Anti-amoebic effects of selected herbal extracts against Acanthamoeba species isolated from different borehole water samples from Budiriro District in Harare, Zimbabwe

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Abstract

Acanthamoeba species are the major cause of Acanthamoeba Keratitis (AK) of eyes, Acanthamoeba meningoencephalitis of the central nervous system, and fatal Granulomatous Amoebic Encephalitis (GAE) of the brain in humans. These diseases are difficult to treat due to their resistance to extreme temperatures, pH, alcohol, and pressure. This research aimed to determine the anti-amoebic effects of some selected plant extracts against Acanthamoeba species isolated from borehole water samples from Budiriro District, Harare, Zimbabwe. *Acanthamoeba castellannii* species were isolated and confirmed present in Budiriro borehole water samples using non-nutrient agar, microscope, real-time Polymerase Chain Reaction (PCR), and gel electrophoresis. The selected plant samples, Murumanyama (*Xeroderris stuhlmannii*) bark extract, Munhundurwa (*Solanum incanum*) fruit extract and Mufandichimuka (*Myrothamnus flabellifolius*) stem and leaf extracts were then investigated for their anti-amoebic effects against the isolated *Acanthamoeba castellannii,* using agar well diffusion method. Chlorhexidine gluconate antibiotic was used as a control. The results show that *Acanthamoeba castellannii* is the most common Acanthamoeba species in borehole water in Budiriro District in Harare and all the tested plant samples had no anti-amoebic effects against this isolated *Acanthamoeba castellannii.*

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Introduction

The genus Acanthamoeba has become a major threat to human health in Zimbabwe.[1] It has become the cause of life-threatening Granulomatous Amoebic Encephalitis (GAE), pulmonary infections, cutaneous lesions, rhinosinusitis, and osteomyelitis in immunocompromised people and those who wear contact lenses.[2] It is also the cause of Acanthamoeba Keratitis (AK), which causes severe corneal damage to those who put on contact lenses and swim in hot pools and to immunocompromised patients, such as those with Acquired Immune Deficiency Syndrome (AIDS), systemic lupus erythematosus, transplanted organ and those undergoing chemotherapy for cancer.[3] AK leads to permanent impaired vision and sometimes complete eye damage. In many researches from different countries, different genus of Acanthamoeba have been isolated from different environmental surfaces such as tap water, borehole water, soil, air, swimming pools, ocean sediments, fresh water, *etc*.[4] There are many cases of AK in Zimbabwe each and every year. Eye infections have been reported to be caused by *Acanthamoeba castellanii, Acanthamoeba polyphaga, Acanthamoeba rhysodes, Acanthamoeba culbertsoni, Acanthamoeba lugdunensis, Acanthamoeba griffini, Acanthamoeba hatchetti, Acanthamoeba quina, Acanthamoeba lenticulata, and Acanthamoeba triangularis.* Central nervous system diseases have been caused by *A. castellanii, A. culbertsoni, Acanthamoeba astronyxis, A. rhysodes, Acanthamoeba healyi,* and *A. lenticulata*.[4] There are also cases of GAE in Zimbabwe, which continues to rise each year.[1] Several diagnostics are done by the Zimbabwean health sector but mainly to identify the causing pathogen, which includes Polymerase Chain Reaction (PCR) with 18s rDNA primers, which specifically amplifies the Acanthamoeba amino acids.

The treatment of Acanthamoeba-related diseases has become a challenge in Zimbabwe due to a lack of enough and better med-

ication. AK has been treated with chlorhexidine gluconate, which has, in some cases, a better prognosis but, to some patients, lesser effects. This research aimed at finding the antimicrobial activities of locally used traditional herbs namely Murumanyama buck extract (*Xeroderris stuhlmannii*), Munhundurwa fruit extracts (*Solanum incanum*) and Mufandichimuka stem and leaf extracts (*Myrothamnus flabellifolius*). These herbs have been tested against many bacteria strains and proved to have good antimicrobial activities due to the presence of many phytochemicals.[5] They have also proved to have better antimicrobial activities to fungi species with higher minimum inhibitory concentrations from the local research.[6] Antibiotics that have been discovered have little success rates during Acanthamoeba treatments, making it difficult to completely heal. Much research is still needed to discover potential herbs and ways to help fight the Acanthamoeba species.[7]

Materials and Methods

Sampling and data collection

The research was done at the Department of Biotechnology and Biochemistry at the University of Zimbabwe. Acanthamoeba species were isolated from water samples collected from 5 boreholes in Budiriro 5 district in Harare. The collected water samples were put in 5 different sterilized 500 mL Scot bottles and transported to the laboratory within 2 hours of collection. Plant samples used for this research were selected by contacting questionnaires to local herbalists in Harare. Many herbalists highlighted the use of Murumanyama *(Xerroderris stuhlmanni)*, Munhundurwa (*Solanum incanum*), and Mufandichimuka (*Myrothamnus flabellifolius*) in the treatment of various diseases. The herbalists had no actual knowledge of the effects of the stated herbs on acanthamoeba species; therefore, the researcher took it to test their effects on the acanthamoeba isolates isolated from selected borehole water.

Isolation and identification of Acanthamoeba species from borehole water samples

Preparation of non-nutrient agar

Non-nutrient agar plates were prepared by first preparing 2 stock solutions of *E. coli* and pipetting 5 mL of each stock solution into 1000 mL of distilled water and 15 g of agar bacteriology media. The media was autoclaved for 15 minutes at 1200 rpm. Media was left to cool down to 45ºC in a water bath. After cooling, the media was poured into every petri dish in a fume hood.

Culturing of Acanthamoeba

Water samples were streaked on non-nutrient agar plates and cultured for 2 weeks to one month. Plates were constantly observed for colony growth and colony morphology in order to identify the Acanthamoeba strain. Other methods that were used to identify and confirm the Acanthamoeba were the PCR method, gel electrophoresis, and identification on the microscope.

Giemsa staining and microscope identification

Thick and thin smears were prepared on microscopic slides. The plates were first heat-fixed using a burner and then treated with methanol by immersing for 5 minutes to dissolve excess colonies on the smears. Giemsa solution was then flooded on top of the slides on a slide rack for 30 minutes. The slides were then rinsed off the excess Giemsa solution for 10 seconds and were left to air dry. Air-dried plates were then viewed using a light microscope at X100 magnification using immersion oil to identify the Acanthamoeba and their developmental stage.

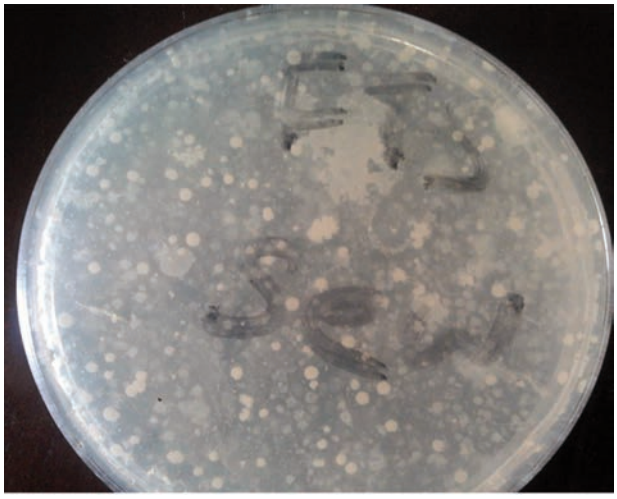

Figure 1. Creamy and rounded colonies on non-nutrient agar media. These colonies were seen on the culture plates of all borehole water samples.

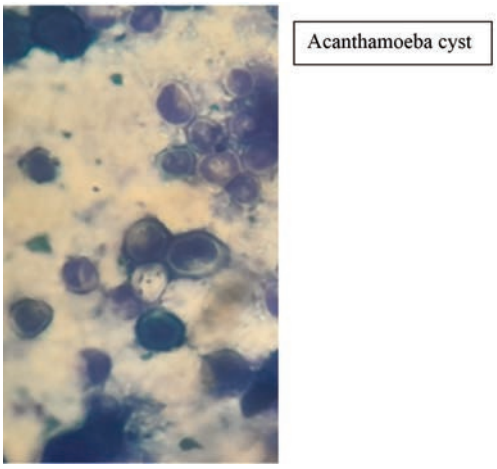

Figure 2. Picture of cysts on Giemsa-stained slides viewed on a light microscope at X100 magnification. Cysts are seen as rounded cells with dark stains inside, as shown by an arrow in the image.

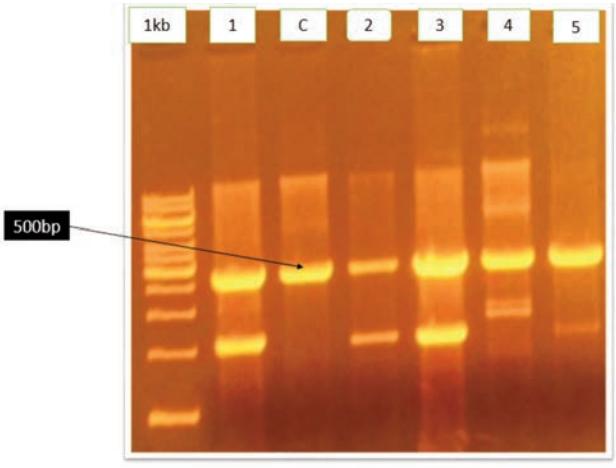

Figure 3. Gel image of Polymerase Chain Reaction (PCR) products. 1kb is the 1kb ladder; C is *Acanthamoeba castellannii* positive control; 1, 2, 3, 4, and 5 are the amplified DNA from borehole samples.

DNA preparation and Polymerase Chain Reaction

Acanthamoeba colonies were picked from non-nutrient plates and prepared to isolate DNA to use for PCR and agarose-gel electrophoresis. DNA was extracted from the non-nutrient agar colonies with the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The 18S rDNA of Acanthamoeba, which distinguishes it from other amoeba genera (Hartmannella, Naegleria, Balamuthia, Nuclearia, and Vahlkampfia), was amplified using reported primers and probe sets; Forward: 5'-CGACCAGCGATTAGGAGACG-3'; Reverse: 5'-CCGACGCCAAGGACGAC-3'; Taqman probe -FAM-TGAATACAAAACACCACCATCGGCGC-BHQ.

Real-time PCR was performed and analyzed using the Light Cycler (Roche, Basel, Switzerland) under the following conditions: 95°C for 15 minutes, followed by 50 cycles at 95°C for 10 seconds, and 60°C for 1 minute. Amplification products were fractionated using 2% agarose electrophoresis gel stained with a solution of 20.000X of REALSAFE Nucleic Acid Staining Solution and visualized under ultraviolet light. *Acanthamoeba castellannii* Neff ATCC 30010 strain was used as a positive control, and distilled water was added to the reaction mixture (instead of DNA) as the negative control.

Plant sample preparation and determination of anti-amoebic effects

Plant sample preparation

Collected plant samples were sun-dried for 3 weeks on top of a shed. Dried barks of Murumanyama *(Xerroderris stuhlmannii)* tree were ground into fine powder using a mortar and pestle. The ground powder was stored in sterilized beakers covered with aluminium foil pending extraction. Mufandichimuka (*Myrothamnus flabellifolius*) stems were cut into smaller pieces using a blade. Small cut pieces were ground in a separate mortar and pestle until a fine powder was produced. Powdered products of both herbs were stored in respective beakers pending extraction. Munhundurwa (*Solanum incanum*) fruits were cut into smaller pieces and ground to fine powder.

Plant sample extraction

Extraction for phytochemicals was done using Soxhlet extraction. A mass of 300 g was weighed for each prepared plant sample powder and put each in separate thimbles. Thimbles were put in Soxhlet extractor, and for each sample, methanol was used as the extraction solvent. A single extraction process was done for 3 hours at 64ºC. The methanol-plant extract was dried up using a Rota-vapor in vacuum at 60°C. A mass of 2000 µg dried powder of each plant extract was added to 20 mL of methanol in order to obtain 200 µg/mL of solvent extracts in closed 50 mL test tubes. Four different concentrations were then prepared by serial dilutions from each plant extract which are 25 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL.

Anti-amoebic determination assay

An antimicrobial assay was done to determine the anti-amoebic effects of the plant extracts against the isolated Acanthamoeba. A sterilized cock borer was used to punch small holes on non-nutrient agar plates inoculated with Acanthamoeba. A volume of 1 mL for all serial dilutions of plant extracts (25 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL) was put into punched holes of inoculated non-nutrient agar plates inoculated with Acanthamoeba and seeded with *E. coli* as food for the Acanthamoeba species. Chlorhexidine gluconate was used as a positive control, and non-inoculated plates as negative controls. Media plates were cultured for up to 4 weeks at 22°C. Using a ruler, the Kirby-Bauer method was used to measure and calculate the diameter of the zone of inhibition in millimeters. The mean and standard deviation were calculated on all 3 replicates per test using a calculator.

Results

Isolation of Acanthamoeba from borehole water

Acanthamoeba was isolated from collected borehole water samples using non-nutrient agar media. Non-nutrient agar media was used to isolate Acanthamoeba species, for it does not provide nutrients for the growth of other organisms but rather Acanthamoeba, which feeds on only seeded Gram-negative bacteria. After 2 weeks of incubation, small rounded creamy colonies were observed on top of non-nutrient agar media, as shown in Figure 1.

Acanthamoeba confirmation

Giemsa staining, PCR, and gel electrophoresis were done to confirm if the colonies observed were Acanthamoeba species. Forward and reverse 18s rDNA primers were used during the PCR reaction, which only amplifies Acanthamoeba amino acids. Figures 2 and 3 show pictures of Giemsa-stained slides on a microscope and gel electrophoresis of PCR products, respectively.

Anti-amoebic effect determination

The results show that all the tested samples had no anti-amoebic effects compared to the positive control chlorhexidine, as shown in Table 1.

Table 1. Zones of inhibition at different methanol plant extract concentrations (µg/mL) and chlorhexidine.

Zones of inhibition in mm of test antimicrobials (mm in diameter) (M±SD) (n=3)			
Murumanyama (Xeroderris stuhlmannii) bark methanol extract			
25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
2±0.05	2±0.19	3±0.02	3±0.10
Munhundurwa (Solanum incanum) fruits methanol extract			
25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
2±0.11	2±0.22	4±0.02	1±0.05
Mufandichimuka (Myrothamnus flabellifolius) stems and leaves methanol extract			
25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
2±0.38	2±0.10	2±0.06	3±0.02
Chlorhexidine gluconate	28.4±0.12		

M, Mean; SD, Standard Deviation.

Discussion

Acanthamoeba are free-living ubiquitous protozoa that have been isolated from different environmental surfaces, including freshwater environments.[8-10] In this current study, *Acanthamoeba castellannii* was confirmed to be present in all the tested borehole water samples using Giemsa staining, PCR, and gel electrophoresis. The gel bands of samples 4 and 5 real-time PCR products were similar to those of control *Acanthamoeba castellannii* Neff ATCC 30010 (Figure 3), whilst samples 1, 2, and 3 had another second visible and brighter 200bp band, which showed the co-presence of another species on non-nutrient agar colonies which were picked by the primers. Thus, more research is required on gene sequencing of the PCR products to identify the 200 bp amplified genome. These results show that water from these boreholes is not safe to drink as *Acanthamoeba castellannii* species are known to have a symbiotic relationship with bacteria and fungi, which can be life-threatening to humans.[5,11]

Upon confirming that the isolated species were *Acanthamoeba castellannii,* the anti-amoebic effects of some selected plant samples were investigated against *Acanthamoeba castellannii.* The results showed that all the plant extracts had no anti-amoebic effects against *Acanthamoeba castellannii*. The positive control chlorhexidine gluconate, proved to be a good disinfection as it showed anti-amoebic effects against *Acanthamoeba castellannii* in this study. Chlorhexidine gluconate is a broad-spectrum antiseptic and has been widely used in a range of applications, including wound care, hand washes, preoperative body showers, oral hygiene, and general disinfection.

Currently, there are new cases of AK patients recorded at Harare Hospital and Parerenyatwa Groups of Hospitals in Harare, Zimbabwe, which are mainly related to immunocompromised and old aged people. The presence of different species of Acanthamoeba and high concentrations of *Acanthamoeba castellannii* in underground borehole water in Harare justifies a number of new cases of AK recorded each year in Zimbabwe.[12] However, there is a need for more research to identify possible sources and the natural habitat of Acanthamoeba castellannii in Zimbabwe to help fight Acanthamoeba infections, which have become a threat to people.

Conclusions

The most common Acanthamoeba species in borehole water in Budiriro District in Harare, Zimbabwe, is the *Acanthamoeba castellannii*. The southern part of Budiriro District had another Acanthamoeba strain, which must be confirmed using gene sequencing. This strain is absent in the northern part of Budiriro District. Murumanyama (*Xeroderris stuhlmannii*) bark extracts, Munhundurwa (*Solanum incanum*) fruit extract and Mufandichimuka (*Myrothamnus flabellifolius*) leaf and stem extracts had no anti-amoebic effects against *Acanthamoeba castellannii*.

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