye and Ogundipe 2013; Alhaji et al. 2019) has led to the appearance of antimicrobial residue as either parent drug or metabolite in foods of animal origin (Oluwafemi et al. 2018). Annual Antimicrobial Usage (AMU) in food animals was estimated at 63,000 tons globally in 2015 and is projected to increase by about 67% by 2030 (Van Boeckel et al. 2015). Global top consumers of antimicrobials include China, the United States, and Brazil. However, a more than 200% relative increase has been projected in developing countries, with Myanmar, Indonesia, and Nigeria taking the lead (Alhaji et al. 2019). The antimicrobial residue in food has been implicative in toxicity (carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, and bone marrow toxicity) and humans immunological responses (allergy) (Kabrite et al. 2019). Other implications include gut flora modification and increasing antimicrobial resistance (Olatoye and Ehinmowo 2010; Samandoulougou et al. 2015). These are health hazards to humans and animals. Milk sales from animals treated with antibiotics in developed countries have been banned until the withdrawal period is appropriately observed (Oluwafemi et al. 2018). Therefore, to ensure food safety, global regulatory authorities such as World Health Organization (1999) and the Food and Agricultural Organization (2008) have set Maximum Residue Limits (MRLs) and acceptable daily intake for several veterinary drugs in foods. The Food and Agricultural Organization, the World Health Organization (FAO/WHO), and the European Union (EU) have recommended a Maximum Residue Limit (MRL) of 100 µg/kg for tetracycline, oxytetracycline and chlortetracycline (singly or in combination) in milk.

Meanwhile, the US Food and Drug Administration (FDA) has set an upper legal level of 300 µg/kg for the combined residues of tetracycline, oxytetracycline, and chlortetracycline. The Joint Expert Committee on Food Additives has recommended an Acceptable Daily Intake (ADI) for tetracyclines residues at a concentration of 0-30 µg/kgBW/day (Aalipour et al. 2015). Studies revealed that tetracycline is one of the most commonly used antimicrobials in cattle husbandry in Northern Nigeria, and it persists in the body over a long period after administration (Kurjogi et al. 2019). Hence, there is the possibility that tetracycline's continuous passage through milk in lactating cows. Consumption of tetracycline-contaminated milk has been reported to have implications in primary and permanent teeth discoloration and pigmentation among infants and children under twelve due to short or long-term consumption (Alanazi et al. 2021). In addition, it has also been reported that tetracycline contamination poses the risk of teratogenicity when administered in the first trimester of pregnancy (Aalipour et al. 2015). Liu et al. (2017) also mentioned that consumption of tetracycline, even at a low concentration over a long period, can lead to the development of antibiotic resistance. Due to the limited information about residual antimicrobial levels in local milk consumed around Northern Nigeria, this work aimed to assess the tetracycline residue in local milk consumed in Yola Adamawa State, Nigeria.

MATERIALS AND METHODS

Study area

Yola, Northeastern Nigeria, is located on the Benue River at the latitude of 9°12'30.20" N and longitude of 12°28'53.26" E.

Sampling method and sample collection

Milk samples were collected from local milk retailers in Yola. One hundred and twenty-two (122) local milk samples were collected through simple random sampling in separate, sterile, well-labeled plastic containers. Samples were placed in an ice-cold box and transported to Chevron Biotechnology Center, Modibbo Adama University Yola, Nigeria, for analysis. The samples were stored at 4°C before being analyzed.

Screening test

Milk samples were screened for antimicrobial residue using the microbiological method described by Kurjogi et al. (2019) with some modifications (wells were used instead of sterile paper discs). *Bacillus subtilis* culture was grown in nutrient broth (Lifesave Biotech, USA) at 37°C for 16 hours. After overnight incubation, the culture was adjusted to 0.5 McFarland standard and inoculated on nutrient agar (Lifesave Biotech, USA) plates. Wells were bored on the Petri dish's surface using sterile micropipette tips. Then, 200 µL of the milk samples were dispensed into the wells and incubated at 37°C overnight. Zones of bacterial growth inhibition were measured in mm (Figure 1). In addition, a zone of inhibition with an annular diameter ≥ 1 mm was recorded as positive, and the results are presented in Table 1.

High-Performance Liquid Chromatography (HPLC) assay

The positive samples in the microbiological test were further analyzed using HPLC to identify and quantify tetracycline residue.

Preparation of standard solution

A standard solution was prepared as described by Marinou et al. (2019) as follows: 10 µg/mL stock solution was prepared by dissolving 100 µg of tetracycline analytical grade in 10mL methanol and refrigerated at 4°C. Working methanolic standards were subsequently prepared within the 0.08 -10 µg/mL concentration range.

Table 1. Antimicrobial residue detected in milk sample

Total sample size	Number/percentage of antimicrobial positive samples (Zone of inhibition with annular diameter ≥ 1mm)	Number/percentage of antimicrobial negative samples (Zone of inhibition with annular diameter < 1mm)
122	28/122 (23%)	94/122 (77%)

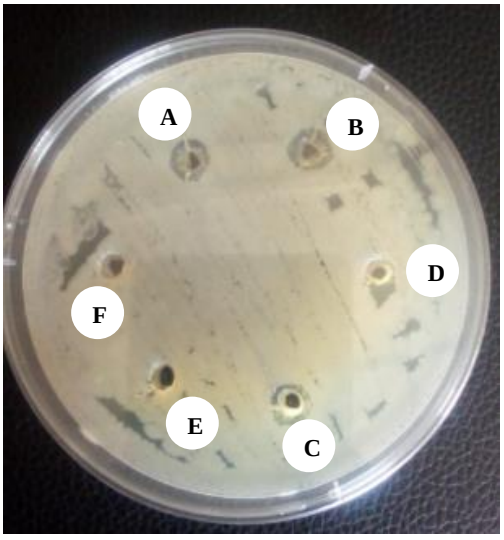

Figure 1. Antimicrobial assay for positive and negative milk samples based on inhibition zone. A, B, and C are positive antimicrobials; D, E, and F are samples with negative antimicrobials

Sample preparation

The sample preparation technique employed in this study is the optimized Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) technique as described in Marinou et al. (2019) work. According to the optimized protocol: an aliquot of 125 mg of QuEChERS material (25 mg of primary and secondary amines, 25mg of C18EC, and 75 mg of magnesium sulfate) was placed in a falcon tube with 1 mL methanol, 1 mL $C_2H_2O_4$ 0.01M and 0.5 g milk sample (stored at 4°C in the fridge). The sample was then vortexed for 30s and centrifuged at 3,500 rpm for 10 min. The supernatant was evaporated to dryness in a water bath at 40°C under a light stream of nitrogen. The dry residue was dissolved in 500 µL ultrapure water and filtered before the HPLC analysis.

Chromatographic conditioning

An orbit 100C_4 (µm, 250×4.0 mm) analytical column at ambient temperature separated the chemical compounds. The analytes were monitored at 355nm. The elution system consisted of 0.01M $C_2H_2O_4$ – 10^{-4}M Na_2EDTA/ACN, delivered at a low rate (0.9mL/minute), according to the gradient program: 0 min. 82:18v/v, 20min 60:40v/v. The dwell volume of the system used was 1.6mL. Inlet pressure was between 215 and 230 bar, and the injection volume was 100 µL.

Data analysis

Data obtained were analyzed using SPSS version 25. Student t-test was used to determine the significant difference ($P<0.05$) between positive and negative concentrations. In contrast, one-sample chi-square and one-sample binomial tests were used to analyze the proportion of positive to negative and the probabilities of their occurrences.

RESULTS AND DISCUSSION

Antimicrobial-positive samples were reported based on their ability to produce a zone of inhibition (≥1 mm annular diameter) on the nutrient agar plates (Figure 1). 23% (28/122) of the local milk samples were positive for antimicrobial residue Table 1. Furthermore, out of the 28 samples positive for antimicrobial residue, 19 (68%) were specific for tetracycline as confirmed by High Performance Liquid Chromatography (HPLC), constituting 15.6% (19/122) of the total milk samples in the study Table 2. The overall mean tetracycline residue concentration detected was 432.64 µg/L, with the lowest and highest concentrations at 52.91 µg/L and 1,597.29 µg/L, respectively. The standard calibration curve and HPLC chromatogram report of tetracycline-positive samples are presented in Figures 2 and 3, respectively.

Even though the proportion of tetracycline residue-positive samples reported in the present study was significantly lower than that of the negative ($P<0.05$), a high proportion of 89.47% (17/19) of tetracycline-positive samples had residue levels above the tolerance limits set by Codex Alimentarius Commission of 100 µg/L in milk.

Table 2. HPLC identification of tetracycline from antimicrobial positive samples

Total number of antimicrobial positive samples	Number/percentage of tetracycline positive samples	Number/percentage of tetracycline negative samples
28	19/28(68%)	9/28 (32%)

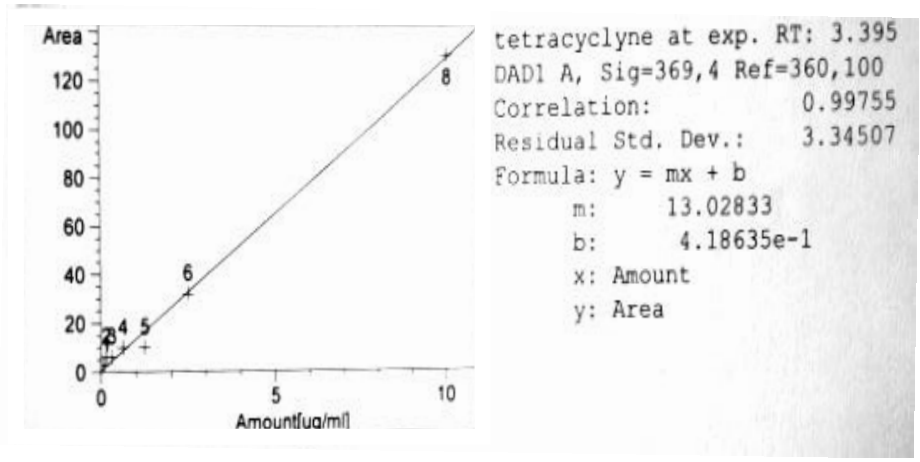

Figure 2. Standard calibration curve for tetracycline

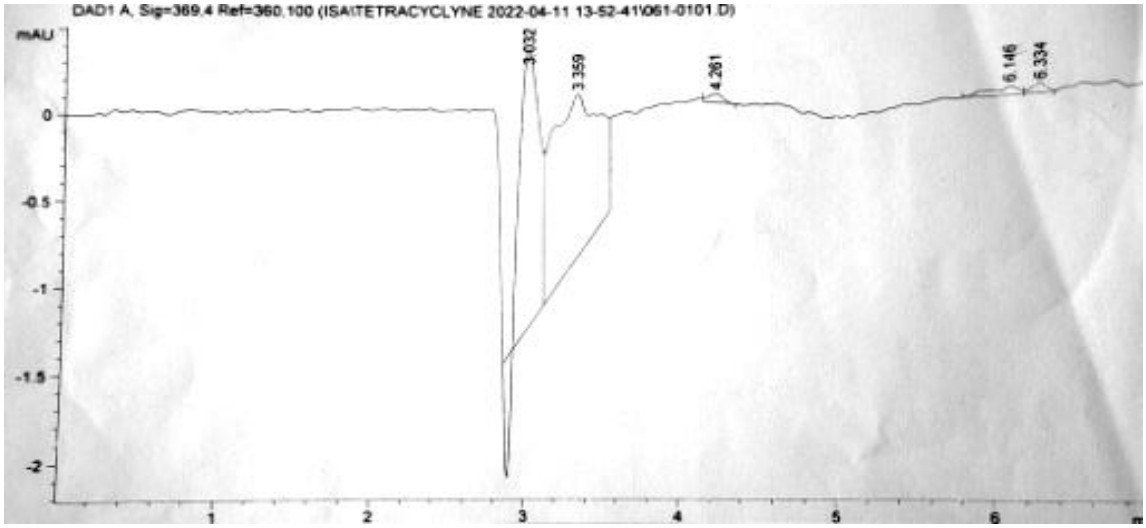

Figure 3. High-performance liquid chromatography chromatogram of tetracycline-positive milk sample. Note: TET: Tetracycline, Retention time: 3.359min

Discussion

This study revealed 23% (28/122) of local milk consumed in the study area contained antimicrobial residue. A higher prevalence of antimicrobial residue has been reported in other studies, such as a 25% prevalence reported by Yusuf et al. (2017). In similar studies in Kano City, Nigeria,40.8% and 62.3% of antimicrobial residue in raw and fermented milk were reported by Olatoye et al. (2016). Stella et al. (2020) also reported 76% and 85% antimicrobial residue prevalence in raw and fermented milk in Delta State, Nigeria. Another study reported 24% of antimicrobial residue in Bendar, Somalia (Mohamed et al. 2020). The 23% (28/122) antimicrobial residue prevalence reported in this study is higher compared to 0.69% of antibiotic residue in Croatia (Bilandzic et al. 2011) and 11-19% in Sweden (Shitandi and Sternesjo 2001). Differences in antimicrobial residue levels across reports and studies reveal differences in the degree of compliance with laws guiding antimicrobial usage by farmers and the implementation of existing laws guiding antimicrobial residue levels in food by regulatory agencies in the respective countries. Regulations such as withdrawal or withholding periods have been formulated for animal antimicrobials to protect the public from the health hazards associated with antimicrobial residues in foods of animal origin. Although there are laws against antimicrobials in food of animal origin in Nigeria, enforcement of such laws rarely occurs, especially with the prevailing traditional animal husbandry system. The findings of this study imply that consumers of local milk in the study area are exposed to a low risk of consuming one or more antimicrobial residues through the consumption of local milk. However, the results confirm non-compliance with the withdrawal periods by some local dairy farmers during the treatment of infections, as it is one of the most common ways antimicrobial residues are passed down to humans through the food chain.

Moreover, out of the 28 milk samples reported positive for antimicrobial residue in the screening test, 68% (19/28) were identified as tetracycline. That provides credence to the previous report, which mentioned tetracycline as one of Nigeria's most commonly used antibiotics, followed by penicillin, streptomycin, and sulfonamides (Alhaji et al. 2019). However, this study's overall prevalence of tetracycline was 15.6% (19/122). Additionally, the proportion of positive to negative tetracycline is significantly low ($P<0.05$), with probabilities of 0.16 and 0.84, respectively ($P<0.05$). From these findings, it can be inferred that local milk consumers in the study area are less likely to be exposed to tetracycline residue. In addition, the prevalence of residual tetracycline (15.6%) in this study is lower than that reported by Oluwafemi et al. (2018) (50%) in a similar study carried out in Abeokuta, Ogun State of Nigeria. The findings in this study were also lower than the prevalence in the Czech Republic (Navratilova et al. 2009), showing that all samples (100%) contained tetracycline, ranging from 5 to 24.47 µg/L. A study by Olatoye and Ehinmowo (2010) showed that tetracycline residue in meat samples in Akure, Nigeria had a higher prevalence of 54.44%, with the mean value of tetracycline residues in muscle, kidney, and liver were 51.8 µg/kg, 372.7 µg/kg and 1,197.7 µg/kg, respectively. Another study on seafood in Saudi Arabia by Alanazi et al. (2021) reported a 24% tetracycline prevalence, which is also above the findings of this present study. Although compliance with antimicrobial regulations in Nigeria is still low, the prevalence of tetracycline in this study is lower than in many previous reports. It can be due to antimicrobial residues other than tetracycline contaminating the food chain. Therefore, there is a reasonable level of compliance to antimicrobial regulations and responsible antimicrobial usage by most dairy farmers in the study area; there is a much higher use of traditional/alternative medicine or seasonal sample collection.

The concentration of tetracycline residue in this study ranged between 52.91 µg/L and 1,597.29 µg/L with a mean of 432.64 µg/L. This value is much higher than Navratilova et al. (2009) reported, which is between 5 and 24.47 µg/L.

It is also much higher than the tetracyclines concentration range in a previous study by Oluwafemi et al. (2018). Variations in the antibiotic levels in the present study can be attributed to the different health statuses of the dairy cattle from which the milk was collected, varying veterinary practices of the farmers, or milk processing (Oluwafemi et al. 2018). It is also interesting to report that the overall mean tetracycline residue level in the positive samples of this study was 432.64 µg/L which is much higher (four folds) than the maximum residue limits (MRLs) (100 µg/L in milk samples) recommended by the codex alimentarius commission maximum residue limits (MRLs). It suggests unprofessional administration of tetracycline in practice, which might have led to over or misuse. The presence of antimicrobial residue above MRLs in foods of animal origin is a global health hazard to human health (Stella et al. 2020).

In conclusion, this study revealed the occurrence of antimicrobial residue, including tetracycline, in the local milk consumed in the study region. That occurrence, though at a low prevalence but at dangerous levels. It could be related to the indiscriminate antimicrobial use in dairy cattle in the study area; therefore, the local milk consumers could be exposed to the potential hazard of tetracycline residue.

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Antibacterial activity on hand sanitizer spray of ethanolic leaf *Sambiloto* extract (*Andrographis paniculata*) against *Staphylococcus aureus*

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Abstract. *Aminah D, Susilowati A, Setyaningsih R. 2023. Antibacterial activity on hand sanitizer spray of ethanolic leaf Sambiloto extract (*Andrographis paniculata*) against* Staphylococcus aureus*. Asian J Trop Biotechnol 20: 50-55. Sambiloto* leaves, known as the king of bitters, contain andrographolide and flavonoid compounds that can be antibacterial. *Sambiloto* leaf extract is formulated into spray preparations as antibacterial active ingredients derived from herbal plants and is an alternative to alcohol-based antiseptics. This study aimed to determine the characteristics of a hand sanitizer spray preparation of *Sambiloto* leaf extract and its activity in inhibiting the growth of *Staphylococcus aureus* bacteria. Bitter leaf extract was added to the formulation of hand sanitizer spray with concentrations of 10, 15, and 20% with carbopol 940 base, triethanolamine, propylparaben, propylene glycol, and methylparaben. Physical evaluation of hand sanitizer spray was examined based on color, texture, aroma, pH, homogeneity, and adhesion. The inhibitory power of spray hand sanitizer was determined based on the diffusion method's diameter of the inhibition zone. The physical evaluation showed that the hand sanitizer results followed the good spray criteria. The three spray formulations can inhibit S. aureus with a weak inhibitory category (<0.05); the highest reduction in microbes was recorded in formulation 3 (59.25%). Based on ANCOVA analysis, there were significant differences between spray treatments, which were influenced by differences in covariates. The result of paired t-test analysis showed that there was a significant difference between the number of bacteria before and after spray treatment.

Keywords: Antibacterial, hand sanitizer, *Sambiloto* leaves, *Staphylococcus aureus*

INTRODUCTION

Infectious diseases are a problem in the health sector that keeps on increasing from time to time and are most common in everyday life (Hossain et al. 2021). Skin is a part of the body that is very susceptible to infection because it is the outermost layer covering the body, always in contact with the external environment, be it sunlight, climate, or chemical factors. Skin is also a medium for transmitting microbes through the hands that occur from humans to the environment or vice versa. *Staphylococcus aureus* is a bacteria that often causes skin infections (Bloom and Cadarette 2019).

The *S. aureus* is a gram-positive bacterium that is round and usually arranged in irregular clusters like grapes (Hossain et al. 2021). The *S. aureus* is a major pathogen for humans. Nearly everyone experiences some S. aureus infection, ranging from food poisoning or mild skin infections to severe, life-threatening infections (Mussard et al. 2020). The *S. aureus* grows rapidly on several types of bacteriological media under aerobic or microaerobic conditions. It actively metabolizes, ferment carbohydrates, and produce a variety of pigments from white to dark yellow. The *S. aureus*, in general, often causes skin infections characterized by redness, swelling, pain, and pus in skin wounds (Sri et al. 2018).

Preventing infectious diseases due to *S. aureus* requires the improvement of the environment and skin that makes direct contact so that it is always sterile at all times. Hand sanitizer is more effective than washing hands with soap because it is more practical and efficient, absorbs, and dries quickly (Lestari et al. 2020). Spray preparation is one of the new preparations that has the advantage that it can be applied directly to the skin to reduce the possibility of contamination or other infections. Spray preparations can last longer when applied to the skin due to the gelling agent (Estikomah et al. 2021). Hand sanitizer spray consists of two or more substances, mainly a homogeneous solute and a solvent (Lestari et al. 2020). Hand sanitizer spray products generally contain the active ingredient alcohol, which triggers microbial resistance. Furthermore, using alcohol in hand sanitizer is also less safe because alcohol is flammable and causes skin irritation with repeated use (Sahabuddin et al. 2017). Hence, an innovation in the form of a hand sanitizer spray, whose active ingredients come from herbal plants, namely *Sambiloto*, known as bitters. Natural active ingredients derived from the bitter plant replace alcohol in a spray formulation as an antibacterial agent.

Sambiloto is a medicinal plant used in traditional medicine that can thrive and is cultivated in various parts of the world, including Indonesia. *Sambiloto* is an annual plant with a 50-90 cm height and many rectangular branches (Wilyawan 2018). The main compounds in the leaves of the bitter plant are andrographolide and flavonoids, which act as antibacterial. In addition, bitter

leaves also contain saponins, alkaloids, and tannins (Islam et al. 2018).

The ethanolic extract of *Sambiloto* leaves is formulated into a spray preparation using a hand sanitizer spray formulation, which refers to research by Puspita et al. (2020). This study aimed to determine the characteristics of a hand sanitizer spray preparation of *Sambiloto* leaf extract and its activity in inhibiting the growth of *S. aureus* bacteria.

MATERIALS AND METHODS

Study area

This research was conducted from March to June 2022 at the Laboratory 3 and 4 of the Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

Instruments and materials

The equipment used in this research were: petri dishes, stir bar, test tube, volume pipette, micropipette, incubator, autoclave, dark bottle, oven, blender, rotary evaporator, bunsen lamp (spirtus lamp), analytical balance, hot plate stirrer, Erlemeyer, beakers, measuring cups, spray bottles and needles, pH strips, Biology Safety Cabinet (BSC), and colony counter.

The materials used were bitter leaf (*Andrographis paniculata* (Burm.fil.) Nees), 70% ethanol, aquadest, Mueller-Hinton Agar (MHA) media powder, carbopol 940, pure culture of *S. aureus*, filter paper, aluminum foil, and commercial hand sanitizer spray (Antis brand) as the positive control, triethanolamine, propylene glycol, propyl paraben, methyl paraben, citri oleum, cotton swab, disc paper, erythromycin, plate-count agar media powder, and Nutrient Broth media powder, and Plate Count Agar (PCA) media.

Preparation of bitter leaf (*Andrographis paniculata*) simplicia

Good quality *Sambiloto* leaves were taken as much as 3 kg. *Sambiloto* leaves were washed with running water and drained. After that, bitter leaves were chopped and dried using an oven at 50ºC for 2x24 hours and blended until smooth. The simplicia was then stored in a dark, closed container to avoid light (Brigitta et al. 2021).

Preparation of ethanolic extract from bitter leaf

The extraction method used in this study was maceration with 70% ethanol as solvent. The simplicia of bitter leaves was weighed as much as 500 grams and transferred into a beaker with a capacity of 1 L. The simplicia was then dissolved in 70% ethanol and tightly covered with aluminum foil. Soaking was carried out for 48 hours without exposure to sunlight while being stirred every 8 hours, and then the mixture was filtered and separated thrice until the color faded. The filtrate obtained was then put in a rotary evaporator at 70 rpm at a temperature of 60°C for one hour and then increased to a temperature of 70°C to evaporate the solvent and obtain a concentrated extract (Mardiana and Handayani 2016).

Antibacterial test of ethanolic extract of bitter leaf

This was a preliminary test to determine the variation in the concentration of bitter leaf's ethanol extract, formulated into a hand sanitizer spray preparation. The *S. aureus* bacteria were subcultured into MHA media using the streak method and then incubated for 24 hours in an incubator at 37°C. Two up to three colonies of *S. aureus* bacteria were taken using an ose needle and transferred into a 0.9% physiological saline solution of ±2 mL in a test tube. The suspension was vortexed so that bacterial cells were evenly distributed. The suspension turbidity was compared with a standard McFarland solution of 0.5. Furthermore, a sterile cotton swab was dipped into the suspension and then pressed against the tube wall above the surface of the suspension to remove excess fluid. The cotton swab was applied evenly to the entire surface of the MHA media, and then a well was made (5 mm) with the help of a cork borer. Each hole was filled with 50 microliters of ethanol extract of bitter leaf (extract concentrations of 5%, 10%, 15%, and 20%), DMSO as a negative control, and 15 g erythromycin as a positive control. Variations in the extract concentration were tested to determine whether it could be formulated into a hand sanitizer spray and inhibit the activity of *S. aureus* bacteria. Then, Petri plates were incubated for 24 hours at 37°C. Determination of the antibacterial activity of ethanolic extract of bitter leaf was measured by calculating the clear zone area around the well with a caliper. This test was repeated 3 times. The concentration of ethanolic extract that can inhibit the growth of *S. aureus* bacteria was used to determine the effective concentration variations in the hand sanitizer spray formulation of *Sambiloto* leaf extract.

Sambiloto leaf extract hand sanitizer spray formulation

In this study, ethanolic extract of *Sambiloto* leaves with different concentrations of 10%, 15%, and 20% were arranged according to the method of Pushpita et al. (2020). The hand sanitizer spray formulation was made with a combination of carbopol 940 as a gelling agent, triethanolamine as a base, propylene glycol as a humectant, methylparaben, and propylparaben as a preservative, citri oleum as a flavoring, and aquadest as a solvent (Table 1).

Table 1. Formulation of hand spray sanitizer of *Sambiloto* leaf extract

Ingredients	Concentrations (%)		
	Formula I (FI)	Formula II (FII)	Formula III (FIII)
Bitter leaf ethanolic extract	10%	15%	20%
Carbopol 940	0.5	0.5	0.5
Triethanolamine	0.5	0.5	0.5
Propyleneglycol	10	10	10
Methyl paraben	0.18	0.18	0.18
Propyl paraben	0.02	0.02	0.02
Citri oleum	0.5	0.5	0.5
Aquadest (total volume)	100	100	100

Preparation of hand sanitizer spray preparation of *Sambiloto* leaf extract

This carbopol was dispersed in cold water, and hot water was added until the carbopol was completely dispersed; then, triethanolamine was added to form a transparent gel mass. Propylene glycol, methylparaben, propylparaben, citri oleum, and ethanol extract of bitter leaf were added to the mixture and stirred until all ingredients were mixed. The preparation was placed into a glass beaker to which 100ml of distilled water was added, stirred until homogeneous, and poured into a spray bottle.

Physical test of hand sanitizer spray

Physical properties testing of hand sanitizer spray of *Sambiloto* leaf extract included organoleptic tests, homogeneity, pH, and adhesion (Anindhita and Oktaviani 2020). An organoleptic test was conducted to observe hand sanitizer's physical appearance (color, aroma, and texture). The homogeneity of hand sanitizer spray was tested by spraying the sanitizer onto a piece of transparent glass and then observing the presence or absence of particles or compounds that were not mixed evenly. The preparation mixture was categorized as homogeneous, with no solid particles and agglomerates. The pH test of the spray preparation was carried out using a universal pH indicator; ideally, the pH value of the mixture was 4.5-7. The adhesion test was carried out on the skin by spraying it on the arm from a distance of 3 cm, and the result was observed after 10 seconds (Anindhita and Oktaviani 2020).

Antibacterial activity test of hand sanitizer spray of ethanolic extract of *Sambiloto* leaves

The *S. aureus* bacteria were subcultured on MHA media using the streak method. 2-3 colonies of *S. aureus* were taken using an ose needle and transferred into a 0.9% physiological saline solution of ±2 mL in a test tube. The suspension was vortexed so that bacterial cells were evenly distributed. The turbidity of the suspension was compared with a standard McFarland solution of 0.5. Furthermore, a sterile cotton swab was dipped into the suspension and pressed against the tube wall above to drain excess fluid. The cotton swab was applied evenly to the entire surface of the MHA media, and then a well was made with a cork borer with a size of 5 mm. Each hole was filled with 50 microliter hand sanitizer-spray preparation of *Sambiloto* leaf extract (formulation 1, formulation 2, formulation 3). The spray formulation without adding ethanol extract of *Sambiloto* leaf was the negative control, and the commercial hand sanitizer spray with Antis brand was the positive control. Then, the plates were incubated for 24 hours at 37°C. Determination of the antibacterial activity of ethanolic extract of bitter leaf was measured by calculating the clear zone area around the well with a caliper. This test was repeated 3 times. Antibacterial activity was determined by calculating the clear zone area around the well with a caliper.

Hand microbial reduction test

The test for reducing the number of microbes on the hands was carried out to determine the number of microbial colonies before and after being treated with spray hand sanitizer. Therefore, swabbing was done with 3 respondents for each hand sanitizer spray formulation. The criteria for respondents being swabbed was that they did not have open wounds or indications of irritation and other skin diseases in the area of the palms and fingers of both hands. First, respondents were asked to wash their hands with running water for 20 seconds and then rub their palms together to make the microbes on their hands uniform. Then, a sterile cotton swab was dipped into the NB medium and rubbed firmly on one finger from the distal to the proximal direction 3 times. The cotton swab was then streaked evenly on Plate Count Agar media on a petri dish (Radji et al. 2007; Nurwaini and Saputri 2018; Situmeang and Sembiring 2019).

Afterward, each formulation of the hand sanitizer spray and positive control was sprayed evenly on the fingers. Hand sanitizer spray Formula I was sprayed on the middle finger, Formula II on the ring finger, and Formula III on the little finger. Swabbing with cotton buds was done again on each finger after hand sanitizer spray dried. Next, the cotton swab was scratched evenly on the PCA media, and all petri dishes were incubated at 37°C for 24 hours. Plate Count Agar (PCA) is a bacteriological medium used to determine the total number of viable aerobic bacteria in a sample. These general-purpose media are routine culture media that microbiology laboratories use to cultivate a broad spectrum of microorganisms. The number of bacteria is expressed per ml (CFU/mL) in liquid samples. In addition, microbial cultures from swabbing hands before and after each treatment that grew on petri dishes were counted using a colony counter, and each calculated microbial number was recorded.

RESULTS AND DISCUSSION

Antibacterial activity of ethanolic leaf extract of *Sambiloto* against *staphylococcus aureus*

The ethanolic extract of *Sambiloto* leaves obtained from the maceration process was 36.4 grams from 165 grams of simplicia *Sambiloto* leaves. The resulting extract was dark green, with an extract yield of 22%. The inhibitory power of ethanolic extract of *Sambiloto* leaves against *S. aureus* can be observed in Table 2. The antibacterial activity of ethanolic extract of *Sambiloto* leaves against *S. aureus* showed the extract's average diameter of inhibition zone was weak at 5%, 10%, 15%, and 20% concentrations.

All variations of the concentration of bitter leaf extract can inhibit the growth of *S. aureus* bacteria in the category of weak inhibition. The 5% concentration extract had the lowest inhibitory power of 12.51 mm, and the 20% concentration extract had the highest inhibition of 15.90 mm. Furthermore, it can be observed that the higher the concentration of the extract, the greater the antibacterial inhibition produced. The ethanolic extract of *Sambiloto* leaves with higher concentrations had a better inhibitory ability against the growth of *S. aureus* bacteria due to the high content of extract active compounds.

Physical characteristics of spray hand sanitizer ethanolic extract of *Sambiloto* leaves

The physical characteristics of spray hand sanitizer ethanolic extract of *Sambiloto* leaves at the beginning and end of storage for 4 weeks are shown in Table 3.

The preparation of spray handsanitizer ethanolic extract of *Sambiloto* leaves was observed to have good physical characteristics at the beginning and end of storage for 4 weeks. The organoleptic characteristics of the three hand-sanitizer spray formulations were as follows: ethanol extract of *Sambiloto* leaves were dark green, a liquid texture like water due to the aquadest composition in the spray, and a citrus aroma that comes from the addition of citri oleum essence. Furthermore, the homogeneity of the three formulations was homogeneous, indicated by the absence of agglomerated particles on the slide. The homogeneity of preparation indicates that the ingredients used in the dosage formula were mixed well. The homogeneity of the mixture produces a good preparation because the active substance in the preparation is dispersed with other ingredients in the formula so that the preparation contains the same amount of active ingredient (Suena et al. 2017).

The pH of all three spray formulations was 6. The pH of spray gel preparation must comply with the pH requirement range for topical application, i.e., 4.5-7; if the pH is too alkaline, it causes scaly skin, while skin infection may occur if the pH is too acidic (Suyudi 2004). The stickiness of hand sanitizer spray preparation of ethanol extract of *Sambiloto* leaves can be seen in Table 3. The three formulations were attached to the skin for more than 10 seconds after being sprayed on the skin of the upper arm. The ability to adhere to the skin shows that spray preparation can maintain the active substance on the skin to increase its effectiveness. Based on the results of the physical quality test of the preparation, the hand sanitizer spray formula of *Sambiloto* leaf extract can be used as an alternative hand sanitizer.

Antibacterial activity of spray hand sanitizer ethanolic extract of *Sambiloto* leaf

The antibacterial activity of hand sanitizer spray preparation of ethanolic extract of *Sambiloto* leaves against *S. aureus* is shown in Table 4 and Figure 1.

Table 2. Antibacterial activity of ethanolic extract of bitter leaf against *Staphylococcus aureus*

Treatments	Zone inhibitory diameter (mm)	Inhibitory power category
Extract 5%	12.51	Weak
Extract 10%	13.60	Weak
Extract 15%	15.65	Weak
Extract 20%	15.90	Weak
Control – (DMSO)	Not detected	Weak
Control + (Erythromycin)	32.44	Strong

Table 3. Physical characteristics of spray hand sanitizer ethanolic extract of *Sambiloto* leaves

Test parameters	Formulations		
	I (10%)	II (15%)	III (20%)
Color	Dark green	Dark green	Dark green
Smell	Orange	Orange	Orange
Texture	Liquid	Liquid	Liquid
Homogeneous	Homogenous	Homogenous	Homogenous
pH	6	6	6
Adhesion (seconds)	19.36	18.54	19.14

Table 4. Inhibitory power of spray hand sanitizer ethanolic extract of *Sambiloto* leaves against *Staphylococcus aureus*

Treatments	Zone inhibitory diameter (mm) ± SD	Inhibitory power category
Spray **Formula I**	9.01 ± 0.50^{a}	Weak
Spray **Formula II**	9.23 ± 0.86^{a}	Weak
Spray **Formula III**	11.42 ± 0.73^{b}	Weak
Negative control	0.00 ± 0.00^{c}	Weak
Commercial spray (Positif control)	7.07 ± 0.84^{d}	Weak

Note: a, b, c, d: The same letter rank in the same column shows that the mean is not significantly different between treatment groups based on Tukey HSD post hoc ($P<0.05$)

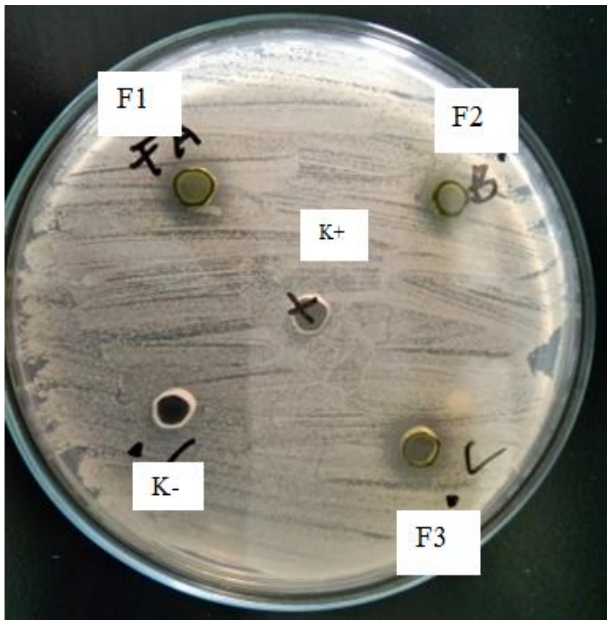

Figure 1. Inhibition zone of hand sanitizer ethanolic extract of *Sambiloto* leaves against *Staphylococcus aureus*. (K+): Positive control; (K-): Negative control; F1: Formula I; F2: Formula II; F3: Formula III

All formulations of hand sanitizer spray can inhibit the growth of *S. aureus* bacteria in vitro and have a higher inhibitory power than commercial hand sanitizer spray. Formula I with 10% ethanolic extract concentration had the lowest inhibitory diameter of 9.01 mm, and Formula III with 20% extract concentration had the highest inhibitory diameter of 11.42 mm. The mean inhibition zone diameter on *S. aureus* was increased with higher concentrations of ethanolic extracts of the three formulations. This indicates that spray hand sanitizer with a higher concentration of ethanolic extract of *Sambiloto* leaves had a better inhibitory ability against the growth of *S. aureus* bacteria. The inhibition of spray hand sanitizer treatment of *Sambiloto* leaf extract was higher than the inhibition of the commercial spray hand sanitizer treatment because the active compound of the ethanolic extract of *Sambiloto* leaves contained in the formulation was able to inhibit the growth of *S. aureus* bacteria better than commercial sprays. The commercial spray product used as a positive control contains alcohol as the active ingredient and DP 300 irgasan as an antibacterial agent. This composition could evaporate during the incubation process, reducing its antibacterial activity and resulting in weaker inhibition. Based on its inhibitory activity against *S. aureus*, this formulation of the spray hand sanitizer extract of *Sambiloto* leaves has the potential to be developed as an alternative to hand sanitizer products with natural non-alcoholic active ingredients.

The difference in the concentration of the ethanolic extract of *Sambiloto* leaves formulated into hand sanitizer spray, i.e., Formula I, Formula II, and Formula III, significantly affected the diameter of the inhibition zone of *S. aureus*. This was indicated by a significance value of 0.000 <0.05 in the one-way ANOVA statistical analysis test. Furthermore, based on Tukey HSD statistical analysis, the Formula I treatment was not significantly different from Formula II; in contrast, the Formula III treatment was significantly different from the treatment of Formula I and 2.

Reduction of microbes on hands before and after using hand sanitizer spray

The test of decreasing the number of microbial colonies after using the hand sanitizer spray formulation of ethanolic extract of *Sambiloto* leaves on 3 respondents. The data on the percentage decrease in the number of microbial colonies on the hands is shown in Table 5.

Table 5. The number of microbial colonies on hands decreases before and after being treated with spray hand sanitizer

Treatments	Percentage of decline (%)
Basic formulation of spray hand sanitizer (Negative control)	4.58
Formula I (10%)	30.23
Formula II (15%)	53.81
Formula III (20%)	59.25
Commercial hand sanitizer spray (Positive control)	77.51

The concentration of ethanolic extract of *Sambiloto* leaves in the formulation of spray hand sanitizer was directly proportional to the percentage of microbial decline. Hand sanitizer spray with Formula III (20% concentration) resulted in the highest (59.25%) reduction in the microbial colony. The less decline in microbial colonies was observed in formulas 1 and 2, i.e., 30.23% and 53.81%, respectively. The decrease in microbes showed that the active ingredient of ethanolic extract of *Sambiloto* leaves contained in the spray hand sanitizer preparation could kill bacteria on the hands. It was observed that only a few bacteria live on the hands while wiping or swabbing after using the spray hand sanitizer of ethanol extract of *Sambiloto* leaves. The percentage decrease in microbial numbers in commercial sprays was higher than the decrease in microbial numbers from the three spray formulations (not following the inhibition test results) because the spray was applied directly to the hand media in this test. The types of bacteria on the hands were very diverse, causing the spray of *Sambiloto* leaf extract to decrease their effectiveness.

Statistical analysis showed a significant difference between the spray treatments given (as evidenced by the sig value or probability of 0.011), with the covariate also having a significant difference (as evidenced by the sig value or probability of 0.008). This means that the difference in treatment between spray formulations of ethanol extract of *Sambiloto* leaves resulted in differences in the number of microbial colonies. Yet, the number of microbial colonies influenced this difference before spray treatment (covariate differences influenced differences between spray treatments). Based on the statistical analysis of the T-test, it can be observed that there was a significant difference between the number of microbial colonies before and after spray treatment (as evidenced by a significance value of 0.000 <0.05).

Spraying hand sanitizer with ethanolic extract of *Sambiloto* leaves can reduce the number of microbes on the hands because it contains active compounds. *Sambiloto* leaves contain large amounts of andrographolide and flavonoid compounds that can be antibacterial to inhibit bacterial growth (Pujiasmanto 2008). The flavonoid compounds work as antibacterial with the mechanism inhibiting nucleic acid synthesis, inhibiting cytoplasmic membrane function, and inhibiting energy metabolism of bacteria (Ansel 2008). Due to the inhibition of microbial growth on the hands, when swabbing was done, spraying hand sanitizer with ethanol extract of Sambilotto leaves on the skin surface of the hands reduced the number of germs as they died (Dewi 2013).

In conclusion, hand sanitizer of ethanolic extract of *Sambiloto* leaves showed good physical characteristics and stability. The spray hand sanitizer of ethanolic extract of *Sambiloto* leaves can inhibit the growth of *S. aureus in vitro* with weak inhibitory power. Formula III, with a 20% concentration, showed a higher inhibitory power of 11.42 mm. Formula III also resulted in a percentage reduction in the number of microbial colonies, 59.25%, but the reduction effectiveness was less than commercial spray (77.51%).

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Effect of chromated copper arsenate on protein, carbohydrate, and chlorophyll content of tropical *Eucalyptus* and *Acacia* species

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Abstract. *Kumari BMR, Nagaraja N. 2023. Effect of chromated copper arsenate on protein, carbohydrate, and chlorophyll content of tropical* Eucalyptus *and* Acacia *species. Asian J Trop Biotechnol 20: 56-61.* Contamination of heavy metals could cause morphological, physiological, and biochemical changes in plants. Chromated Copper Arsenate (CCA) is a wood preservative that contains Cr, Cu, and As. Leaching these heavy metals into agricultural fields from moderate to higher levels causes a serious threat to the ecosystem. A pot experiment was conducted to evaluate the effect of various concentrations of CCA (250-2,500 mg kg^{-1} soil) on the protein, carbohydrate, and chlorophyll content of *Eucalyptus* and *Acacia* seedlings using commercially available C-type CCA with a proportion of Cr (CrO_3- 47.5%), Cu (CuO-18.5%), and As (As_2O_5- 35%). The quantitative estimation of total carbohydrate, protein, and chlorophyll content in control and treated seedlings was carried out by spectrophotometric methods. Results showed that the high concentrations of CCA significantly ($p \leq 0.05$) reduced protein, carbohydrate, and chlorophyll content of *Eucalyptus* and *Acacia* seedlings. The lowest amount of total carbohydrates, proteins, and chlorophyll content found in *E. citriodora* Hook. and *E. tereticornis* Sm. were 0.97±0.05 and 1.70±0.17 mg g^{-1}, 0.42±0.08 and 0.45 ±0.02 mg g^{-1} and 0.11±0.04 and 0.07±0.03 mg g^{-1} at 2,500 mg kg^{-1} soil CCA respectively. Furthermore, the lowest total carbohydrates, proteins, and chlorophyll content in *A. mangium* seedlings were 1.91±0.43, 0.52±0.13, and 0.13±0.01 mg g^{-1} at 2,500 mg kg^{-1} soil CCA, respectively. The present study advocates that the higher concentrations of CCA affect the biochemical parameters of *Eucalyptus* and *Acacia* tree species.

Keywords: *Acacia*, biochemical parameters, CCA, *Eucalyptus* spp, heavy metals

INTRODUCTION

Heavy metal contamination of the environment is one of the main threats affecting fauna and flora worldwide (Briffa et al. 2020; Mitra et al. 2022). Heavy metals are non-biodegradable and remain in the soil for a long time, thus posing a long-term environmental threat (Suman et al. 2018). Chromated Copper Arsenate (CCA) is a wood preservative that protects timber from microbial decay and insect damage (see reviews by Morais et al. 2021). The CCA-treated woods are widely used as construction material, resulting in the release of its components viz Cr, Cu, and As into agricultural fields (Kumpiene et al. 2008), which raises concern about food safety (Saleem et al. 2020; Kumari 2022). Several methods have been adopted for the remediation of soils contaminated with heavy metals. Several biological approaches have proven promising remediation tools for their cost-effective and eco-friendly properties (Yan et al. 2020).

Phytoremediation effectively reduces heavy metal contamination in soil (Ashraf et al. 2019; Yan et al. 2020). However, phytoremediation efficiency is related to the nature of heavy metals found in the soil and the physiological characteristics of plants (Bhat et al. 2022). Plants for phytoremediation should produce relatively high biomass, be tolerant to metal toxicity, and have high metal absorption capacity (Kumari and Nagaraja 2023). *Eucalyptus citriodora* Hook. and *E. tereticornis* Sm. have a shallow root system and produce a high amount of biomass in short periods, even in dry conditions (Luo et al. 2016). Similarly, *Acacia mangium* Willd is a medium-sized, fast-growing tree across India. It is a potential candidate for producing high biomass and is well-adapted to poor soil conditions. Furthermore, these species can accumulate large amounts of heavy metals such as Cd, Cu, Pb, Ni, Zn, and Cr in their tissues (Motesharezadeh et al. 2017; Kumari and Nagaraja 2023). Since these plant species have better tolerance to heavy metal stress, they have the potential to be used to remediate CCA-contaminated areas (Usman et al. 2012).

Cu is an essential metal required for plants at a certain level. However, it is toxic at higher concentrations and affects various biochemical processes in plants (Marastoni et al. 2019; Rather et al. 2020). A study by Feigl et al. (2015) showed a reduction in photosynthetic pigments in Indian mustard plants in increasing concentrations of Cu (10-50 µM). Cr is considered a non-essential metal for plant growth and development. Cr contamination in agricultural soils at a concentration of 350 mg kg^{-1} causes a serious threat to the ecosystem and induces oxidative stress in plants (Ertani et al. 2017). Cr interferes with photosynthetic mechanisms in plants (Bashir et al. 2021), and its toxicity highly depends on the duration of exposure and its concentration in plants (Christou et al. 2020).

The permissible arsenic (As) level in dry fodder was 4 mg kg^{-1} (Adamse et al. 2017). Arsenic (As) causes many

morphological, physiological, and biochemical changes in plants (Abbas et al. 2018). It also affects the metabolic functions of the plant by inducing stress (Kalita et al. 2018). Limited studies have been conducted on the impact of different heavy metals on biochemical parameters. Little information is available on the effect of CCA compounds on the *Eucalyptus* and *Acacia* species (Kumari 2022). The objective of this study was to evaluate the impact of CCA on protein, carbohydrate, and chlorophyll content in the seedlings of *E. citriodora, E. tereticornis,* and *A. mangium* on various concentrations of CCA.

MATERIALS AND METHODS

Plant material and experimental design

Studies on the effect of components of chromated copper arsenate, namely Cr, Cu, and As, on biochemical parameters (protein, carbohydrate, and chlorophyll) were conducted using the seedlings of *E. citriodora* and *E. tereticornis* and *A. mangium* in Bengaluru, India (12.9716° N, 77.5946° E). Healthy seeds of *E. tereticornis* and *A. mangium* were procured from the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, India, and *E. citriodora* from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Bengaluru, India. The seeds were surface sterilized with bavistin (50% WP), followed by a double wash with deionized water. After pre-germination treatment, seeds were placed in the Petri dish (9 cm diameter) and treated with 10 ml of five different concentrations (50,100, 500, 1,000, and 5,000 mg kg^{-1} soil) of CCA. Every treatment contained 20 seeds. CCA was prepared from commercially available C-type CCA with a proportion of Cr (CrO_3- 47.5%), Cu (CuO-18.5%), and As (As_2O_5- 35%). Seeds were soaked in distilled water as a control treatment. Every treatment has four replications. The Petri dishes with different treatments were kept in a germination chamber. Seed germination rates were observed and recorded for 21 days (Kumari and Nagaraja 2023). Seeds with visible protruded radicals and plumules were considered germinated seeds.

Pot experiments

The healthy seedlings were transplanted to root trainer pots filled with sand, soil, and compost in the ratio of 2:1:2, followed by deoiled neem cake (10 kg/m^3) and single phosphate (4.5 kg/m^3) incorporated with fungicide (Indofil M-45), and pesticide (Phorate (0.25 kg/m^3 each) as a prophylactic measure. Furthermore, one-month-old seedlings (3 to 5 cm long) were transplanted in plastic pots (2,000 cc) filled with sufficient air-dried soil mixed with compost. All the pots were arranged in the greenhouse in four replications. Planting media was given at respective concentrations (250, 500, 750, 1,000, 1,250, 1,500, 2,000, and 2,500 mg kg^{-1} soil) of CCA separately based on the pilot experiments conducted on seed germination in Petri dishes. Regular management practices, including sufficient irrigation and weed control, were performed during the experiments.

Biochemical analysis (protein, carbohydrate, and chlorophyll content)

The seedlings of each species (6 months old) were harvested and washed thoroughly with deionized water. The spectrophotometric methods were used to estimate total carbohydrates, protein, and chlorophyll. The leaves from each treatment were cut into fine pieces and then ground with mortar and pestle. 0.5 g of fresh leaf powder from each treatment was homogenized with 5 µL of 80% acetone, incubated overnight at room temperature, and centrifuged at 5,000 rpm for 5 min. The supernatant was added with 80% acetone to 5 µL. The optical densities were measured at 645 and 663 nm wavelengths using a UV-VIS Spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). Total carbohydrates were analyzed using the Anthrone method (Hedge et al. 1962). Lowry's method (1951) determined the total protein of the seedlings. Total chlorophyll was estimated using Arnon's method (1949). The results of different treatments were compared with the control.

Statistical analysis

The experimental data on changes in total carbohydrates, proteins, and chlorophyll content of tree species on exposure to different CCA concentrations (250-2,500 mg kg^{-1} soil) were statistically analyzed by one-way analysis of variance (ANOVA). Biochemical parameters of CCA treatments were compared to the control and analyzed statistically for significant differences at $p \leq 0.05$ by Duncan's multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The effect of CCA on the biochemical parameters of *Eucalyptus* seedlings

Nutrient sufficiency is required for the healthy growth and development of plants. However, soil contaminated with toxic heavy metals may affect morphological and biochemical parameters. The effect of different concentrations of CCA (250-2,500 mg kg^{-1} soil) on the biochemical parameters of the seedlings of *E. citriodora* and *E. tereticornis* is presented in Table 1. The results showed a gradual decrease in total carbohydrate, protein, and chlorophyll content with increased CCA concentrations. Lower concentrations (250-500 mg kg^{-1}) of CCA had no significant effect. Total carbohydrates were significantly ($p \leq 0.05$) decreased in CCA concentrations of ≥2,000 mg kg^{-1} soil. The total carbohydrates in the seedlings of *E. citriodora* in the control treatment and treatment of 2,500 mg kg^{-1} soil were 2.71±0.15 mg g^{-1} and 0.97±0.05 mg g^{-1}, respectively. The treatment of CCA ≥750 mg kg^{-1} soil significantly ($p \leq 0.05$) reduced the protein and chlorophyll content in *E. citriodora*. The lowest and highest amounts of proteins and chlorophyll content were in the control, and the treatment of 2,500 mg CCA kg^{-1} soil were 1. 6±0.12, and 0.42±0.08 mg g^{-1} and 0.21±0.02 and 0.11±0.04 mg g^{-1}, respectively.

Table 1. Effect of Chromated Copper Arsenate (CCA) on total carbohydrate, protein and chlorophyll content of seedlings of *Eucalyptus citriodora* and *Eucalyptus teretonicornis* at six-months age

Treatments (CCA in mg kg^{-1} soil)	*E. citriodora*				*E. teretoricornis*		
	Carbohydrate (mg g^{-1})	Protein (mg g^{-1})	Chlorophyll (mg g^{-1})	Dry Weight (g)	Carbohydrate (mg g^{-1})	Protein (mg g^{-1})	Chlorophyll (mg g^{-1})
Control	2.71±0.15^{a}	1.16±0.12^{a}	0.21±0.02^{a}	10.84±1.23^{ab}	2.76±0.17^{a}	1.66±0.03^{a}	0.24±0.01^{a}
250	2.72±0.16^{a}	1.15±0.11^{a}	0.20±0.01^{a}	11.88±1.30^{a}	2.73±0.18^{ab}	1.54±0.05^{a}	0.25±0.02^{a}
500	2.62±0.13^{a}	1.05±0.11^{a}	0.19±0.03^{ab}	10.15±1.02^{bc}	2.53±0.15^{abc}	1.41±0.06^{a}	0.23±0.01^{ab}
750	2.60±0.11^{a}	0.97±0.09^{b}	0.17±0.02^{b}	10.03±1.29^{bcd}	2.48±0.13^{abc}	1.25±0.04^{ab}	0.22±0.02b
1000	2.58±0.09^{a}	0.92±0.10^{b}	0.16±0.03^{b}	9.37±0.90^{cd}	2.42±0.14^{abc}	0.99±0.05^{ab}	0.17±0.03^{c}
1250	2.39±0.10^{a}	0.87±0.08^{c}	0.15±0.02^{b}	8.74±0.81^{de}	2.39±0.16^{bc}	0.94±0.03^{ab}	0.16±0.02^{cd}
1500	2.15±0.07^{ab}	0.85±0.09^{c}	0.14±0.01^{bc}	7.96±0.78^{ef}	2.23±0.19^{cd}	0.68±0.02^{ab}	0.15±0.04^{d}
2000	1.78±0.09^{b}	0.78±0.07^{d}	0.12±0.02^{c}	7.00±0.75^{fg}	1.97±0.20^{de}	0.49±0.01^{bc}	0.10±0.01^{e}
2500	0.97±0.05^{c}	0.42±0.08^{e}	0.11±0.04^{c}	5.79±0.70^{g}	1.70±0.17^{e}	0.45±0.02^{c}	0.07±0.03^{f}

Note: Data are means ± SE of four replications in each experiment. Data with the same letter are not significantly different. Different letters indicate significant differences at p≤0.05 level according to the Duncan's multiple range test

Table 2. Effect of Chromated Copper Arsenate (CCA) on total carbohydrate, protein and chlorophyll content of seedlings of *Acacia mangium* at six months age

Treatments (CCA in mg kg^{-1} soil)	Carbohydrate (mg g^{-1})	Protein (mg g^{-1})	Chlorophyll (mg g^{-1})
Control	3.87±0.90^{a}	1.56±0.40^{a}	0.31±0.02^{a}
250	3.57±0.87^{a}	1.48±0.30^{a}	0.27±0.03^{ab}
500	3.25±0.75^{ab}	1.40±0.51^{ab}	0.27±0.02^{ab}
750	2.95±0.68^{ab}	1.21±0.62^{bc}	0.25±0.03^{ab}
1000	2.90±0.65^{bc}	1.15b±0.41^{c}	0.22±0.02^{bc}
1250	2.70±0.51^{cd}	1.03±0.32^{cd}	0.21±0.03^{c}
1500	2.57±0.49^{a}	0.78±0.34^{d}	0.20±0.02^{cd}
2000	2.30±0.36^{a}	0.72±0.22^{de}	0.15±0.01^{d}
2500	1.91±0.43^{b}	0.52±0.13^{e}	0.13±0.01^{d}

Note: Data are mean ± SE of four replications in each experiment. Data with the same letter are not significantly different. Different letters indicate significant differences at p≤0.05 level according to the Duncan's multiple range test

The amount of total carbohydrates, proteins, and chlorophyll content was significantly different in the seedlings of *E. tereticornis* treated with various concentrations of CCA (Table 1). The results showed a significant ($p \leq 0.05$) decrease in the total carbohydrates with increased CCA concentrations at ≥1,250 mg kg^{-1} soil. Total carbohydrate content in the treatment of 2,500 mg CCA kg^{-1} soil and control were 1.70±0.17 mg g^{-1} and 2.76±0.17 mg g^{-1}, respectively. Protein content in the leaf at various concentrations of CCA differed significantly. Protein content was decreased significantly ($p \leq 0.05$) in the treatment of CCA concentrations ≥2,000 mg kg^{-1} soil (0.45±0.02 mg g^{-1}), and protein content in control was 1.66±0.03 mg g^{-1}. Chlorophyll content in the leaves decreased with increasing CCA concentration compared to the control. Chlorophyll content in control and treatment of 2,500 mg CCA were 0.24±0.01 mg g^{-1} and 0.07±0.03 mg g^{-1}, respectively.

Toxicity of CCA on biochemical parameters of *Acacia* seedlings

The biochemical parameters of the seedlings of *A. mangium* treated with CCA at various concentrations (250-2,500 mg kg^{-1} soil) were presented in Table 2. The carbohydrate content of the seedlings was significantly ($p \leq 0.05$) decreased in the treatment of 2,500 mg kg^{-1} soil. The highest carbohydrate was in control (3.87±0.90 mg g^{-1} and the lowest was in the treatment of 2,500 mg CCA kg^{-1} (1.91±0.43 mg g^{-1}). It is apparent that the CCA concentrations of ≥1,000 mg kg^{-1} in soil significantly reduced the protein and chlorophyll content in the seedlings of *A. mangium*. The highest and lowest protein and chlorophyll content were in control (1.56±0.40, 0.31±0.01 mg g^{-1}) and 2,500 mg CCA kg^{-1} soil (0.52±0.13 mg g^{-1}, 0.13±0.02 mg g^{-1}).

Discussion

Heavy metals and metalloids are essential in plant development for metabolic processes and as micronutrients (Rahman and Singh 2019). Nevertheless, heavy metals that exceed the threshold are toxic to plants. Despite plants having developed several biochemical and molecular strategies to cope with heavy metal stress (Naila et al. 2019, Kapoor et al. 2019), exceeding heavy metals can impair plants' morphological, biochemical, and physiological processes (Thakur et al. 2016). This study showed that CCA consisting of Cu, Cr, and As affected the carbohydrate, protein, and chlorophyll of *Eucalyptus* and *Acacia* seedlings due to their toxicity.

In excess, Cu inhibits respiration, affects nitrogen and protein metabolism, and causes a reduction of chlorophyll contents in plants (Chen et al. 2022). Cu may also destabilize membrane integrity, decrease photosynthesis, and alter enzyme activity (Shabbir et al. 2020). Oxidative stress caused by Cr inhibits plant development and ion liberation from cells, which initiates damage to proteins and nucleic acids (Pizzino et al. 2017), thereby inhibiting their metabolic processes. Similarly, arsenic (As) is one of the most potent non-metalloid environmental contaminants that damage plant growth.

Carbohydrates play an essential role in plant growth and immunity. Results of the study showed that carbohydrate content decreased with increased CCA concentrations in the seedlings of *Eucalyptus* and *Acacia* species due to stress induced by CCA components. Exposure to heavy metals in plants produces Reactive Oxygen Species (ROS) through oxidative stress that degrades carbohydrates in the plants (Yang et al. 2023). This study's results align with the findings of Jha and Dubey (2004), who reported a decreased ratio of reduced and non-reduced sugars in shoots of As-supplemented *Oryza sativa*. A previous study by Wiszniewska et al. (2019) showed that the soluble sugar content of *Aster tripolium* was significantly decreased under Cd and Pb stress. The results of this study showed that increasing chromium concentrations decreased the carbohydrate content of the seedlings. Correspondingly, Cr also reduced the lateral roots of alfalfa and white clover (Wakeel et al. 2018). A study by Finnegan and Chen (2012) reported that arsenic (As) stress may also lead to reduced water availability by hindering carbohydrate metabolism.

Proteins are essential components of the cell that are prone to environmental stress. Consequently, any changes in these compounds indicate oxidation stress (Pant and Tripathi 2014). Increasing concentrations of CCA reduced the protein content of the seedlings. The study's results confirm the findings of Tripathi and Tripathi (1999) that reduced protein content in *Albizia lebbeck* on exposure to heavy metal. Adekunle et al. (2019) also observed a decrease in protein content of up to 33 % in *Zea mays* exposed to Cd (44 mg/kg). Ugwu and Agunwamba (2020) showed that excessive Cr inhibits the cell cycle, enzyme activity, nitrogen assimilation, and other vital metabolic processes. Arsenic (As) in high concentration decreased total protein content in *Pteris vittata* (Singh et al. 2006) and *Vigna radiata* (Ismail 2012). The reactive oxygen species (ROS) cause severe changes in protein structure, altering the proteins' functions and subsequently affecting metabolic pathways (Gan et al. 2023).

Chlorophyll is sensitive to heavy metal stress and is used as an indicator of toxicity evaluation caused by different pollutants (Gan et al. 2023). Our study showed a decrease in the chlorophyll content in the seedlings exposed to various concentrations of CCA. Reduced chlorophyll synthesis in metal-stressed plants was possibly due to imbalanced ion homeostasis (Acemi et al. 2017). Excessive amounts of Cu caused damage to the chloroplast's ultrastructure, directly changing the thylakoid membrane composition (Rahman and Singh 2019). Feigl et al. (2015) noted reduced photosynthetic pigments in young rapeseed and Indian mustard plants due to exposure to higher Cu concentrations. In addition, increasing Cr concentration may lead to the deterioration of the chlorophyll in many plants (Sharma et al. 2020).

Plants exposed to Cr stress showed depleted chlorophyll contents that might be due to the disrupted chlorophyll biosynthesis (Muhammad et al. 2021). Cr-induced toxicity decreased chlorophyll in several plant species, such as *Pistia stratiotes* (Sinha et al. 2005), *Citrus limonia*, and *Citrus reshni* (Balal et al. 2017). The decrease in chlorophyll contents under Cr toxicity could be due to the impairment of chlorophyll biosynthesis enzymes, which are compromised under Cr toxicity (Singh et al. 2020). A reduction in chlorophyll synthesis was reported on exposure to arsenic (As) in *Zea mays* (Emamverdian et al. 2015) and *Trifolium pratense* (Hasanuzzaman et al. 2017). The proline amount in plant species increases under heavy metal stress by decreasing the chlorophyll concentration (Ahmed et al. 2021).

In conclusions, The findings suggest that high concentrations of CCA ($\geq$ 1,000 mg kg^{-1} soil) reduced the total carbohydrate, protein, and chlorophyll content in *E. citriodora*, *E. tereticornis*, and *A. mangium* seedlings. Further research on the effect of CCA contamination of *Eucalyptus* and *Acacia* under long-term field conditions must be done.

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Profiling of phytochemicals and evaluation of antifungal activity of *Piliostigma* species on *Aspergillus* species causing tomato fruit rot

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Abstract. *Mahmud AA, Danladi MD, Adi BA, Abdulrahman MD, Nafi'u BS, Mohammed UA. 2023. Profiling of phytochemicals and evaluation of antifungal activity of* Piliostigma *species on* Aspergillus *species causing tomato fruit rot. Asian J Trop Biotechnol 20: 62-68.* Medicinal plants, such as *Piliostigma reticulatum* (DC.) Hochst. and *Piliostigma thonningii* (Schumach.) Milne-Redh. from the family Fabaceae are considered therapeutic due to the bioactive compounds present in their organs. These medium-sized, leguminous trees, native to the tropics and common in Nigeria, are rich in antifungal and antioxidant compounds. The study focused on the phytochemical components of these plants and evaluation of antifungal activity which was tested against *Aspergillus* fungi species isolated from rotten tomatoes. Identification and quantification of the plants' phytochemical constituents of ethanolic and aqueous extracts were conducted, assessing the antifungal activity of the extracts and determination of the extracts Minimum Fungicidal (MFC) and Inhibitory Concentrations (MIC). From the study, these extracts contained biologically active compounds, including alkaloids, flavonoids, tannins, saponins, cardiac glycosides, and anthraquinones. The inhibitory effect of the ethanolic extracts ranged between (4-42mg/mL) and (5-31mg/mL) for *Aspergillus niger* and *Aspergillus fumigatus*, respectively. While aqueous extracts was recorded (6-20mg/mL) and (7-15mg/mL). The MIC values of ethanolic extracts have (6-8mg/mL) and (7-26mg/mL), and MFC values of (28-30mg/mL) and (22-28mg/mL) against *A. niger* and *A. fumigatus*. The aqueous extracts have MIC values (32-38mg/mL) and (36-59mg/mL), MFC values ranged (50-56mg/mL) and (38-65mg/mL) against *A. niger* and *A. fumigatus*, respectively. The study has shown that the plant extracts contained antifungal properties based on bioactive compounds found in their leaves and antifungal effects demonstrated against the tested organisms. The findings suggest the use of these plant extracts, which could serve as potential bio-fungicides when explored further. The study of the bioactive compounds of these plants provides a foundation for creating natural, safer medications and innovative bio-fungicide products. Subsequent investigations on the specified plants will be based on the framework established by this study. Future research, including in vivo testing and exploration of the mechanism of action, is recommended.

Keywords: Agriculture, extraction, isolation, medicinal plants, tomato

INTRODUCTION

Medicinal plants are globally targeted in agriculture and health to limit the usage of harmful chemicals in food. The World Health Organization defines a medicinal plant as any flora containing therapeutic substances in one or more organs (Behbahani et al. 2020; Memory et al. 2021). Medicinal plants are a rich source of bioactive compounds such as alkaloids, flavonoid, tannins, and phenolic compounds. These compounds can exert various therapeutic effects, from anti-inflammatory and antimicrobial to anticancer and antioxidant properties. Approximately 80% of individuals in developing countries employ non-modern medicine otherwise traditional medicine (Ali et al. 2018; Abdulrahman et al. 2022; 2023). As the world increasingly seeks sustainable solutions, using plant extracts as natural alternatives for managing fungal diseases in agriculture has gained significant attention (Abdulrahman et al. 2019; Abdulrahman et al. 2022).

Plants extracts contain bioactive compounds with a broad spectrum of antifungal activity, targeting multiple fungal species. This versatility is advantageous as it reduces the need for specific fungicides tailored to individual pathogens, thereby simplifying disease management strategies (Da Silva et al. 2021). Furthermore, unlike synthetic chemical fungicides, plant extracts are generally considered safe for the environment. They are biodegradable and do not persist in the soil or water, minimizing the risk of long-term contamination and ecological disruption. Additionally, plant extracts often are non-toxic to beneficial organisms such as pollinators, predators, and decomposers, thus supporting the overall ecosystem (Ginovyan and Trchounian 2019).

Piliostigma species, such as *P. reticulatum* (DC.) Hochst. and *Piliostigma thonningii* (Schumach.) Milne-Redh., are examples of medicinal plants (Memory et al. 2021; Ogbiko et al. 2021). These species belong to the Sub-family; Caesalpinioideae, in the Family; Fabaceae. These medium-sized, leguminous trees grow wild in the tropics and are common in Nigeria. Despite their similar morphology, *P. thonningii* and *P. reticulatum* have distinct characteristics, such as leaf size and texture (Ogbiko et al.

2021). The local names of *P. reticulatum* and *P. thonningii* are Kargo (or Kalgo) in Hausa (Northern Nigeria), Abafe in Yoruba language (Western Nigeria) and Okpo-atu in Igbo (Eastern Nigeria). Other names include Camel food (English), Pied de Chameau, Semalier (France), and Musacanca (Portugal) (Musa et al. 2015). These plants are also known as Barkee-hi in Fulfulde by Fulani in Northern Nigeria (Musa et al. 2015). These plants' leaves and pods are used as medicine in Nigeria to treat various diseases, including cough, diarrhea, and ulcers. Plant extracts, derived from various parts, offer a promising avenue for sustainable disease management, providing effective control while minimizing negative impacts on the environment and human health.

In agriculture, plant diseases caused primarily by various fungi result in considerable economic losses and pose health risks due to the mycotoxins produced. Fungal diseases significantly threaten global Agriculture, causing substantial early yield losses. Animal pests contribute to approximately 18% of agricultural crop losses, while microbial diseases and weeds account for 16% and 34%, respectively (Pawel et al. 2020). Fungal pathogens alone are responsible for about 70-80% of crop losses. The yearly economic impact of crop losses caused by fungal pathogens has reached a staggering £200 billion (Singh and Pulikkal 2022). Traditional disease control methods such as synthetic fungicides have proven effective but often with undesirable environmental and health consequences. Continuous and intensive use of synthetic fungicides has led to the emergence of resistant fungal strains, rendering many chemical control options ineffective. Among the fungal pathogens, *Aspergillus niger* and *Aspergillus fumigatus* are particularly destructive to tomatoes. *Aspergillus*, a genus with over 200 species, is economically significant despite not being a major cause of plant diseases. Some species, such as *A. niger* and *A. flavus*, can cause diseases in susceptible plant varieties (Abdulrahman et al. 2019). These pathogens can contaminate crops at various stages, forming harmful mycotoxins (Nogueira et al. 2021). Tomatoes (*Solanum lycopersicum* L.), a significant crop worldwide and notably in Nigeria, are particularly vulnerable to fungal pathogens. These pathogens cause considerable spoilage and decrease in tomato Production, especially in comparison to temperate zones (Pawel et al. 2020).

Based on the search from the available databases such as Google Scholar, Hindawi, Pub Med, the majority of the available studies on pharmacological properties of these plants focused on antimicrobial effects of the leaves (Ogbiko et al. 2021). Hence, the study was necessary to fill the existing research gap on the antifungal effect of the plants. The present study was designed to profile some phytochemicals and evaluate antifungal activity (in vitro) of *Piliostigma* species leaves extracts on *Aspergillus* fungi, which could be a promising alternative to chemical fungicides. The successful application of the extracts can lead to a significant advancement in sustainable agriculture.

MATERIALS AND METHODS

Study area

Dutsin-Ma Local Government Area, Katsina State, Nigeria was the site where the research was conducted (Figure 1). The Local Government Area is located 60 Kilometers from Katsina Metropolis, it has an area of about 527km^2, and a population of 169,671 according to 2006 Nigerian Census. In terms of geographical coordinates, Dutsin-Ma lies between Latitude 12^o 27′ 18″ North and 7^o 29″ East (Fanen and Olalekan, 2014).

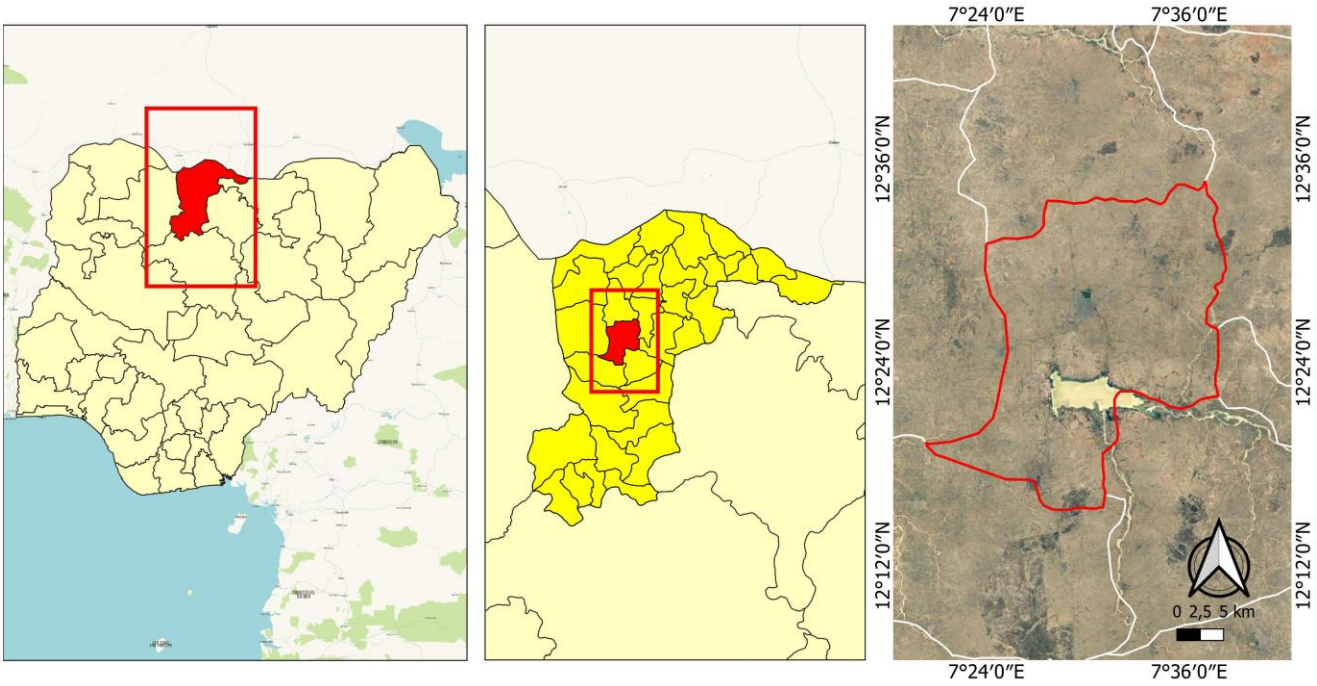

Figure 1. Dutsin-Ma local government area map, Katsina State, Nigeria

Collection of plants, identification and preparation

The *P. reticulatum* and *P. thonningii* leaves were collected from natural habitat at Dabawa Area, in Dutsin-Ma (12° 27′ 18″ N and 7° 29″ E), Katsina State, Nigeria. The leaves were authenticated and identified by Plant Scientist at Department of Plant Science and Biotechnology, Federal University Dutsin-Ma, Katsina State. Nigeria. The voucher numbers for *P. reticulatum* FUDMA/PSB/00136 and *P. thonningii* FUDMA/PSB/00140 were obtained and deposited at the herbarium unit of Plant Science and Biotechnology Department, Federal University, Dutsin-Ma, Katsina State, Nigeria. The leaves were washed with distilled water, and then, air dried for 21 days. Finally, ground into a fine uniform powder using mortar and pestle following the procedure of (Ginovyan and Trchounian 2019).

Extraction of plants' Leaves

Cold maceration technique was employed during the extraction of the plants' leaves. Two solvents, ethanol and water were opted for the extraction of *P. thonningii* and *P. reticulatum* leaves. About 100g of the leaves powder of each plant was immersed with 500 mL of each solvent separately. Each suspension was prepared in a bottle with intermittent shaking and soaked for 24 hours. All extracts were filtered separately using a Whatman No.50 filter paper, the water filtrates were heated on a hot plate at 100°C to allow the water vapor to evaporate, while the ethanol extracts were kept for a week to allow the ethanol to escape leaving behind the solid extracts. All the extracts were put to an oven at 25°C for a week, after which they were subject to phytochemical tests to identify and quantify some selected phytochemical constituents of the plants following the procedure (Oladunmoye et al. 2018). Thus, the percentage yield of the extracts was calculated:
Percentage (%) = weight of dried extract (g) ⁄weight of dried plant sample (g) ×100. For *P. reticulatum* ethanol extract= 50/100×100=50%, for *P. thonningii* ethanol extract= 45/100×100=45%, for *P. reticulatum* water extract= 30/100×100=30%, for *P. thonningii* water extract= 25/100×100=25%.

Qualitative and quantitative phytochemical tests

Plant aqueous and ethanolic extracts were tested to identify the presence of five (5) phytochemical constituents, such as flavonoids, alkaloids, saponins, tannins, and cardiac glycosides followed by the quantity identification (Afolayan et al. 2018).

Collection of tomato samples and isolation of fungi

Rotten tomatoes were purchased from Dutsin-Ma Wednesday Market in Dutsin-Ma, Katsina State, Nigeria. It was then transported to the Federal University Dutsin-Ma Biology laboratory under a cold chain. The edges of the rotting tomato samples were cut into thin sections (2mm in diameter), which were then sterilized for 2 minutes with 1% Sodium hypochlorite, immersed in 70% ethanol for an additional minute, and then rinsed in distilled water. The thin slices were placed on Petri dish with Potato Dextrose Agar (PDA) media that have been mixed with the antibiotic Gentamycin, and they were cultured for 7 days at a temperature of 28°C. Then, aseptically performing multiple colony development transfers from PDA plates resulted in the creation of a pure culture (Asma et al. 2018).

Morphological and molecular identification of the fungi

For macroscopic identification, the appearance, variation in medium color and development of the colony were noted on the Petri dish. For microscopic identification; a sterile inoculating loop was used to apply a thin smear of fungal isolates from 5-7 day old culture aseptically to a clean glass slide. The mixture was then combined with a drop of Lacto phenol cotton blue, coated with a cover slip, and examined with a ×100 magnification light microscope. The conidia, spores, and hyphae shapes were noted according to Yau et al. (2020). Molecular identification of the fungi at the species level, DNA extraction, gene amplification, and sequencing were performed (Iyanyi et al. 2021).

Determination of antifungal activity

About 10,000 µg/mL of the stock solutions of the leaves extract (both ethanolic and aqueous) of each plant was dissolved in Dimethyl Sulfoxide (DMSO) in a 1% (weight/volume) concentration, the following concentrations were prepared from the stock solutions 10, 50, and 100mg/mL, respectively (Afolayan et al. 2020). The antifungal activity of the fungi was assessed using the poisoned food method (Tasneem et al. 2021). Potato Dextrose Agar (PDA) was employed as the culture medium. Dimethyl sulfoxide (DMSO) at a concentration of 1% (weight/volume) was used to dissolve the plant extracts. Following this, 0.1mL (containing 1mg of the specific plant extract being tested) was transferred to a sterile Petri dish and allowed to harden. Each Petri dish's centre was inoculated with a 5mm block of each fungus's mycelium. The mycelium block was established by applying a corkscrew to the developing region of a 5-day-old culture of the tested fungi on a PDA medium. To maximize contact between the culture medium and the fungal mycelium, the fungi blocks were positioned at the centre of each Petri dish in an inverted posture. The fungi's mycelium growth was then gauged. This experiment was carried out thrice (Erhonyota et al. 2020; Tasneem et al. 2021).

Determination of MIC and MFC of plants' extracts

Broth dilution method determined the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). All antifungal tests were performed in Potato Dextrose Broth (PDB) mediums. Serial dilutions of plant extracts, starting from 1-60 mg/mL, were used. Tubes were inoculated with 1 mL of fungal spore suspension and incubated at a temperature 28±2°C for 72 hours. The MIC and MFC were determined according to changes in optical density following conidial germination 72 hours after inoculation using a UV-Spectrophotometer (Model AE-450, 2003, Japan). Fungal growth was indicated by turbidity and a "pellet" on the bottom of the tube. The MIC value was determined as the lowest plant extract concentration that inhibits the fungi's visible

growth. At the same time, MFC was the concentration by which the extracts inhibited the growth of the fungi at 99.9% level. The experiment was conducted in triplicate (Babatunde et al. 2023).

Statistical analysis

Variance Analysis (ANOVA) was employed to analyze the data, a General Linear Model with the MINITAB Statistical Package version 17. Means separation was done using the Tukey Kramer comparison test at ($P \leq 0.05$).

RESULTS AND DISCUSSION

High yield of ethanolic extracts

Several technical procedures have been used to extract medicinal plants. Some new methods are still being developed and existing methods are being modified (Sasidharan et al. 2011; Azwanida 2015). Choosing an appropriate extraction method is very important and in some cases depends on the intended use of the extract. The extraction method used; cold maceration, was opted because it mirrors traditional herbalist techniques. The choice of water and ethanol in the current study was based on the fact that, ethanol and water are being used in preparation of food for consumption (Abdulrahman et al. 2023). The high polarity of ethanol, one of the solvents used for the extraction, could explain its polarity when compared with aqueous extracts. The highest yield was recorded from ethanol extraction (50% and 45%) from *P. reticulatum* and *P. thonningii* ethanol extracts (Table 1). This might be attributed to high polar solvents' ability to extract more phytochemical constituents from plant materials. Ethanol's strong polarity allows it to extract more phytochemicals than water. The difference in phytochemicals identified from the plant extracts is insignificant, as only alkaloids were recorded to be absent in the aqueous extracts (Table 1). The research agrees with Abdulrahman et al. (2022) who reported a high yield of ethanol extract from the study of in vitro biological investigations on *Syzygium polyanthum* (Wight) Walp. cultivars. Similarly, the research disagrees with Namadina et al. (2020), who reported a high yield of aqueous extract against ethanol extract in the study of the phytochemical and antibacterial activities of *Vitellaria paradoxa* C.F.Gaertn. stem bark and root extracts against some clinical isolates of respiratory tract infections. According to Abdulrahman et al. (2022) and Ferreira et al. (2022), the variation in the extract yield might be due to the extraction type, the extraction solvent's nature, and the solvent's ability to extract more compounds from the extract.

Phytochemical constituents of the plant extracts

A qualitative preliminary test was carried out on some selected phytochemicals of *Piliostigma* species leaves extracts, which revealed the following phytochemicals, namely flavonoid, alkaloids, saponins, tannins, cardiac glycosides and anthraquinones except for alkaloids, that have been absent in both aqueous extracts of the plants (Table 1). On the other hand, the quantity of these phytochemicals has been reported, with flavonoid recording the highest quantity in both plants (150mg/mL and 136mg/mL), followed by Tannins, which also recorded (130mg/mL and 128mg/mL) (Table 2). The high Flavonoid content in the plants might be the reason why the plants leaves are being used to treat cough, and various transmissible diseases in Dutsin-Ma. Because it is a well-known fact that flavonoid have been known to treat various antimicrobial activities according to researchers such as, Mithilesh et al. (2013), Ruvimbo and Stanley (2017), Pratiwi et al. (2021), Aboudou et al. (2022), and Ferreira et al. (2022). In addition, tannins have also been reported to possess in vitro antimicrobial activities on various organisms (Wada et al. 2019).

Minimal inhibitory and fungal concentrations

All the test organisms responded to the extracts. The values ranged from (6-38 and 7-59 mg/mL) for *A. niger* and *A. fumigatus.* At the same time, the MFC recorded is (28-56 to 22-65mg/mL), respectively (Table 3). The lowest recorded MIC value of (6.00 mg/mL) was achieved on *A. niger*, followed by the value of (7.00 mg/mL) for *A. fumigatus.* The highest values of (56 mg/mL and 65 mg/mL) were observed on *A. niger* and *A. fumigatus*. The MIC/MFC tests demonstrated in the study has shown that, the ethanolic and aqueous extracts of *P. reticulatum* and *P. thonningii* have antifungal properties based on the *invitro* testing of various concentrations of the plant leaves 10, 50, and 100mg/mL (Table 3). The findings of the study support the conclusion of Babatunde et al. (2023) that, the antimicrobial effectiveness of plant extracts depends on concentration. Furthermore, when comparing the MIC and MFC values, a lower value suggests a strong capacity of the plant extracts to combat fungi (Babatunde et al. 2023). It is apparently clear from the study that, the MIC values were consistently lower than the MFC values, suggesting that, the extracts have antifungal properties. Distilled water was used as the control in the experiment which exhibited no inhibition against the fungi (Table 3).

Table 1. Some selected phytochemical constituents of the plants' extracts

Plants extracts	Plant phytochemicals					
	Flavonoids	Alkaloids	Saponins	Tannins	Cardiac glycosides	Anthraquinones
PREE	+	+	+	+	+	+
PTEE	+	+	+	+	+	+
PRAE	+	-	+	+	+	+
PTAE	+	-	+	+	+	+

Note: PREE: *Pilisostigma reticulatum* ethanol extract, PRAE: *Piliostigma reticulatum*aqueous extract, PTEE: *Piliostigma thonningii* ethanol extract, PTAE: *Piliostigma thonningii* aqueous extract. The +/- represent presence or otherwise absence of the phytochemical

Antifungal activity

Antifungal activity of three (3) concentrations of the plants leaves extracts (10,50, and 100 mg/mL) were evaluated against the tested fungi which was determined by measuring the diameter of mycelia growth inhibition zone of the fungi by poisoned food technique on PDA medium (Table 4). The results showed the extracts' inhibitory effect, ranged from (4-30 mm) against *A. niger* and (4-31 mm) against *A. fumigatus*. Ethanol extracts of *P. reticulatum* and *P. reticulatum* aqueous extracts were the most effective antifungal, followed by aqueous extract of *P. reticulatum* and aqueous extract of *P. thonningii.* A significant difference was recorded from the inhibition of the various extracts against the tested fungi. The study agrees with Perveen et al. (2020) on the study of green and sol-gel synthesis of ZnO nanoparticles and evaluation of antibacterial activity against various pathogens and phytochemical, evaluation of toxicity, antioxidant and antibacterial activities against active secondary metabolites of *P. thonningii* stem bark.

PCR amplification and sequencing

Genomic DNA from the fungal isolates was extracted with an Accu Prep Genomic DNA extraction kit from Bioneer. The genomic DNA of the fungal isolates shows clear bands on the gel when viewed under UV light (Figure 2). ITS1-forward (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-reverse (5'-TCCTCCGCTTATTGATATGC-3') primer pairs were used to amplify the DNA fragments through Polymerase Chain Reaction (PCR). The expected amplicon size was between 500-700bps. The band size for ITS is 580. The fungal isolates' ITS 1 and 4 gene sequences were determined to be 543 and 586 base pairs. The alignment scores of the sequences were greater than 200 base pairs, which are ideal for identifying of microorganisms. Alignment scores were represented by the red lines in each chromatogram. The isolates were blasted on the NCBI database and identified as: *Aspergillus* species.

DNA sequence produces a nucleotide base sequence of a particular organism and a chromatogram that tells how good the Sequencing is conducted. Chromatogram produces from the sequencing is a a four-color chromatogram that is usually generated from the sequence sequence that displays the sequencing run results, where a specific color indicates each nitrogenous base; a chromatogram shows how good a sequence is.

The green color represents the base Adenine, the blue color represents Cytosine, and blackcolor represents Guanine and the red color represents Thymine.

The results of the blasts showed the identity of the fungal organisms as *A. niger* and *A. fumigatus* from isolate 1 and 2. Table 5 shows the taxonomic affinities of the fungal isolates retrieved from Basic Local Alignment Search Tool (BLAST) database.

Macroscopic and microscopic identification of the organism used in the study were not entirely relied upon during the course of the study. The morphological characteristics noted cannot be successfully identified and characterized the fungal organisms up to specie level. This is why the Polymerase Chain Reaction (PCR) and Sequencing were necessary to concisely reveal the identity of the *Aspergillus* species. Molecular techniques of identification are the most suitable method to provide a complete data on any organism.

Table 2. Quantity of some selected phytochemical constituents of the plant extracts

Phytochemicals	*P. reticulatum* (mg/mL)	*P. thonningii* (mg/mL)
Flavonoid	150.00	136.00
Alkaloids	120.00	97.60
Saponins	115.80	125.00
Tannins	130.00	128.00
Cardiac glycosides	93.40	86.50
Anthraquinones	90.20	62.40

Table 3. Determination of MIC and MFC of plants' extracts in mg/mL

Plant Extracts	*A. niger*		*A. fumigatus*	
	MIC	MFC	MIC	MFC
PREE	6.00	28.00	7.00	22.00
PTEE	8.00	30.00	26.00	28.00
PRAE	38.00	50.00	59.00	65.00
PTAE	32.00	56.00	36.00	38.00
Distilled Water (Control)	0.00	0.00	0.00	0.00

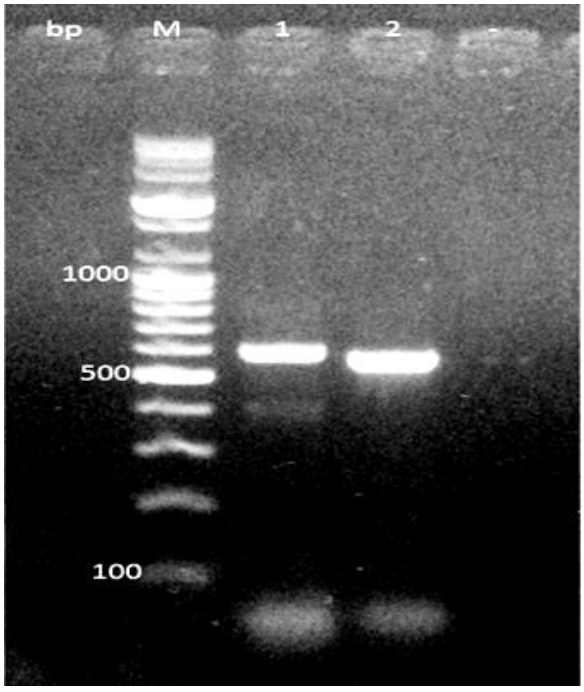

Figure 2. Agarose gel electropherogram showing PCR amplicon. Note: M; represents the DNA ladder, the numbers 1 and 2 represent the fungal isolates, 1and 2 represents *A. niger* and *A. fumigates*, respectively

Table 4. Determination of antifungal activity in mg/mL

	A. niger			*A. fumigatus*		
Concentrations(mg/mL)	**10**	**50**	**100**	**10**	**50**	**100**
Plant extracts						
P. reticulatum ethanol	$4.0.0^{a}$ ± 0.9129	20.0^{a} ±1.041	42.0^{a} ±0.6455	13.0^{a}±0.7638	23.0^{a}± 1.041	31.0^{a}±0.7638
P. thonningii ethanol	7.0^{b} ± 0.9129	16.0^{ab}±1.041	30.0^{b}±0.6455	5.0^{b}±0.7638	18.0^{b}± 1.041	21.0^{b}±0.7638
P. reticulatum aqueous	6.0^{b} ± 0.9129	12.0^{b}±1.041	20.0^{c}±0.6455	9.0^{c}±0.7638	15.0^{bc}± 1.041	14.0^{c}±0.7638
P. thonningii aqueous	12.0^{b} ± 0.9129	7.0^{c}±1.041	10.0^{d}±0.645	7.0^{c}±0.7638	12.0^{c}±1.041	10.0^{d}±0.763
Distilled Water (control)	0	0	0	0	0	0

Note: Values are means ± standard error. Means followed by different letter within a column (superscripts) are significantly different at $P \leq 0.05$

Table 5. Taxonomic affinities of the sequences retrieved from NCBI database

Specie name	**% identity**	**Accession number**
A. niger	99.63%	MH0375421
A. fumigates	97.97%	MT4974471

To conclude, the study revealed some phytochemicals in both aqueous and ethanolic leaves extracts of *P. reticulatum* and *P. thonningii* with only Alkaloids that recorded absent in all the aqueous extracts. Similarly, the antifungal activity tested *in vitro* on the fungi has shown the efficacy of the extracts against the tested fungal species. The ethanolic extracts were the most effective antifungal followed by the aqueous extracts. The ethanol was the best solvent for the extraction of the plants leaves due to the high extract recorded from the extraction. From the available data obtained from the antifungal activity of the extracts, the study suggests the use of these plants extracts as potential biofungicides when exploration of mechanism of action is investigated.

Recently, there is an absurge of deteriorating activity from the fungal species on plant produce worldwide according to Memory et al. (2021). However, the primary motivation for seeking a safer, natural alternative to synthetic chemical fungicides currently used to control fungal pathogens stems from the considerable risks the chemical fungicides pose to human health, plant life, animals, and the environment. Additionally, the development of resistance strain by fungi has rendered the available synthetic chemicals increasingly ineffective. Consequently, the quest for a safer alternative has become necessary. Plants have long been used in disease management due to the vast array of secondary metabolites they produce, making them a promising option. While the use of plant extracts for fungal disease management holds immense promise, several challenges must be addressed. Standardization of extraction methods, formulation development, and optimization of application techniques are crucial to ensure consistent efficacy and practical implementation. Furthermore, more research is needed to identify and understand the specific bioactive compounds responsible for antifungal activity in these plants. The study recommends a thorough research on the use of medicinal plants extracts for the treatment of various agricultural diseases. This needs to be explored further in our quest for a more effective and safer biochemical fungicides. The current study is a stepping stone and examined the chemical element and antifungal effect of *P. reticulatum* and *P. thonningii* against *Aspergillus* fungi. To our knowledge, there is no previous documentation in the literature regarding the specific antifungal activity of these two *Piliostigma* species in Dutsin-Ma, Katsina State Nigeria, which underscores the need for this research. Given their richness in secondary metabolites and the demonstrated antifungal activity against the tested fungi as shown from this research, these plants may serve as safer antifungal alternatives to synthetic chemical fungicides. The study recommends further research on antifungal effects on the other plant parts aside from the leaves. In addition, more in vivo studies need to be conducted in order to ascertain the plant potential as well as to develop the most effective extracts. Efforts should also be made to isolate and characterize more biologically active compounds from other parts of the plant.

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Antimicrobial potential and cytotoxicity of endophytic fungi crude extracts from *Ricinus communis* of Tanzania

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Abstract. *Shemnkande NS, Lyantagaye SL, Mpenda FN. 2023. Antimicrobial potential and cytotoxicity of endophytic fungi crude extracts from* Ricinus communis *of Tanzania. Asian J Trop Biotechnol 20: 69-78.* The study aimed to assess the antimicrobial potential and cytotoxicity of crude extracts from endophytic fungi found in *Ricinus communis* L. from Tanzania. The researchers isolated and identified fifty-one fungi species from the leaves and roots of *R. communis*. The isolates were morphologically characterized, considering color, size, shape, elevation, margin, and density parameters. The isolates were further analyzed using Sanger sequencing and bioinformatics tools to determine their phylogenetic relationships and identify the isolates. Two techniques were deployed to evaluate the antimicrobial potential: disc diffusion and microdilution. The disc diffusion method measured the inhibition zones formed by the fungi extracts against selected bacterial strains (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. In comparison, the microdilution technique determined the crude extracts' Minimum Inhibitory Concentrations (MIC) against the bacterial strains. Cytotoxicity of the crude extracts was evaluated using the brine shrimp lethality assay; Brine shrimp larvae were exposed to the extracts to evaluate their potential toxicity. The study successfully isolated 51 fungi species from *R. communis* and observed significant morphological variation. Four endophytic fungi (*Penicillium menonorum, Curvularia verruculosa, Aspergillus niger* and *Aspergillus aculeatinus*) exhibited potent antibacterial activity against *B. subtilis, E. coli, P. aeruginosa,* and *S. aureus*, with low minimum inhibitory concentrations (3.125 µg/mL to 0.098 µg/mL). Cytotoxicity tests on brine shrimp larvae indicated the crude extracts' non-toxicity, suggesting their potential as safe therapeutic agents. The findings suggest that endophytic fungi from *R. communis* possess potential therapeutic applications against drug-resistant pathogens, warranting further investigation of their bioactive compounds and broader biotechnological uses.

Keywords: Antimicrobial activity, cytotoxicity, endophytes, *Ricinus communis*, Tanzania

INTRODUCTION

The United Nations targets 3.3 to end the epidemic and neglected tropical diseases (Fitzpatrick and Engels 2016; Bangert et al. 2017; Raviglione and Maher 2017). In developing countries, the majority of deaths, around 90%, are caused by various infectious diseases such as tuberculosis, malaria, and HIV/AIDS (Gavazzi et al. 2004). For instance, Tanzania reports approximately 18% and 15% of airborne and waterborne infections annually. Current control measures for infectious diseases heavily rely on antifungal, antimalarial, and antibacterial drugs (Fair and Tor 2014). However, these available control options are compounded by the emergence and reemergence of drug-resistant infectious agents, which entails searching for alternatives like exploring the untapped potential of medicinal plants and their related endophytes (Anand et al. 2019).

Plants are valuable sources of organic compounds, most of which possess medicinal properties (Anand et al. 2019). Secondary metabolites produced by plants, including coumarins, alkaloids, essential oils, flavonoids, lectin, phenolics, terpenoids, tannins, polypeptides, and acetogenins, are utilized in the synthesis of antibiotics for the curing of infectious ailments (Gurib-Fakim 2006; Gautam and Avasthi 2019). According to Elijah et al. (2020), medicinal plants are those whose components, such as leaves, seeds, stems, roots, and fruits, are used to treat various human and animal ailments. Approximately 80% of the global population relies on herbal medicines for medical care, with people in underdeveloped countries extensively using medicinal plants (Mahady 2005; Gurib-Fakim 2006). These plants control various illnesses, including cough, diarrhea, dysentery, malaria, smallpox, syphilis, taeniasis, cholera, tuberculosis, and fever (Rakotoarivelo et al. 2015).

Although it is not widely supported by empirical evidence, communities of Tanzania have long been using *Ricinus communis* L. leaves, seeds, and roots to cure microbial infections and non-infectious illnesses. However, in other regions of the world, *R. communis* is recognized for its potential in numerous areas, including antifertility, anticancer, antioxidant, antidiabetic, antiulcer, antimicrobial, anti-asthmatic, cytotoxic, anti-inflammatory, and wound healing effects (Elijah et al. 2020). Nevertheless, the direct harnessing of medicinal plants has environmental implications, and therefore, exploring alternative sources of secondary metabolites, such as tapping into the potential of endophytes, has garnered significant attention.

Endophytes are microorganisms living in the tissues of various plant parts, such as roots, stems, seeds, and leaves,

establishing a mutualistic relationship without causing disease symptoms (Marwat and Fazal-Ur-Rehman 2017). Endophytes inhabiting medicinal plants have been observed to produce secondary metabolites with biological activities akin to those generated by the host plants (Gautam et al. 2013; Gautam 2014; Gouda et al. 2016). For instance, the endophytic fungus *Diaporthe* sp. is isolated from the medicinal plant *Cinchona ledgeriana* (Howard) Bern.Moens ex Trimen has yielded compounds such as quinine, quinidine, and cinchonine; plants own secondary metabolites (Rahmawati et al. 2021). A similar pattern emerged with the endophytic fungus *Fusarium solani* from the *Camptotheca acuminate* Decne. produces the effective antineoplastic drug camptothecin, a compound also present in the host plant (Rahmawati et al. 2021). Another notable example is the identification of podophyllotoxin, a precursor for essential anticancer medications, not only derived from the medicinal plant *Podophyllum peltatum* L. Still, it has also within the endophytic fungus *Phialocephala fortinii* isolated from the same plant (Shah et al. 2021).

Based on these facts, it is likely that endophytes living in symbiotic association with *R. communis*, including those associated with the *R. communis* used by Tanzanian communities, may produce secondary metabolites with remarkable biological applications. Furthermore, the antimicrobial properties of endophytes found in *R. communis* used by Tanzanian communities have not yet been isolated, and the characterization of these endophytes from *R. communis* is yet to be conducted. Therefore, the present study aimed to investigate the chemical profile and biological activity of endophytes from *R. communis* found in Tanzania.

MATERIALS AND METHODS

Sample collection

Samples were collected from Lushoto District in the Tanga region located at 4° 57' 54.3168" S and 38° 30' 5.7132" E and around the University of Dar es Salaam, Tanzania main campus located at S 06°78'122" and E 039° 20'574". Visually healthy-looking leaves and roots of *R. communis* were carefully collected in sterile polyethylene bags and stored. Voucher samples, required for proper identification and documentation, were promptly delivered to a botanist at the Botany Department, University of Dar es Salaam, and given a voucher number NSS 01. Following the authentication of the voucher samples, the collected plant materials were transported for further processing to the Department of Molecular Biology and Biotechnology laboratory at the University of Dar es Salaam.

Isolation and morphological characterization of endophytic fungi

Endophytic fungi were isolated following the procedures outlined in the study conducted by Mwanga et al. (2019) with minor modifications. Samples (plant parts) were subjected to sterilization steps to eliminate any external contaminants. Tap water was used to remove debris on the plant parts and soil contaminants, followed by air drying. Next, samples were sequentially immersed in 70% ethanol for 3 minutes to ensure sterility and 0.4% NaOCl for 1 minute. Samples were rinsed thrice with sterile distilled water for 1 minute and gently dried using sterile tissue paper. Then, processed plant parts were cut into small segments of approximately 1 cm in length. Subsequently, 4 segments were placed in Petri dishes containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (250 mg/mL) to prevent bacterial growth. Petri dishes were then incubated in the dark at 30°C for 4-6 days. Regular observations were made to monitor the growth of fungal colonies. Fungi isolates were purified by cutting the tips of the growing fungal hyphae using a sterile blade. Next, transfer them to sterile Petri dishes containing PDA supplemented with 250 mg/mL chloramphenicol. The petri dishes were carefully sealed with cling film and incubated at room temperature for 5 days. Pure cultures of the endophytic fungi were obtained through serial sub-culturing. These cultures were maintained in glycerol at 4°C for consequent screening and analysis.

Morphological characterization

Morphological characterization of endophytic fungi isolates involves an examination of various parameters such as mycelia types, isolate color, size, elevation, density, and margin of the isolates. These observations were made to assess the visual characteristics of the fungal isolates.

Molecular characterization

The collection of fungal mycelia was performed according to the protocol outlined by Aboul-Maaty and Oraby (2019). Fungal mycelia were transferred from Potato Dextrose Agar (PDA) to Potato Dextrose Broth (PDB) in 250 mL Erlenmeyer flasks. The flasks containing mycelia were placed on a shaker and incubated at 28°C for four days to allow growth. After incubation, approximately 100 mg of mycelia were collected by centrifugation at 12,000 rpm for five minutes, followed by genomic DNA extraction. The DNA extraction was conducted using the CTAB method, as explained by Aboul-Maaty and Oraby (2019), with minor modifications. DNA concentration and purity were determined using a Nanodrop spectrophotometer (Nanodrop One, Thermo Scientific, USA) at 260/280nm absorbance.

Amplification of rDNA Internal Transcribed Spacer (ITS) regions of the isolates was employed through the PCR method as described by Martin and Rygiewicz (2005) using primer pairs ITS1 (5’-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5’-TCCTCCGCTTATTGATATGC-3'). Next, PCR products were run on agarose gel electrophoresis, and the gel was analyzed using a gel documentation system (Shiva Scientific Company).

Bioinformatics analysis

The PCR products of fungal isolates (four isolates, Table 5) that were selected following initial screening were Sanger sequenced at Macrogen Europe (Amsterdam Medical Center), and the sequences were analyzed by

bioinformatics tools as previously described by Jeewon et al. (2013). Geneious Bioinformatics software was then used to trim and generate consensus sequences. A similarity search was done using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI). Closely related sequences were retrieved from GenBank of NCBI, and Molecular Evolution Genetic Analysis (MEGA X) software was used on multiple sequence alignment using the MUSCLE algorithm. Then, using aligned sequences, a neighbor-joining phylogenetic tree was constructed using MEGA X to depict genetic relatedness between isolates found in the present study and the previous isolates retrieved from GenBank of NCBI.

Mass cultivation and endophytic fungi crude extract harvesting

The primary screening was conducted to identify endophytic fungi with antimicrobial activity. Of the 51 isolated endophytic fungi, only 4 endophytes exhibited antimicrobial activity. The 4 fungal isolates were subsequently subjected to mass cultivation to produce metabolites. Media and mycelia for mass cultivation were prepared following the protocol outlined in the study conducted by Martin and Rygiewicz (2005). Briefly, mycelia on PDA agar were cut using a sterile blade and then placed into multiple 1,000 mL conical flasks containing 500 mL of sterile potato dextrose broth media to increase the potential of higher production of secondary metabolites. Subsequently, the flasks were incubated at room temperature with regular shaking for 4 weeks. After 4 weeks of incubation period, the cultures of endophytic fungi were filtered to eliminate the mycelia mats using cotton gauze. A solvent extraction procedure was employed to prepare crude extracts (Hajrah et al. 2018). Equal filtrate and ethyl acetate volumes were carefully measured and placed in a separating funnel. The mixture was shaken vigorously for 10 minutes and then left to settle, allowing the separation of cell masses from the solution. The aqueous solution was discarded, and a sterilized flask collected the organic solution. Any excess solvent was removed using a rotary vacuum evaporator (BUCHI Rota vapor Model R-210) at a temperature of 45°C under reduced pressure. The resulting fungal crude extracts were weighed, dissolved in Dimethyl Sulfoxide (DMSO), and stored for subsequent antimicrobial assay at 4°C.

Antimicrobial activity screening of crude extracts

With some modifications, antimicrobial activity was performed through the disc diffusion method, as explained by Mwanga et al. (2019). Inoculums of test organisms (Table 1) in normal saline were prepared from an overnight nutrient agar bacterial culture based on 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL). Six mm diameter sterile Whatman discs were soaked with 10 µL of each crude extract, and the crude extracts were at the concentration of 200 mg/mL. After soaking, the discs were allowed to dry, placed on Mueller-Hinton agar plates, and then pre-inoculated with test microorganisms (bacteria, Table 1).

Table 1. List of selected test microorganisms for antimicrobial activity screening

Microorganism name	Culture number	Gram's reaction
Staphylococcus aureus	ATCC 29213	Gram-positive
Escherichia coli	ATCC 8736	Gram-negative
Pseudomonas aeruginosa	ATCC 6539	Gram-negative
Bacillus subtilis	ATCC 6051	Gram-positive

The control experiments followed the same procedures, but gentamicin and Dimethyl Sulfoxide (DMSO) were used as positive and negative controls instead of crude extracts; an incubation was followed on plates at 37°C for 24 hours. The growth inhibition zones after incubation were measured and recorded. Notably, all the antimicrobial test experiment results were presented as the (mean ± standard deviation) and performed in duplicate.

Minimum Inhibitory Concentration (MIC) testing

Minimum Inhibitory Concentrations (MICs) were determined by the microdilution method using 96-well microtitre plates (Lema et al. 2022). Initially, the plates were pre-loaded with 50 µL of Muller-Hinton broth media in each well; next, to make a total volume of 100 µL in the first wells, followed by the addition of 50 µL of the fungal crude extracts (100 mg/mL) into the first wells of each row tested. After thoroughly mixing, 50 µL was drawn from each well of the first row and put into wells of the next row. Then, to the last wells at the bottom, the process was repeated down the columns, where 50 µL was discarded. After that, 50 µL of the bacterial and fungal suspension 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL) was added to make the final volume of 100 µL in each well. Rows containing gentamicin (50-0.024 µg/mL) were used as a positive drug, while Dimethyl Sulphoxide (DMSO) was used as a negative control. Plates were then incubated for 24 hours at 37°C. Each extract's MIC was determined by adding 30 µL of 0.05% p-Iodonitrotetrazolium (INT) chloride in each well, followed by incubation for 30 minutes bacteria; a color change in pink indicated bacterial growth. The lowest concentration, which showed no bacterial growth, was considered MIC.

Cytotoxicity assay

Brine Shrimp Lethality Assay (BSLA) was utilized to assess the presence of bioactive compounds in the extracts and evaluate their toxicity, following the protocol described by Nondo et al. (2011). Initially, 40 mg of each crude extract was accurately measured and dissolved in 1 mL of Dimethyl Sulfoxide (DMSO), resulting in a 40 mg/mL stock solution. For the brine shrimp hatching, 3.8 g of sea salt was measured and dissolved in 1 L of distilled water, creating a media solution with a concentration of 3.8 g/L. A tank was prepared, divided into two compartments by a perforated polythene wall. One part of the tank, covered by the wall, was filled with 1 L of the media solution, and 0.5 g of brine shrimp eggs were added. The left was uncovered, and the other part of the tank was illuminated with a lamp.

The tank was then left undisturbed for 36-48 hours to allow for the hatching of brine shrimp eggs.

After hatching, different concentrations of the extract were prepared. Varying volumes were drawn from the stock solution to achieve concentrations of 240, 120, 80, 40, 24, and 8 µg/mL. Each concentration was added to separate vials, with each vial containing 10 brine shrimp larvae. The volume in each vial was adjusted to 5 mL using artificial seawater prepared in 1 L of distilled water by dissolving 3.8 g of sea salt. Each concentration was tested in duplicate. The negative control consisted of vials containing brine shrimp larvae, artificial seawater, and DMSO only. The vials were then incubated under light for 24 hours. Following incubation, the number of dead larvae in each vial was counted, and the mean mortality was calculated.

Statistical analysis

Moreover, R software (4.3.0 version) was used to perform descriptive and inferential statistics. Next, the Shapiro-Wilk test was used to determine the normality of the inhibition zone and MIC data, and it found that data were not normally distributed. Therefore, the Kruskal-Wallis test was used to determine significant differences between the data. In addition, the correlation between the inhibition zone and MIC data was determined using Spearman rank correlation, with the significant difference set at $P<0.05$ levels.

RESULTS AND DISCUSSION

Abundance and morphological appearance of endophytic fungi from *R. communis*

Moreover, 51 endophytic fungi were isolated from the leaves and roots of *R. communis*. Therefore, 29 isolates were from roots, while 22 were from leaves. Figure 1 depicts the morphological appearance of 4 endophytes selected after initial screening for antimicrobial activity.

Fungi isolates were characterized based on their morphological features. There was a significant variation among the fungal isolates regarding color, size, shape, elevation, margin, and density (Table 2).

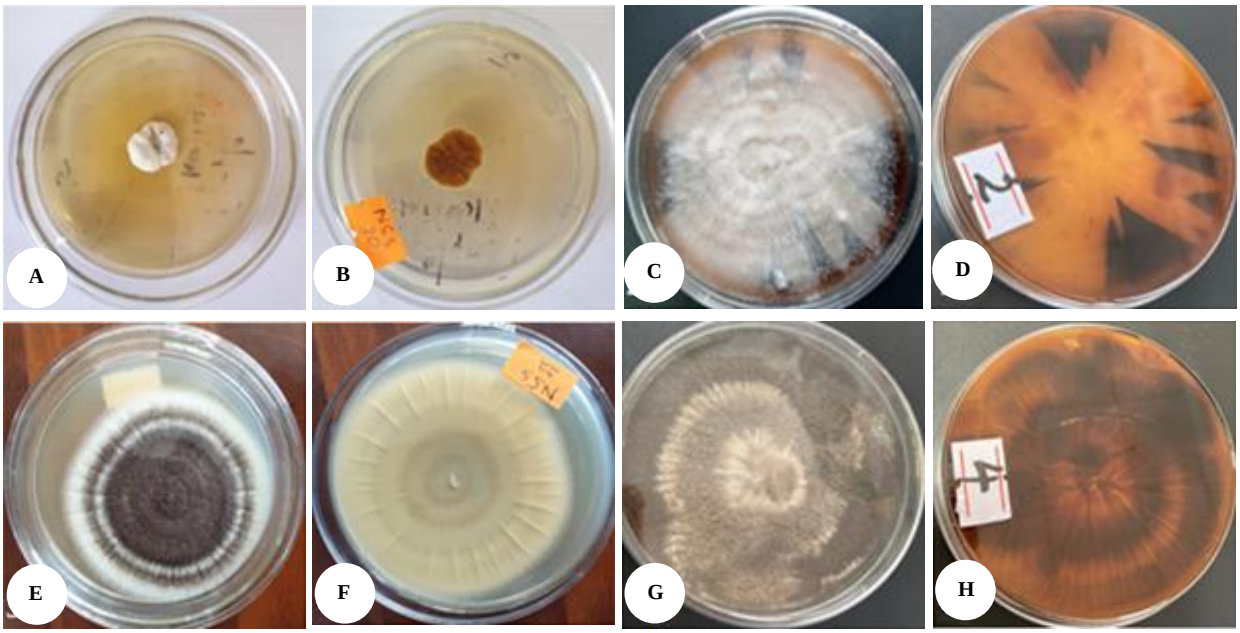

Figure 1. In the image, A and B are front and back views of *Penicillium menonorum* (MT529969.1), C and D are front and back views of *Curvularia verruculosa* (MF568070.1), E and F are front and back view of *Aspergillus niger* (LC496501.1), G and H are front and back view of *Aspergillus aculeautinus* (MT023714.1)

Table 2. Growth and morphological characteristics of endophytic fungi isolated from *Ricinus communis*

Isolate ID	Color		Size (mm)	Shape	Elevation	Margin	Density
	Front view	Back view					
1	Whitish	Brownish	23	Irregular	Raised	Undulate	Dense
2	whitish brownish	brownish blackish	50	Circular	Flat	Filamentous	Medium
3	whitish blackish	Grayish	57	Circular with margins	Flat	Filamentous	Dense
4	whitish blackish	Brownish blackish	60	Circular	Flat	Filamentous	Dense

Molecular identification of endophytic fungi from *R. communis*

The 51 isolates were confirmed to be fungal endophytes by PCR amplification of genomic DNA using ITS 1 and ITS 4 fungal-specific primers. As expected, the amplicon size was around 650 bp (Figure 2). The amplicon size ranged from 500 bp to 700 bp using ITS 1 and ITS 4 specific primers.

Furthermore, 4 isolates *Penicillium menonorum* (MT529969.1), *Curvularia verruculosa* (MF568070.1), *Aspergillus niger* (LC496501.1) and *Aspergillus aculeautinus* Noonim, Frisvad, Varga & Samson (MT023714.1) found in Figure 1, which were mass cultivated for fully antimicrobial test, were characterized by Sanger sequencing of PCR products of ITS 1 and ITS 4. Specifically, the GenBank database sequences were compared with the resulting sequences using the BLAST tool on NCBI. The analysis revealed that all the endophytic fungal sequences had similarity levels greater than 90% compared to the database sequences (Table 3). Also, the phylogenetic tree (Figure 3) displayed the molecular relatedness of fungal isolates in the present study. The four endophytes were of the genera *Penicillium, Aspergillus*, and *Curvularia.*

Antimicrobial activity of fungi crude extracts

Based on the initial screening observation, 4 out of 51 isolates were selected for mass cultivation and harvesting crude extracts, which were then tested for antimicrobial.

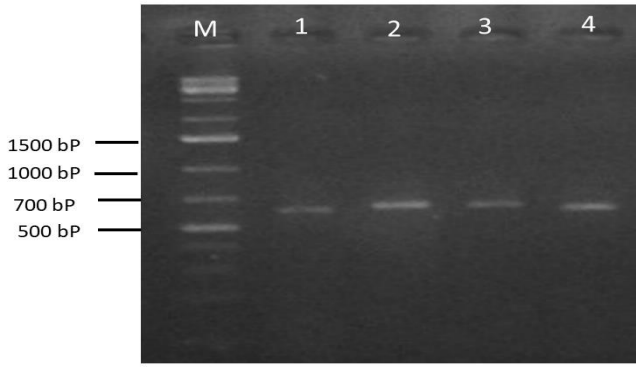

Figure 2. A gel picture of amplified DNA samples. M represents 1kb Plus DNA ladder (*New England Biolabs*), lane 1: *Pennicillum menonorum* (MT529969.1), lane 2: *Curvularia verruculosa* (MF568070.1), lane 3: *Aspergillus niger* (LC496501.1) and lane 4: *Aspergillus aculeautinus* (MT023714.1)

activity against selected organisms (Table 1) obtained in the Microbiology Laboratory. The inhibition zone was measured to provide insights into antimicrobial effectiveness against tested organisms. Interestingly, except for the crude extract of *A. niger* (LC496501.1), the overall antimicrobial activity of crude extracts was promising (Figure 3). The highest inhibition zone was 32.5 mm for the crude extract of *C. verruculosa* (MF568070.1) and *A. aculeautinus* (MT023714.1) against *B. subtilis*. In contrast, the crude extract of *A. niger* (LC496501.1) exhibited relatively weaker ($P<0.05$) antimicrobial activity against all tested organisms. For example, the lowest antimicrobial activity was 8 mm for crude extract of *A. niger* (LC496501.1) against *S. aureus*.

Table 3. Identified fungi and the percentage identity of isolated endophytic fungi from *R. communis* and that of the taxa found in the NCBI

Isolate	Name of species	Accession number	% identity
1	*Penicillium menonorum*	MT529969.1	100.00
	Penicillium menonorum	MT529566.1	99.83
	Penicillium pimiteouiense	MH045589.1	99.66
	Penicillium pimiteouiense	MH045584.1	99.66
	Penicillium pimiteouiense	MH029828.1	99.49
	Penicillium pimiteouiense	OQ874536.1	99.32
2	*Curvularia verruculosa*	MF568070.1	100.00
	Curvularia verruculosa	MH375716.1	99.45
	Curvularia verruculosa	OP412958.1	99.44
	Exserohilum rostratum	MN599601.1	99.07
	Curvularia americana	OP526931.1	99.07
	Curvularia verruculosa	OP412957.1	99.81
3	*Aspergillus niger*	LC496501.1	100.00
	Aspergillus niger	MT675916.1	97.77
	Aspergillus niger	LC496503.1	93.34
	Aspergillus niger	LC496502.1	92.73
	Aspergillus niger	MN593010.1	92.72
	Aspergillus niger	KU171053.1	97.42
4	*Aspergillus aculeatinus*	MT023714.1	100.00
	Aspergillus sp.	OQ913870.1	98.79
	Aspergillus aculeatinus	MK392046.1	98.95
	Aspergillus niger	FJ037755.1	99.64
	Aspergillus sp.	MT645654.1	98.78
	Aspergillus japonicas	OM096161.1	98.78

Note: % identity: percentage identity. Species with 100% identity are isolates from *Ricinus communis* of the present study

Table 4. Antimicrobial activities of the crude extracts (200 mg/mL) of endophytic fungi isolated from *Ricinus communis* against the pathogen of medical importance

Isolate	Test organisms			
	E. coli	***B. subtilis***	***S. aureus***	***P. aeruginosa***
Penicillium menonorum (MT529969.1)	25.0±0.71	31.5±0.71	28.0±0.41	31.5±0.71
Curvularia verruculosa (MF568070.1)	23.0±0.41	32.5±0.71	25.5±0.71	31.0±0.00
Aspergillus niger (LC496501.1)	12.0±0.41	15.0±0.00	8.0±0.41	10.0±0.00
Aspergillus aculeautinus (MT023714.1)	24.5±0.71	32.5±0.71	24.5±0.71	31.0±0.00
Positive control	27.0±0.00	25.0±0.00	26.0±0.00	25.0±0.00

Note: Positive control: Gentamicin (4 mg/mL); Numbers in the table are zones of inhibition in mm expressed as mean ± standard deviation

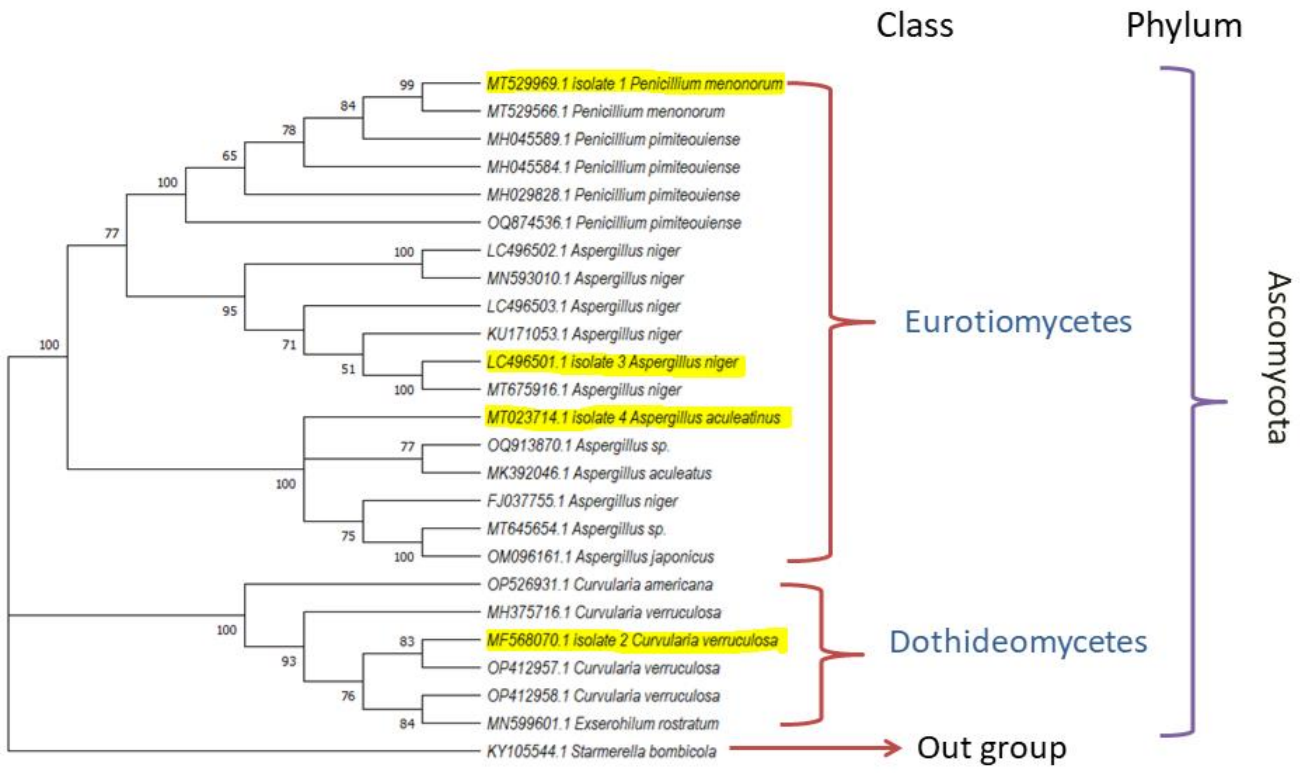

Figure 3. A Neighbor-Joining phylogenetic tree depicting the relationship of *Pennicillum menonorum* (MT529969.1), *Curvularia verruculosa* (MF568070.1), *Aspergillus niger* (LC496501.1), and *A. aculeautinus* (MT023714.1) with other related fungi found in the NCBI. The *Starmerella bombicola* C.A.Rosa & Lachance (KY1055441) was used as the outgroup. Branches represent values based on 1000 replications of Felsenstein's bootstrap method

Additionally, the crude extracts *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. aculeautinus* (MT023714.1) displayed a comparable antimicrobial activity pattern against all the organisms tested (Figure 4). When arranged in descending order, the test organisms exhibited varying levels of susceptibility to the crude extracts, with *B. subtilis* being the most susceptible, followed by *P. aeruginosa*, *S. aureus*, and *E. coli*. However, this pattern was not observed for crude extract 3 (*A. niger*, LC496501.1), as illustrated in Figure 4. In the bar graph in Figure 4, for example, the zone of inhibition of *A. niger* crude extract against *E. coli* was higher compared to that of *P. aeruginosa*, which was not the general trend for the rest of the crude extracts.

Minimum inhibitory concentrations

The Minimum Inhibitory Concentration (MIC) values of crude extracts from *R. communis* were determined against four test organisms, as shown in Table 5. Regarding Figure 5, among all crude extracts, the crude extract of *P. menonorum* (MT529969.1) exhibited the lowest MIC value against *B. subtilis*. Both crude extract of *P. menonorum* (MT529969.1) and crude extract of *A. niger* (LC496501.1) had the highest MIC values when tested against *E. coli* and *S. aureus*. The crude extracts of *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. aculeautinus* (MT023714.1) exhibited the same MIC value against *P. aeruginosa*. Crude extracts of *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. niger* (LC496501.1) displayed similar MIC values when tested against *S. aureus*.

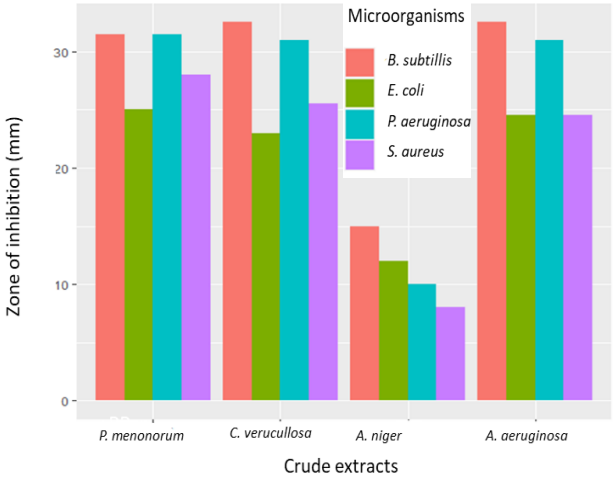

Figure 4. Antimicrobial activity pattern of endophytic fungi crude extracts isolated from *Ricinus communis* against tested organism based on inhibition zone

Table 5. Minimum Inhibitory concentrations (mg/mL) of four endophytic fungi ethyl-acetate crude extracts from *Ricinus communis* against tested microorganisms

Isolate	Test microorganism			
	E. coli	***B. subtilis***	***S. aureus***	***P. aeruginosa***
Penicillium menonorum (MT529969.1)	3.125	0.195	1.563	1.563
Curvularia verruculosa (MF568070.1)	0.781	0.781	1.563	1.563
Aspergillus niger (LC496501.1)	1.563	1.563	3.125	0.781
Aspergillus aculeautinus (MT023714.1)	1.563	0.391	0.781	1.563
Positive control	0.098	0.098	0.098	0.098

Note: gentamicin was used as a positive control

Table 6. Cytotoxic activities of endophytic fungi crude extracts from *Ricinus communis*

Isolate	LC_{50} (µg/mL)	95%CL	Regression equation	R^2
Penicillium menonorum (MT529969.1)	1011.58	214-513	y=27.345logx-32.178	0.854
Curvularia verruculosa (MF568070.1)	382.82	94-107	y=32.832logx-34.811	0.936
Aspergillus niger (LC496501.1)	170.88	46-625	y=44.926logx-50.307	0.863
Aspergillus aculeautinus (MT023714.1)	382.82	94-107	y=32.832logx-34.811	0.936

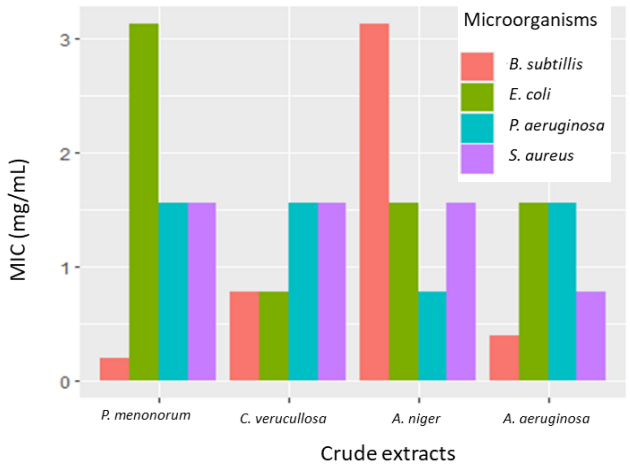

Figure 5. Antimicrobial activity pattern of endophytic fungi isolated from *Ricinus communis* against tested organism based on minimum inhibitory concentration

Cytotoxicity assay

All crude extracts from endophytic fungi of *R. communis* were not toxic to brine shrimp larvae since LC_{50} values for all extracts were above 100 µg/mL, as illustrated in Table 6.

Discussion

Due to an expected global challenge of antimicrobial resistance to most antibiotics currently in use (Cassell and Mekalanos 2001), there is an endeavor to search for novel drug structures or compounds from various sources, including plants (Anand et al. 2019). Although plants have been a good source of various anti-pathogenic agents (Manandhar et al. 2019), the direct harnessing of plants has environmental implications (Mehta et al. 2022). Therefore, alternative options like bio-prospecting of endophytes are of great interest. This is based on the ground that endophytes inhabiting medicinal plants have been found to produce secondary metabolites with biological activities similar to those produced by the host plants (van Wyk and Prinsloo 2018). The present study hypothesized that endophytes living in symbiotic association with *R. communis*, including those associated with the *R. communis* used by Tanzanian communities, may produce secondary metabolites with remarkable biological applications. Interestingly, the present study's findings supported the hypothesis because ethyl acetate crude extracts of endophytes isolated from *R. communis* had outstanding antimicrobial activity, as displayed in Table 4. Most importantly, the MIC for most extracts was very low, and the extracts did not demonstrate cytotoxicity upon BSLA assay.

This study represents the first report of isolation of endophytic fungi from *R. communis* of Tanzania. In this study, 51 endophytic fungi were isolated from the leaves and roots of *R. communis*. Morphological characterizations were employed in the identification of endophytic fungi isolates. However, there was a significant variation among the fungal isolates regarding color, size, shape, elevation, margin, and density, as depicted in Table 2. This may be due to genetic variability among the fungal isolates and different stages of fungal growth and development (Hallmann et al. 2007). Fungi isolates can exhibit considerable morphological diversity, and these variations provide valuable information for characterizing and distinguishing between different isolates (Hallmann et al. 2007). Based on the phylogenetic relationship (Figure 3), the isolates were found to be in 4 species (*P. menonorum*, *C. verruculosa*, *A. niger* and *A. aculeatinus*), three genera (*Penicillium*, *Curvularia*, and *Aspergillus*) and two classes (Dothideomycetes, Eurotiomycetes) under the phylum Ascomycota. The isolation of the four species in the present study is not surprising because the species have been isolated from other plants elsewhere. For example, in

the study conducted in Pudukkottai South India, *C. verruculosa* was isolated from the leaves of *Catharanthus roseus* (L.) G.Don (Parthasarathy et al. 2020). Furthermore, Díaz et al. (2019) isolated *A. niger* from rotten wood in the subtropical rainforest of Misiones, Argentina. The *P. menonorum* was also reported to be isolated from rhizosphere soil in Korea by Babu et al. (2015). Then, *A. aculeatinus* was isolated from the soil sample in Malaysia (Aziz and Zainol 2018). In addition, most of the endophytes are members of the phylum Ascomycota. Taken as an example in a study conducted by Abdulwehab et al. (2015), from different soil crust microhabitats and rhizosphere soils around the native bunchgrass in the USA, five Ascomycota fungi were isolated.

The crude extract of *A. niger* showed the lowest zone of inhibition (8 mm) when tested against *Staphylococcus aureus*. This may be caused by resistance of *S. aureus* towards crude extracts of *A. niger*. Similar to our results, Amina et al. (2017) conducted a study on the antibacterial activity of *Aspergillus* species; they reported that *A. niger* had a 7.33 mm zone of inhibition against *S. aureus*. Furthermore, crude extracts of *P. menonorum* (MT529969.1), *C. verruculosa* (MF568070.1), and *A. aculeatinus* (MT023714.1) displayed a comparable antimicrobial activity pattern against all the microorganisms tested. This observation is challenging; however, it may be explained by the adaptability of the fungi species to the host plant (*R. communis).* The observed trend has been reported in a previous study by Idris et al. (2013), which indicated that *Aspergillus* species and *Curvularia lunata* (Wakker) Boedijn had comparable inhibition zones against tested microorganisms.

The lowest MIC value obtained in the present study was 0.195 µg/mL for *P. menonorum* against Gram-positive bacterium *B. subtilis*, indicating that crude extracts of *P. menonorum* were most active against *B. subtilis.* Comparison of this observation with the previous results is difficult with two-fold explanations. First, *P. menonorum* is a novel species that has not been extensively studied, and such information is limited. Second, the inoculum of *P. menonorum* isolated from soil samples in Korea was evaluated for plant growth promotion (Babu et al. 2015), and therefore it is challenging to compare. However, the present information is very interesting as it demonstrates the wide biotechnological application of the species (*P. menonorum)* from medicine to agriculture applications.

The MIC value of *A. niger* was higher than *B. subtilis* compared to other crude extracts (Table 5). The information indicates that *B. subtilis* was less susceptible to ethyl acetate extract of *A. niger*. This observation was unexpected, considering that the *B. subtilis* possesses a thicker peptidoglycan layer on its cell wall, allowing antibiotic permeability. However, this may be explained by the chemical nature of the extract, which may be less effective against *B. subtilis.* Several antibiotics are effective against Gram-negative bacteria but less effective against Gram-positive bacteria. For example, ciprofloxacin is effective against *Pseudomonas aeruginosa*, a Gram-negative bacteria, but less effective against *S. aureus*, a Gram-positive bacteria (Baggio and Ananda-Rajah 2021).

MIC results did not depend on whether the test microorganisms were Gram-positive or Gram-negative. For instance, the crude extract of *P. menonorum* had a high MIC value of 3.125 µg/mL against the Gram-negative bacterium *E. coli*, and the crude extract of *A. niger* had a high MIC value of 3.125 µg/mL against gram-positive bacterium *B. subtilis*. These results contrast the common understanding that Gram-negative bacteria have reduced antimicrobial potency compared to Gram-positive bacteria due to their morphological differences in cell walls (Mpenda and Mkangara 2022). Gram-negative bacteria possess an additional outer membrane comprising lipopolysaccharides and proteins, serving as a protective barrier and making them less susceptible to the penetration of antimicrobial agents. In contrast, Gram-positive bacteria possess a thicker peptidoglycan layer, making it easier to penetrate antimicrobial agents (Sosovele et al. 2012). Sosovele et al. (2012) reported that actinomyces crude extracts showed a high MIC value of 5 µg/mL against *P. aeruginosa* while *B. subtilis* has MIC value of 0.1563 µg/mL. This understanding may not always be accurate because crude extracts are effective in Gram-positive and Gram-negative. For example, Yimgang et al. (2022) studied the antimicrobial activity of endophytic fungi inhibiting Cameroonian *Annona muricata* L.; they reported most MIC values similar to Gram-positive and Gram-negative. Additionally, Njeru et al. (2015) conducted a study on the antimicrobial activity of the crude extracts of *Premna resinosa* (Hochst.) Schauer, a Kenyan traditional medicinal plant; they reported that the antibacterial activity was high and broad spectrum, inhibiting both Gram-positive and Gram-negative bacteria. Based on these observations, the present results may be attributed to some extracts possessing secondary metabolites, which are effective against Gram-positive and Gram-negative bacteria.

The non-toxic nature of the crude extracts from endophytic fungi of *R. communis* towards brine shrimp larvae is an interesting finding, as indicated by LC_{50} values above 100 µg/mL (Table 6). According to Hadiza et al. (2014), LC_{50} < 1.0 µg/mL is considered highly toxic, LC_{50} 1.0-10µg/mL is toxic, LC_{50} 1.0- 30µg/mL is moderately toxic, LC_{50} > 30 <100 µg/mL is considered as mildly toxic and LC_{50} > 100 µg/mL is considered as non-toxic. These results suggest that the tested crude extracts do not cause acute toxicity toward brine shrimp larvae. This is an encouraging finding, implying that these endophytic fungi crude extracts may have a favorable safety profile. Previous studies that reported bioactive compounds with no toxicity in endophytic fungi supported the absence of toxicity in the crude extracts. For instance, a study conducted by Tibuhwa (2017) evaluated the cytotoxicity of *Boletus bicolor* Raddi and found that the extracts were significantly non-toxic towards brine shrimp larvae. Additionally, the study conducted by (Sosovele et al. (2012) reported that all ethyl acetate extracts from *Streptomyces* strains were non-toxic. The lack of toxicity in the crude extracts from *R. communis* endophytic fungi indicates the presence of bioactive compounds that are safe for use in further research and development. These extracts

can be valuable for exploring and isolating pharmacologically active compounds with reduced effects.

To conclude, in the present study, findings demonstrate that crude extracts of endophytic fungi from *R. communis* have promising therapeutic potential as they were effective against *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa*, which are currently reported to pose great public health challenges due to drug resistance. However, the crude extracts from the present study also did not exhibit any cytotoxicity against brine shrimp larvae. Further studies should investigate bioactive compounds responsible for observed antibacterial activities. Research should also be conducted on the antimicrobial activity of crude extract from *R. communis* endophytic fungi against resistant bacterial strains like Methicillin-Resistant *S. aureus* (MRSA) and Methicillin-Resistant *P. aeruginosa* (MRPA). Additionally, further research should be conducted to assess if crude extracts from endophytic fungi present in *R. communis* may have other biotechnological applications in medicine, industry, and agriculture.

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Functional properties of bambara groundnut flour fermented with lactic acid bacteria consortium

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Abstract. *Ogodo AC, Agwaranze DI, Opara JI, Iheanacho CC. 2023. Functional properties of bambara groundnut flour fermented with lactic acid bacteria consortium. Asian J Trop Biotechnol 20: 79-84.* The objective of this study was to evaluate the functional properties of bambara groundnut flour fermented with Lactic Acid Bacteria (LAB) consortium isolated from fermenting maize and sorghum. Sorghum was processed into flour and fermented with LAB-consortium previously isolated from maize (*Lactobacillus plantarum* WCFS1 + *Lactobacillus rhamnosus* GG, ATCC 53/03 + *Lactobacillus fermentum* CIP 102980 + *Lactobacillus nantensis* LP33 + *Lactobacillus reuteri* DSM 20016) and sorghum (*Pediococcus acidilactici* DSM 20284 + *Lactobacillus nantensis* LP33 + *Lactobacillus fermentum* CIP 102980 + *Lactobacillus brevis* ATCC 14869 + *Lactobacillus plantarum* WCFS1), respectively and then naturally to evaluate their effects on the functional properties of the bambara groundnut flour at 12 h intervals. Results showed that there was a gradual decrease in Bulk Density (BD), Swelling Capacity (SC), and Water Holding Capacity (WHC) with increasing fermentation period. Oil Holding Capacity (OHC) increased significantly ($p < 0.05$) with increase in the fermentation periods from 8.40±0.00 mL/g to 8.90±0.02 mL/g (spontaneous fermentation), 8.40±0.00 mL/g to 9.20±0.03 mL/g (LAB-consortium from maize fermentation) and from 8.40±0.00 mL/g to 9.70±0.03 mL/g (LAB-consortium from sorghum fermentation). The lowest gelation concentration ranged from 3.0% in the unfermented sample to 7.0% in the various fermentation products. The variations differ significantly ($p < 0.05$) between the unfermented, spontaneously fermented, and LAB consortium fermented samples. Emulsion Capacity (EC) increased with increasing fermentation period from 72.36±2.01% to 87.54±0.36%, from 72.36±2.01% to 87.22±1.44% and from 72.36±2.01 % to 88.56±0.14% in natural, LAB-consortium from maize and LAB-consortium from sorghum fermentation respectively. This result indicates that lactic acid bacteria consortia can potentially improve the functional properties of bambara groundnut flour.

Keywords: Bambara groundnut, consortium, fermentation, functional properties, lactic acid bacteria, nutritional quality

INTRODUCTION

Bambara groundnut (*Vigna subterranea* L. Verdc.) is a crop that is native to Africa and is grown in various countries across the African continent, especially Senegal, Kenya, and South Africa (Atiku et al. 2004; Goudoum et al. 2015) as well as Nigeria. It is regarded as one of the most neglected and underutilized crops. However, it can be used as a complete food (Ogodo et al. 2018a). The report of Aremu et al. (2006) revealed that bambara groundnut contains 64.9% carbohydrate, 9.7% moisture, 16% protein, 5.9% fat, and 2.9% ash. Similarly, Olanipekun et al. (2012) reported that bambara groundnut contains an appreciable amount of some amino acids, such as lysine, trypsin, and chymotrypsin.

In sub-Saharan Africa, leguminous crops indigenous to the region can reduce the vulnerability of rural households to food insecurity and malnutrition, as revealed through impact assessment (Matsa and Mukoni 2013; Mubaiwa et al. 2018). Moreover, efforts are geared towards processing legumes to make proteins available due to the high cost of animal protein, thereby combating malnutrition (Goudoum et al. 2015). Bambara groundnut is an important legume that is a source of cheap and affordable protein, especially in areas where animal proteins are expensive (Mubaiwa et al. 2018). The report of Plahar and Yawson (2004) showed that bambara groundnut is rich in essential amino acids and has a protein score of 80%, which is higher than other legumes such as soybean (74%) and cowpea (64%). This indicates that bambara groundnut contains more available human proteins than other legumes in Africa, although it does not contain sulfur-rich amino acids like other legumes (Brough and Azam-Ali 1992; Schaafsma 2012). However, it can be mixed with cereals like maize, which is rich in methionine and cysteine, and can form good nutritional fortification strategy (Akpapunam and Darbe 1994; Mubaiwa et al. 2018). Fermentation of food substrates leads to improvement in their nutritional composition, such as increase in protein and amino acids as well as improvement in the protein and starch digestibility with concomitant decrease in the antinutritional factors, leading to bioavailability of minerals (Singh et al. 2012). Fermentation contributes to the valuable properties of food products as well as in the production of beneficial products, including biomass protein, amino acids, minerals, vitamins, aroma, and flavor compounds as well as products of biosynthetic pathway (lactic acid, ethanol, pyruvate, acetaldehyde etc.) which contributes to the reduction of

food pH leading to the control of the growth of pathogens, which enhances the shelf-life of food and food safety and preservation (Onyango et al. 2013; Ojokoh and Bello 2014).

Lactic Acid Bacteria (LAB) are large groups of non-sporing, non-motile firmicutes, Gram-positive cocci, and rods. They are catalase and oxidase-negative and utilize carbohydrates to produce lactic acid during fermentation (Masood et al. 2011). LAB includes *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc* species, etc. Lactic Acid Bacteria (LAB) fermentation makes food more palatable and increases food's protein and vitamin contents (Masood et al. 2011; Pang et al. 2011; Ogodo et al. 2017). It is one of the major ways of preparing food locally in Africa. Cereals, such as maize and sorghum and legumes such as cowpea and bambara groundnut are fermented using LAB (Ogodo et al. 2016). In addition, lactic acid fermentation helps in food preservation. Moreover, the regular use or consumption of LAB-fermented foods strengthens the immune system in fighting bacterial infections, promoting health (Chelule et al. 2010).

Legumes, like soybean, bambara groundnut, lima beans, cowpea, pigeon pea, African yam beans, etc., serve as sources of nutrients, including proteins, calories, minerals, and vitamins (Olanipekun and Adelakun 2015). These legumes contain healthy proteins, carbohydrates, dietary fiber, and diverse vitamins and minerals. However, their content of antinutritional factors, such as phytic acid, tannins, polyphenols, are associated with fiber, reduces mineral bioavailability (Dueñas et al. 2012; Olanipekun and Adelakun 2015). Bambara groundnut and other legumes have been identified to have a major role in the fight against malnutrition in poor countries, especially in Africa (Yusufu and Ejeh 2018). Hence, it is very important to increase the consumption of legumes in developing countries, which serve as a source of affordable proteins for poor countries (Borget 1992). Therefore, the purpose of present study was to determine the LAB consortium's effect on bambara groundnut flour's functional properties.

MATERIALS AND METHODS

Sample collection

Bambara groundnut seeds (*V. subterranea*) were purchased from Lagos (Yaba market), Nigeria. The seeds were brought to the Federal Institute of Industrial Research Oshodi (FIIRO) for identification, processing and analysis. Lactic acid bacteria were obtained from stored cultures isolated from previously fermented maize and sorghum.

Sample preparation

The sample was prepared following the method described by Ogodo et al. (2018b). The bambara groundnut seeds were sorted, washed, and dried at 60°C for 8 hours in a hot air oven (GL, England). The dried seeds were milled into flour, and then stored in a clean, airtight container for further use.

Inoculum preparation

Inoculum was prepared according to the method described by Ogodo et al. (2017). A consortium of 5 lactic acid bacteria, each from the stock of bacteria isolated from fermented maize (*Lactobacillus plantarum* WCFS1, *Lactobacillus fermentum* CIP 102980, *Lactobacillus rhamnosus* GG, ATCC 53/03, *Lactobacillus nantensis* LP33, *Lactobacillus reuteri* DSM 20016) and fermented sorghum (*Pediococcus acidilactici* DSM 20284, *Lactobacillus nantensis* LP33, *Lactobacillus fermentum* CIP 102980, *Lactobacillus brevis* ATCC 14869, *Lactobacillus plantarum* WCFS1) were selected. The organisms were combined by growing them in a 250 mL conical flask and incubating them in an orbital shaker for 48 h in a co-culture for the inocula to build up. Afterward, the cells were harvested using the centrifuge at 5,000 rpm for 10 min and maintained (before being used for fermentation) in fresh de Manne Rogosa and Sharpe (MRS) broth. The bacteria cells were cleaned with sterile distilled water and standardized to 0.5 McFarland standard (Dajanta et al. 2009).

Fermentation of bambara groundnut flour

The method described by Ogodo et al. (2017) was conducted to carry out the fermentation process. The prepared bambara flour was combined with sterile distilled water in a ratio of 1:2. Exactly 500 g of the bambara flour sample was transferred to a sterile fermentation container and mixed with 1,000 mL of sterile distilled water followed by the addition of 0.5 g/L potassium sorbate to help eliminate microbial contaminants. The mixture was inoculated with 10 mL of 10^8 cells/mL of the consortium of lactic acid bacteria suspension and allowed to ferment. The potassium sorbate and starter organisms were not added to one of the set-ups, which was allowed to ferment spontaneously. The analysis of the functional properties of the flour was carried out at 12 h intervals.

Determination of functional properties

The method of Chau and Huang (2003) was used to determine the bulk density of the flour. Water Holding Capacity (WHC) and Oil Holding Capacity (OHC) were respectively determined following the method of Singh et al. (2012), while the swelling capacity of the flour was determined using the Robertson et al. (2000) method. The gelation properties of the bambara flour were determined following the method given by Aremu et al. (2008). The method of Suresh and Samsher (2013) was used to determine the various flours' Emulsion Capacity (EC).

RESULTS AND DISCUSSION

The results of Bulk Density (BD) of all the fermentation set-ups at different time intervals is presented in Figure 1. The bulk density decreased from 0.78±0.02 g/mL (0 h) to 0.72±0.02 g/mL (48 h) (spontaneous fermentation), 0.78±0.02 g/mL (0 h) to 0.71±0.03 g/mL (48 h) (LAB-consortium from maize fermented), and from 0.78±0.02

g/mL (0 h) to 0.71±0.03 g/mL (0 h) in LAB-consortium from sorghum fermented samples.

The Swelling Capacity (SC) of the LAB-consortium and spontaneous fermented bambara flour, which decreased with increasing fermentation time, is presented in Figure 2. The decreased ranged were 0.46±0.01% (0 h)-0.30±0.02% (48 h), 0.46±0.01% (0 h)-0.29±0.03% (48 h), and 0.46±0.01% (0 h)-0.29±0.03% (48 h) for spontaneous, LAB-consortium from maize and LAB-consortium from sorghum fermented samples, respectively.

Figure 3 shows the fermentation products' Water Holding Capacity (WHC) at different intervals. There was a decrease in the WHC ranging from 1.7±0.02 mL/g (0 h)-1.3±0.03 mL/g (48 h) (spontaneous fermentation), 1.7±0.02 mL/g (0 h)-1.3±0.03 mL/g (48 h) (LAB-consortium from maize fermentation) and 1.7±0.02 mL/g (0 h)-1.2±0.01 mL/g (48 h) (LAB-consortium from sorghum fermentation).

The Oil Holding Capacity (OHC) of all the fermentation products at different time intervals is presented in Figure 4. Results revealed that there was an increase in the OHC ranging from 8.40±0.00 mL/g (0 h)-8.9±0.02 mL/g (48 h), 8.40±0.00 mL/g (0 h)-9. 20±0.03 mL/g(48 h) and 8.40±0.00 mL/g (0 h)-9.70±0.03 mL/g (48 h) for spontaneous, LAB-consortium from maize and LAB-consortium from sorghum fermentations respectively.

The gelation concentration ranged from 3.0% (unfermented sample) to 6.0% in 48 h spontaneous and LAB-consortia fermentations, respectively (Table 1).

The fermented samples' Emulsion Capacity (EC) increased with fermentation time. It ranged from 72.36±2.01% (0 h)-87.54±0.36% (48 h) during spontaneous fermentation, 72.36±2.01% (0 h)-89.22±1.44% (48 h) and 72.36±2.01% (0 h)-88.56±0.14% (48 h) for LAB-consortium from maize and LAB-consortium from sorghum fermentations, respectively (Figure 5).

Table 1. Least gelation concentration (LGC) (%) of LAB-consortium fermented bambara groundnut flour

Concentrations	0hr	12hr			24hr			36hr			48hr		
(%)	F*	NF	MF	SF	NF	MF	SF	NF	MF	SF	NF	MF	SF
1.0	V	V	V	V	V	V	V	V	V	V	V	V	V
2.0	V	V	V	V	V	V	V	V	V	V	V	V	V
3.0	G	G	G	G	V	V	V	V	V	V	V	V	V
4.0	G	G	G	G	G	V	V	V	V	V	V	V	V
5.0	G	G	G	G	G	G	G	G	G	G	V	V	V
6.0	G	G	G	G	G	G	G	G	G	G	G	G	G
7.0	G	G	G	G	G	G	G	G	G	G	G	G	G
8.0	G	G	G	G	G	G	G	G	G	G	G	G	G
9.0	G	G	G	G	G	G	G	G	G	G	G	G	G
10.0	G	G	G	G	G	G	G	G	G	G	G	G	G
LGC	3.0^{a}	3.0^{a}	3.0^{a}	3.0^{a}	$4.0^{a,b}$	$5.0^{b,c}$	$5.0^{b,c}$	$5.0^{b,c}$	$5.0^{b,c}$	$5.0^{b,c}$	$6.0^{c,d}$	$6.0^{c,d}$	$6.0^{c,d}$

Note: F*: Unfermented sample, NF: Spontaneously fermented, MF: Fermented with LAB consortium from maize, SF: Fermented with LAB consortium from sorghum, V: Viscous, G: Gel, LGC: Least gelation concentration. Values with the same superscript are not significantly different (P >0.05)

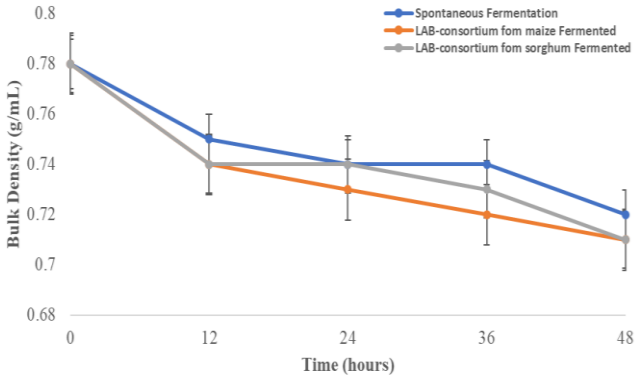

Figure 1. The bulk density (g/mL) of bambara flours fermented with lactic acid bacteria-consortium. The error bars represent standard error; LAB: Lactic Acid Bacteria

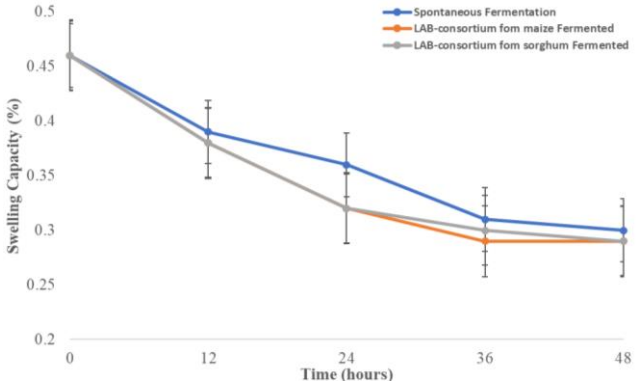

Figure 2. The swelling capacity (%) of bambara flours fermented with lactic acid bacteria-consortium. The error bars represent standard error; LAB: Lactic Acid Bacteria

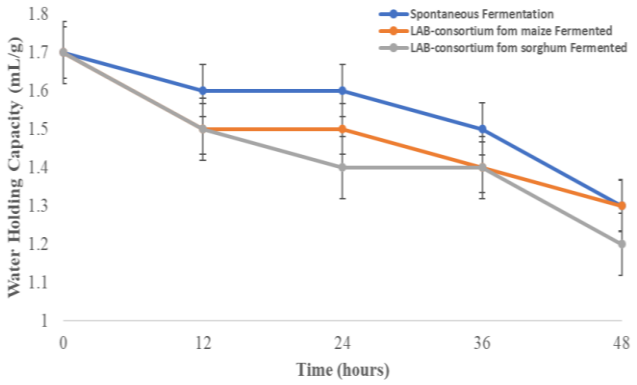

Figure 3. The water holding capacity (mL/g) of bambara flours fermented with lactic acid bacteria-consortium. The error bars represent standard error; LAB: Lactic Acid Bacteria

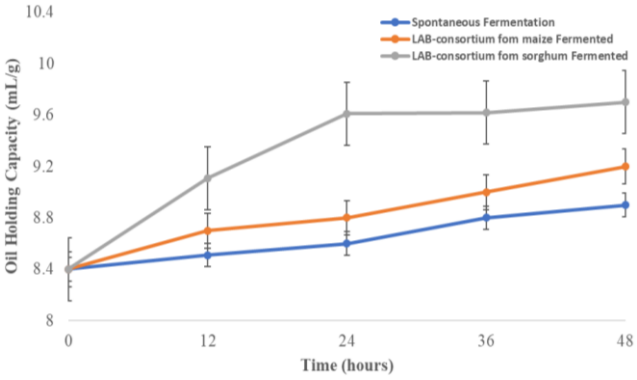

Figure 4. The oil holding capacity (mL/g) of bambara flours fermented with lactic acid bacteria-consortium. The error bars represent standard error; LAB: Lactic Acid Bacteria

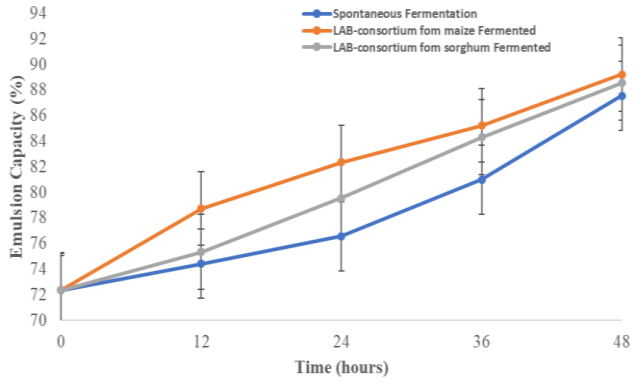

Figure 5. The emulsion capacity (%) of bambara flours fermented with lactic acid bacteria-consortium. The error bars represent standard error; LAB: Lactic Acid Bacteria

Discussion

In the present study, bambara groundnut's Bulk Density (BD) gradually decreased with increasing fermentation time. It decreased from 0.78±0.02 g/mL to 0.72±0.02 g/mL (spontaneous fermentation), 0.78±0.02 g/mL to 0.71±0.03 g/mL (LAB-consortium from maize fermented), and from 0.78±0.02 g/mL to 0.71±0.03 g/mL in LAB-consortium from sorghum fermented samples. The samples' bulk density variations were not differ significantly (P >0.05). Eltayeb et al. (2011) observed higher values of bulk density of bambara groundnut flour and protein. Adebowale and Maliki (2011) reported a gradual decrease in BD in the range of 0.80 to 0.63 g/mL with an increasing fermentation period of pigeon pea flours, comparable to the values obtained in the present investigation. Decreases in bulk density have been reported in products such as maize (Ogodo et al. 2016), sorghum, millet (Singh et al. 2012; Ocheme et al. 2015; Ogodo et al. 2017) and soybean (Ogodo et al. 2018c). The bulk density of the flour represents the load it can carry when allowed to be directly on top of one another (Singh et al. 2012). Moreover, Singh et al. (2012) reported that low-density foods obtained through fermentation are good for preparing weaning foods and infant food formulations (Singh et al. 2012; Ogodo et al. 2017). This indicates that the product of the present study can be used in infant food formulations.

Results of present study showed decrease in bambara groundnut flour's Swelling Capacity (SC) as fermentation progressed. The decreased ranged from 0.46±0.01%-0.30±0.02%, 0.46±0.01%-0.29±0.03%, and 0.46±0.01%-0.29±0.03% for spontaneous, LAB-consortium from maize and LAB-consortium from sorghum fermented samples, respectively. The variations showed significant differences ($p < 0.05$) when the unfermented flour was compared to the fermented flour. The present observation is consistent with that of Singh et al. (2012), who reported a decrease in SC of pigeon peas with increasing fermentation time. Ogodo et al. (2018c) have also reported a decrease in the swelling capacity of fermented soybean flour. Similarly, the swelling capacity of fermented bambara groundnut-ogi formulation decreased with increasing percentage concentration of bambara groundnut flour in a study by Chude et al. (2018). However, the decrease in swelling capacity did not affect the organoleptic properties of the formulated food.

The water holding capacity in bambara groundnut flour decreased significantly ($p < 0.05$) when compared between unfermented and fermented samples. The decreased value ranged from 1.7±0.02 mL/g-1.3±0.03 mL/g (spontaneous fermentation), 1.7±0.02 mL/g-1.3±0.03 mL/g (LAB-consortium from maize fermentation) and 1.7±0.02 mL/g-1.2±0.01 mL/g (LAB-consortium from sorghum fermentation). The result of this study is comparable to the work of Adebowale and Maliki (2011), who reported a decrease in WHC of pigeon peas at the end of a 5-day fermentation period. Similarly, decreases in water holding capacity after fermentation have been reported in products, such as maize (Ogodo et al. 2016), sorghum, millet (Singh et al. 2012), and soybean (Ogodo et al. 2018c). The report of Chude et al. (2018) showed a decrease in the water absorption capacity of bambara groundnut-ogi food formulation following an increase in the percentage of bambara groundnut flour. Similarly, in a study by Elkhalifa et al. (2005), there was a decrease in WHC after 8-24 h fermentation of sorghum. However, the report of an increase in the water absorption capacity of maize from 1.2-1.8 ml/g by Beugre et al. (2014) did not correspond to

the present study. Water binding capacity is useful when flour is added to food formulation, especially when doughs are involved. It is a useful indicator of the quantity of water available for gelatinization. Low water absorption holding capacity is desirable in making thinner gruels (Singh et al. 2012), which agrees with the current findings. This indicates that the fermented flours in this study can be applied in the preparation and formulation of infant weaning foods and the production of product,s such as biscuits, snacks, and other baked foods when blended with other unfermented flours (Singh et al. 2012; Ogodo et al. 2016).

There is a significant increase ($p < 0.05$) in the Oil Holding Capacity (OHC) of the bambara groundnut flour in the present study as fermentation time increased. The OHC increased from 8.60±0.01 mL/g to 9.78±0.02 mL/g (spontaneous fermentation), from 8.92±0.02 mL/g to 9.69±0.03 mL/g (LAB-consortium from maize fermentation) and from 8.40±0.00 mL/g to 9.70±0.03 (LAB consortium from sorghum fermentation). The increase values observed in the present study is higher than the 1.43 ml/g reported by Acuña et al. (2012) for soybeans. This implies that these fermented flours could be used to formulate and fortify food, especially where the limiting factor is OHC (Singh et al. 2012). Moreover, the water and oil holding capacity depends on the food's inherent factors, such as surface hydrophobicity and polarity, protein conformation, and amino acid contents (Suresh and Samsher 2013; Ogodo et al. 2016); also, where oil absorption needs to be optimized in a food system, the binding of the flour proteins with oil makes it useful. This property could make the flour useful if food production includes sausages (Suresh and Samsher 2013).

Gelation power is an index of the gelling tendency of samples, and it is an important factor in food preparations (Adebowale and Maliki 2011). In the present study, the Least Gelation Concentration (LGC) decreased as the fermentation time increased, ranging from 3.0% (unfermented sample) to 6.0% in 48 h spontaneous and LAB-consortia fermentations. This observation is in line with Adebowale and Maliki (2011) and Ogodo et al. (2018c) who observed that fermentation decreased the gelation power of pigeon peas and soybeans, respectively. Gelation power may be affected by the proportion of carbohydrates, lipids, and proteins in the flour, which shows that the way these macromolecules interact within a product influences the behavior of the functional properties of the product greatly (Adebowale and Maliki 2011; Ogodo et al. 2017).

The result of the Emulsion Capacity (EC) of the bambara groundnut flour increased with increasing fermentation period ranging from 72.36±2.01% to 87.54±0.36% (spontaneous fermentation) from 72.36±2.01% to 89.22±1.44% and 72.36±2.01%-88.56±0.14% for LAB-consortium from maize and LAB-consortium from sorghum fermentation, respectively. The values recorded for the fermented products significantly ($p < 0.05$) differ from those from the unfermented samples. However, the values recorded for spontaneous fermentation, LAB-consortium from maize, and LAB-consortium from sorghum fermented products showed no significant difference ($p > 0.05$). The increase in the emulsion capacity of soybeans has been reported in a previous study (Ogodo et al. 2018c). The observation of EC has a relationship with the protein solubility pattern, as Suresh and Samsher (2013) asserted. Similarly, Kaushal et al. (2012) have reported that protein hydrophobicity in a particular product is a contributing factor to the emulsifying properties of the product.

In conclusion, the present study proved that spontaneous fermentation with lactic acid bacteria consortium isolated from maize, and fermentation with lactic acid bacteria consortium isolated from sorghum could improve the parameters of functionalities of bambara groundnut flour. The results of the present study revealed that fermentation with lactic acid bacteria consortium enhanced the functional properties of bambara groundnut flour more than spontaneous fermentation. Hence, this indicates the potential of lactic acid bacteria consortium from cereals in improving bambara groundnut flour's functional and nutritional qualities. This can be applied in natural food fortification by food industries.

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