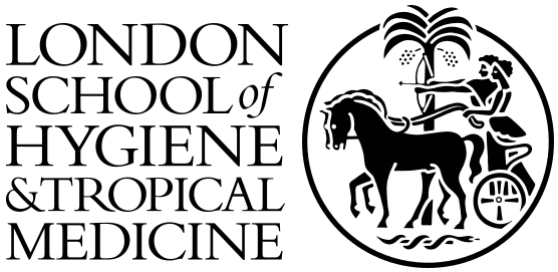

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Evaluation of Key Antimicrobial Properties of *Moringa oleifera* in Relation to Its Use as a Hand-Washing Product

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Abstract: *Moringa oleifera* (*M. oleifera*) is a fast-growing, drought-resistant plant found throughout tropical and subtropical regions. A previous study found dry *M. oleifera* leaf powder to be similarly efficacious to non-medicated soap when used as a hand-wash, even without the use of water. These characteristics suggest that *M. oleifera* could serve as a potential hand-washing product in water and resource-limited contexts, such as humanitarian and emergency settings. The purpose of this study was to assess the efficacy of minimally processed *M. oleifera* sourced locally in Ghana as a hand-washing and antimicrobial product by assessing whether: (1) different preparations of *M. oleifera* have antibacterial properties against potential diarrheal pathogens through set-up of die-off studies; (2) *M. oleifera* is an effective hand-washing product by conducting an in-vivo trial with healthy volunteers; and (3) *M. oleifera* has antimicrobial properties in potentially reusable aqueous solutions, such as rinse water used for hand-washing. *M. oleifera* was found to be significantly less effective than non-medicated soap when tested as a hand-washing product and promoted the growth of bacteria in aqueous solution. Moreover, the *Moringa* used in the study was found to be host to pathogenic bacteria, reinforcing the idea that it is unsuitable to use as a hand-washing product. Accordingly, in its minimally processed form, *M. oleifera* appears to be an ineffective antimicrobial agent and its use as a hand-washing product in water-scarce and resource-limited settings is not recommended.

Keywords: *Moringa oleifera*; diarrhoea; hand-washing; water; filtration; faecal indicator bacteria; antibacterial; Ghana; humanitarian

1. Introduction

Diarrhoeal diseases kill more children than malaria, HIV, and measles combined [1]. Although reductions in diarrhoea-related mortality have been made in recent years, incidence and diarrhoea-attributable morbidity remain high with diarrhoea often leading to serious sequelae including environmental enteropathy of the small intestine, malnutrition, and stunting [2–4]. Significant progress in diarrheal reduction has not been reflected in humanitarian settings where diarrhoea continues to account for 40% of deaths [5]. While a number of factors influence an individual's likelihood to develop diarrhoea, hand-washing with soap has been shown to be exceedingly important in reducing infectious diarrhoeal incidence [4,6]. Specifically, it has been estimated that up to 50% of diarrheal

disease could be avoided if proper hand-washing with soap were consistently employed [7], not to mention reductions in respiratory, skin, and all other faecal–oral infections [8].

Moringa oleifera (*M. oleifera*) is a fast-growing plant, native to the foothills of the Himalayas and now found throughout much of the tropics [9]. The plant is known to be multi-purpose [9–11]. Various parts of the plant have been used in traditional medicine, for food, and even as a water purifier [9,11–13]. Recently, there has been interest in identifying active components of the plant for potential use in the treatment of communicable and non-communicable diseases [9,10]. The plant has been identified as a potent antioxidant, anti-inflammatory, anti-cancer agent, and abundant source of nutrients [10,11,14,15].

The plant's usefulness as an antimicrobial agent has also been evaluated in a number of studies [15,16]. While some have found that different preparations of the plant have broad-spectrum antimicrobial activity [9,10,15,17], others report the plant as active only against Gram-positive organisms [18], and some have suggested that its efficacy is mixed and species dependent [11]. There are several potential explanations for such variation. Namely, the methods used for harvesting, drying, and preparing the plant have varied greatly from study to study. Furthermore, the time of year at which the plant was harvested and the environmental conditions have also been highly divergent.

Although soap is relatively inexpensive, its use and availability in resource-poor and humanitarian settings is often limited [19] and more traditional hand-washing methods, such as the use of mud and ash, are still common [20]. Even when soap is freely and readily available, many individuals continue to wash their hands with water alone despite being aware of the importance of using soap [19,21,22]. Given that using water alone for hand-washing is considerably less effective at removing microbes from hands [23–25], individuals that wash their hands with water alone may be at a higher risk of acquiring or transmitting infections [25]. Studies evaluating the efficacy of *M. oleifera* as a water purifier have found that people were happy to use the plant [26], suggesting that similar attitudes may be displayed toward using *M. oleifera* as a potential alternative hand-washing product. Moreover, considering the plant's widespread availability in the tropics, it was hypothesised that *M. oleifera* may be an effective and acceptable hand-washing product in resource-limited and humanitarian settings. This served as the motivation for a recent clinical study in which *M. oleifera* was evaluated as a potential hand-washing product [27]. The study found 4 g of dry *M. oleifera* leaf powder (sourced commercially in Europe) similarly efficacious to regular, non-medicated soap. Specifically, in both dry and wet forms, *M. oleifera* was able to remove a comparable amount of bacteria from artificially contaminated hands [27].

The current study was therefore designed to further evaluate *M. oleifera*'s potential as a locally-sourced hand-washing product in resource-limited and humanitarian settings. The plant's widespread availability and thus the ability to source it locally is particularly important as it usually takes six weeks or more for basic supplies such as soap to arrive at refugee camps [28]. However, for a soap to simply remove bacteria from hands is not enough. In humanitarian and resource-limited settings where water is routinely scarce, water used for hand-washing is often reused [29–35]. Accordingly, understanding what happens to bacteria that have been removed from hands is of extreme importance. In situations where hands are washed communally in basins or receptacles, something which is common in Ghana and other developing countries [31,33–36], the risk of infection from using communal water, can itself pose a risk due to an accumulation of pathogenic organisms [29,31,32,35]. In fact, some studies have estimated that 80% of schools in Ghana make use of basins for hand-washing [29]. A study conducted at eight different primary schools in Ghana found that only one school had an adequate hand-washing facility with clean running water while the other schools had children make use of communal hand-washing facilities consisting of a receptacle filled with soapy solution, this being mirrored in the homes of the children participating in the study [36]. Further, in such settings, soap is often locked away to prevent misuse and thus children simply wash their hands with the reused basin water alone [29]. Moreover, the water is often only replaced once it appears visibly dirty [37], which may take several days. Accordingly, should *M. oleifera* aid or accelerate bacterial death, it may be advantageous as a hand-washing product

compared to traditional non-medicated soaps that simply act as detergents and remove pathogens but otherwise do not interfere with their integrity. Accordingly, to build off previous research and further assess *M. oleifera*'s potential as a hand-washing product in resource-limited and humanitarian settings, two locally-sourced, simple preparations of *M. oleifera* were evaluated as hand-washing products. To evaluate other properties of the plant in relation to its use as a hand-washing product, die-off experiments were set up to assess whether *M. oleifera* has bacteriostatic or bactericidal properties in aqueous solutions, such as rinse water used for communal hand-washing.

2. Materials and Methods

2.1. Antibacterial Properties of Minimally Processed M. oleifera Against Faecal Indicators Bacteria in Solution

2.1.1. Bacterial Strains

To assess the antibacterial properties of locally-sourced, minimally processed *M. oleifera* against potential diarrheal pathogens, two faecal indicator bacteria, *Escherichia coli* (*E. coli*) (ACTC 25922) and *Enterococcus faecalis* (*E. faecalis*) (ATCC 29212), were used for conducting all experiments [38,39].

2.1.2. *M. oleifera* Preparations

Three different preparations of *M. oleifera* were evaluated: fresh, ground *M. oleifera* leaves; boiled, ground *M. oleifera* seeds; and dry *M. oleifera* leaf powder. All *Moringa* products were purchased locally in Accra from the Ghana Permaculture Institute. As per the Institute's protocol, *M. oleifera* was picked in the morning and washed with saline solution (NaCl). The fresh leaves and seeds were then separately placed inside plastic bags with holes for aeration before distribution. For the preparation of dry *M. oleifera* leaf powder, washed leaves were placed in a drying oven before being ground into a powder.

The dry *M. oleifera* leaf powder was used as purchased. Fresh ground leaves were prepared by blending 500 g of fresh *M. oleifera* leaves and 300 mL of sterile, distilled water for 2 min until a homogenous mixture was achieved. Ground *M. oleifera* seeds were prepared following the same method and after blending the mixture was boiled for 5 min, as it has been suggested that the most potent antimicrobial component of *M. oleifera* seeds is contained within the seed as a water-soluble lectin [40,41]. Both of the prepared mixtures were refrigerated at 6 °C until ready for use.

2.1.3. Set-up and Estimation of Colony Forming Units (cfu/mL)

Seven 2000 mL reagent bottles containing 1190 mL of sterile, distilled water were inoculated with 10 mL of a 1.0 McFarland Standard of *E. coli* and the colony forming units (cfu/mL) was assessed immediately after inoculation using membrane filtration with an OXFAM-DELAGUA Water Testing Kit (DelAgua Water Testing Limited, Marlborough, UK) to provide a Day 1 cfu/mL. Hydrophilic, 0.45 µm mixed cellulose membranes were used for filtration of samples. Membranes were placed onto Brilliance *E. coli*/Coliform Selective Agar (Oxoid, Hampshire, UK, CM1046), a chromogenic agar which distinguishes *E. coli* from other coliforms due to its ability to cleave two substrates: Rose-Gal, which detects ß-galactosidase activity and causes colonies to appear pink, and X-Glu, which detects ß-glucuronidase activity and in turn cause colonies to appear purple/blue in colour [42]. Plates were incubated at 37 ± 1 °C for 18–24 h. Upon removal, colonies on plates were counted within 30 min. The estimated cfu/mL of each bottle was calculated using the following formula:

$$\frac{\text{cfu}}{\text{mL}} \text{ of sample} = \frac{\text{Number of colonies on membrane}}{\text{Volume of sample filtered (mL)}} \quad (1)$$

Two different amounts of each *M. oleifera* preparation were assessed: 10 g and 50 g of the fresh ground leaf mixture; 10 g and 50 g of the boiled, ground seed mixture; and 10 g and 25 g dry leaf powder, the amount for dry leaf powder being lesser due to the preparation being undiluted.

After bottles were inoculated with *E. coli* and the Day 1 cfu/mL was assessed, each respective preparation of *M. oleifera* was added to a separate bottle. One bottle containing only *E. coli* served as control. Bottles were incubated at 37 ± 1 °C for 18–24 h. The Day 2 cfu/mL of each bottle was assessed by preparing serial dilutions. Diluted samples were then processed using membrane filtration. The cfu/mL of each bottle was reassessed in this way every 18–24 h for a total of five days. All preparations were tested in triplicate.

To test the theory that *M. oleifera* may only be active against Gram-positive organisms [18], a brief experiment was set up using the same methods outlined above but using *E. faecalis*. As Brilliance plates are selective and do not support the growth of Gram-positive organisms, Tryptone Soya Agar (Oxoid, CM0131) was used for this experiment. The experiment was not run in triplicate (n = 1) and the cfu/mL of each preparation was assessed for a total of three days instead of five.

2.1.4. Quality Control

To ensure the accuracy and reliability of results, 100 mL of sterile, distilled water was incubated at 37 ± 1 °C and processed as a blank control at the beginning, middle, and end of each day throughout the duration of the study.

2.2. Hand-Washing Trial with Healthy Volunteers

2.2.1. Study Design

The hand-washing trial was conducted using an adaptation of the European Committee for Standardization protocol 1499/1500—a protocol used to evaluate the efficacy of new hygienic hand-washing products in reducing transient microbial flora on artificially contaminated hands [43]. The protocol requires a quantification of the number of bacteria present on hands of participants after artificial contamination with a select non-pathogenic bacterial stock (*E. coli*, ACTC 25922) and again after washing hands with the hand-washing product under evaluation. The recorded $\log_{10}$ reduction in cfu/mL of *E. coli* is then compared to that observed when using a regular, non-medicated reference soap.

Each *Moringa* preparation was tested by fifteen volunteers in total and compared with the efficacy of the non-medicated reference soap in the same fifteen volunteers using a Latin-square design. At the end of the whole series of runs every volunteer used each hand-washing product once, including washing with soap. Only one volunteer participated at a time.

2.2.2. Subjects

Fifteen healthy adult volunteers from the local community in Accra were selected for the study. Volunteers were informed of the purpose and scope of the study verbally and through a written document. Consent was obtained both verbally and in writing.

Volunteers were examined to be physically healthy and did not have any skin disorders. None of the volunteers had taken systemic antibiotics in the two weeks prior to their participation in the study. All participants had short, natural fingernails. All jewellery was removed prior to participation in the study.

The study was carried out at the National Public Health and Reference Laboratory of the Ghana Health Service in Accra, from July to August 2017. The study was approved by the LSHTM Ethics Committee on 13 June 2017. Local ethical approval was obtained from the Ghana Health Service Ethical Review Committee on 30 June 2017 (Reference number: GHS-ERC: 21/05/17).

2.2.3. *M. oleifera* Preparations

Two different preparations of *M. oleifera* were evaluated: 4 g dry *M. oleifera* leaf powder and 5 mL fresh boiled *M. oleifera* leaves. The dry *M. oleifera* leaf powder was used as purchased from the supplier and the fresh boiled *M. oleifera* was prepared by boiling 500 g of fresh *M. oleifera* leaves in 300 mL of

sterile, distilled water for 5 min. The mixture was refrigerated at 6 °C until ready for use. Given that it has been well established that hand-washing with water alone is less effective than hand-washing with soap [23–25], we chose not include hand-washing with water alone in the study.

2.2.4. Contamination Procedure

Participants were asked to wash their hands using non-medicated soap for 1 min as per the standard hand-washing procedure [43] to remove any transient bacteria. Hands were then dried and immersed up to the mid-metacarpal including the thumb in a contamination fluid of Tryptone Soya Broth containing *E. coli* for 5 s. Given that the trial was run over the course of several days, the cfu/mL of the contamination fluid was enumerated each day and ranged between 6.4×10^{10} and 1.73×10^{11} cfu/mL. After contamination, hands were allowed to air-dry for 3 min with care being given to avoid contact with any surfaces.

2.2.5. Pre-Value Estimation

After contamination, participants were asked to rub their fingers and thumbs in a circular motion on the bottom of a two standard 90 mm petri dishes (one for each hand) containing 10 mL of sterile TSB without neutralizers for 1 min. The pre-values for each hand were estimated separately using membrane filtration (see Section 2.1.3). The cfu/mL for each hand was quantified and the results for the right and left hands were averaged to provide the pre-value estimate for each hand-washing procedure.

2.2.6. Hand-Washing Procedure

Once pre-value estimates were obtained, participants were asked to wash their hands for 1 min as per the standard hand-washing procedure with one of three different products: 5 mL of the liquid, boiled *M. oleifera* mixture; 4 g of dry *M. oleifera* leaf powder; and 5 mL of regular, non-medicated soap. After washing their hands with the liquid, boiled *M. oleifera* mixture and the regular, non-medicated soap, 250 mL of sterile, distilled water was dispensed onto hands for rinsing for 15 s. Hands were then allowed to air-dry for 3 min. After washing their hands with the dry *M. oleifera* leaf powder, participants were asked to air-dry their hands for 3 min without rinsing off the powder.

2.2.7. Post-Value Estimation

After air-drying hands for 3 min, participants were asked to rub their fingers and thumbs in a circular motion on the bottom of a two standard 90 mm petri dishes (one for each hand) containing 10 mL of sterile TSB without neutralizers for 1 min as per the pre-value estimation procedure. Post-values were assessed using membrane filtration following the same procedure used for assessing pre-values.

After washing their hands with all three different products, participants were given antibacterial soap to wash their hands followed by a 60% alcohol-based hand-sanitizer.

2.3. Bactericidal or Bacteriostatic Properties of M. oleifera in Potentially Reusable Aqueous Solution

To assess if the *M. oleifera* products had bactericidal or bacteriostatic properties in aqueous solution, the water used for rinsing hands after washing with each different product was collected from three participants (n = 3 for each product) and the cfu/mL was assessed. The water used for rinsing hands after washing with the liquid, boiled *M. oleifera* mixture and the regular, non-medicated soap was collected in a sterile, reagent bottle through use of a sterile, large, plastic filter over which participants rinsed their hands. After post-values were assessed for dry *M. oleifera* leaf powder, participants were given 250 mL of sterile, distilled water with which to rinse their hands and this rinse water was also collected. The cfu/mL of each rinse water sample was assessed using membrane filtration within 1 h of collection to provide the Day 1 cfu/mL. Bottles were stored at room temperature (26 ± 2 °C) to stimulate temperatures of water basins in community settings. The cfu/mL was reassessed every 18–24 h for two more days, resulting in a three-day total.

2.4. Statistical Analysis

All statistical analysis was conducted using the Statistical Package STATA version 15.0.

2.4.1. Die-Off Studies

Paired *t*-tests were conducted to determine if there were significant differences between the change in $\log_{10}$ cfu/mL of Controls compared to the different *M. oleifera* preparations over the course of follow-up.

2.4.2. Hand-Washing Trial

To determine the efficacy of the *M. oleifera* products compared to the reference soap, the arithmetic means of all individual $\log_{10}$ reductions in cfu/mL for each product were calculated. The distribution of the data was assessed using Kurtosis and Skewness tests. Since the data were not normally distributed, the Wilcoxon matched-pair signed ranks test was used to test for differences between each *M. oleifera* preparation and the reference soap. The efficacy of each *M. oleifera* preparation was considered to be the same as the reference soap if the mean $\log_{10}$ reduction factor was not significantly smaller. Considering the confirmatory nature of the protocol used for assessing the efficacy of new hand-washing products, in this case *M. oleifera*, the level of significance is set at $p = 0.1$. The test to be used is two-sided. The discrimination efficiency of the test procedure described has been set to detect a difference between the two mean $\log_{10}$ reduction factors of approximately 0.6 log at a power of 95%.

2.4.3. Rinse Water Bacterial Die-Off

To determine if there were significant differences in the change in $\log_{10}$ cfu/mL of reference soap rinse water samples, compared to boiled *M. oleifera* leaf, and dry *M. oleifera* leaf powder rinse water samples over the days of follow-up, paired t tests were conducted. The mean difference in $\log_{10}$ cfu/mL between consecutive days (Day 1 and Day 2; Day 2 and Day 3) in addition to differences between Day 1 and Day 3 were analysed.

3. Results

3.1. Die-Off Studies

The mean $\log_{10}$ cfu/mL of *E. coli* of Controls and the different *M. oleifera* preparations on Days 1–5 is represented in Figure 1.

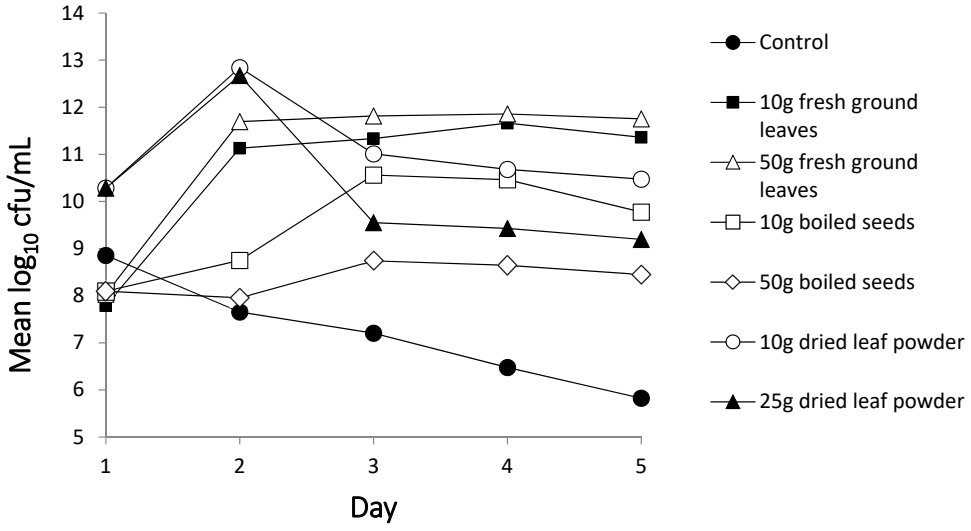

Figure 1. Mean $\log_{10}$ cfu/mL of *E. coli* in bottles containing different preparations of *Moringa oleifera* compared to Controls, Days 1–5.

All the solutions containing *Moringa* preparations, apart from that containing 25 g of dry *M. oleifera* leaf powder, showed an increase of *E. coli* between Day 1 and Day 5, and the cfu/mL of most peaked on Day 2. The $\log_{10}$ cfu/mL of *E. coli* in Controls, however, showed a pattern of decreasing concentration over time.

While Controls saw a 3.031 decrease in mean $\log_{10}$ cfu/mL from Day 1 to Day 5, 10 g dry *M. oleifera* leaf powder resulted in a 0.189 ± 0.180 increase in mean $\log_{10}$ cfu/mL and 25 g resulted in a 1.089 ± 0.301 decrease in mean $\log_{10}$ cfu/mL. Accordingly, dry *M. oleifera* leaf powder was significantly less effective in reducing mean $\log_{10}$ cfu/mL over all days of follow-compared to Controls (10 g: p-value = 0.018; 25 g: p-value = 0.026).

Ten and fifty grams of fresh ground *M. oleifera* leaves resulted in a 3.569 ± 0.291 and 3.719 ± 0.046 increase in mean $\log_{10}$ cfu/mL, respectively, from Day 1 to Day 5. The differences in mean $\log_{10}$ cfu/mL compared to controls were also significant for both 10 g (p-value = 0.001) and 50 g (p-value = 0.003) of fresh ground leaves. A dose–response relationship was also observed as bottles containing 50 g of fresh ground *M. oleifera* leaves had a higher $\log_{10}$ cfu/mL than those containing only 10 g.

Ten and fifty grams of boiled ground *M. oleifera* seeds resulted in a 1.678 ± 0.222 and 0.353 ± 0.131 increase in the mean $\log_{10}$ cfu/mL of *E. coli*, respectively, from Day 1 to Day 5. The differences in mean $\log_{10}$ cfu/mL compared to Controls were also significant for both 10 g (p-value = 0.010) and 50 g (p-value = 0.007) of the boiled ground seed mixtures. Moreover, similar to the dry powder, bottles containing greater amounts of the boiled seed mixture seemed to have a lower $\log_{10}$ cfu/mL than those containing less.

Unexpectedly, it was found that that the fresh ground *M. oleifera* leaves and the dry *M. oleifera* leaf powder were themselves contaminated with bacteria upon arrival into the laboratory and thus introduced other bacterial species into experiment. This was evidenced due to Brilliance plates being able to distinguish *E. coli* from other bacteria chromogenically. On Day 1, all bottles contained only *E. coli*, as the cfu/mL was assessed after inoculation and before the addition of *Moringa* products. Throughout the four days of follow up, controls continued to only show the growth of *E. coli* (which appears as a purple/blue colony). However, starting Day 2, bottles containing fresh ground *M. oleifera* leaves and dry *M. oleifera* leaf powder, showed the growth of *E. coli* in addition to other coliforms which appeared dark pink, light pink, and orange in colour. Such contamination was not found in bottles containing the boiled seed mixture.

3.1.1. Experiment to Determine Extent of *M. oleifera* Contamination

Given the unexpected finding that the *M. oleifera* being used in experiments was contaminated with bacteria upon arrival to the laboratory, two brief experiments were set up to determine if *Moringa* products received from the supplier were contaminated with *E. coli* and/or other coliforms, and confirmatory test were performed to identify the contaminating species.

Six reagent bottles containing 99 mL of sterile, distilled water were set up. A different preparation of *M. oleifera* was then added to each of the bottles: 1 mL of a fresh ground *M. oleifera* leaf mixture; 1 mL of a boiled ground *M. oleifera* leaf mixture; 1 mL of a ground *M. oleifera* seed mixture; 1 mL of a boiled ground *M. oleifera* seed mixture; and 1 g of dry *M. oleifera* leaf powder (all prepared using the method outlined in Section 2.1.2). One bottle in which no *Moringa* was added served as a control. The bottles were incubated at 37 ± 1 °C and the cfu/mL of each preparation was assessed after 18–24 h using membrane filtration.

While the boiled *M. oleifera* leaf and the boiled, ground *M. oleifera* seed mixtures did not show the presence of any bacteria, while the non-boiled ground leaf and seed mixtures and the dry *M. oleifera* leaf powder did, indicating that the fresh leaves, fresh seeds, and the dry *M. oleifera* leaf powder were all contaminated.

To verify the results, another batch of fresh *M. oleifera* leaves, seeds, and dry leaf powder was tested from the same supplier (Ghana Permaculture Institute). The results were found to be the same

for the new batch, establishing that all three forms of *Moringa* (fresh leaves, fresh seeds, and dry leaf powder) were contaminated upon entry into the laboratory.

To determine the species of bacteria present on *M. oleifera*, three reagent bottles containing 100 mL of sterile, distilled water was prepared. Each bottle received a different preparation of *Moringa*: 5 g of dry *M. oleifera* leaf powder; 5 g fresh *M. oleifera* leaves; and 5 g fresh *M. oleifera* seeds. Bottles were incubated at 37 ± 1 °C for 18–24 h. The next day, a 10^{-6} and a 10^{-8} mL dilution of each mixture was processed using membrane filtration. Membranes were placed onto Brilliance plates which were incubated at 37 ± 1 °C for 18–24 h. Several drops of each mixture were also dispensed directly onto a separate Brilliance plate.

All preparations showed contamination as evidenced by the different coloured bacterial colonies present on membranes. Each uniquely coloured bacterial colony was sub-cultured onto separate Brilliance and TSA plates to obtain pure cultures. These plates were incubated at 37 ± 1 °C for 18–24 h. To determine the bacterial species, cultures were then subjected to a number of biochemical tests, including: Gram staining, Tripe Sugar Iron (TSI) (Oxoid, CM0277), Citrate (Oxoid, CM0155), Urea (Oxoid, CM0053), Indole (Becton Dickinson, 261185), and Motility tests [44]. The results of the different tests were read the following day.

Two different colours of bacteria (pink and orange) grew on membranes, whereas only one colour grew on direct drop plates (dark pink). Pure cultures confirmed to the colours of the respective colony that had been picked up. The dark and light pink colonies were confirmed to be *Klebsiella oxytoca* and orange colonies were identified to be *Serratia* spp. [44]. Moreover, the colours of *K. oxytoca* and *Serratia* spp. on Brilliance plates conforms to findings of another study in which the chromogenic properties of the Brilliance agar were evaluated using known reference strains of bacteria [42]. Results are summarized in Table 1.

3.1.2. Antibacterial Activity of *M. oleifera* Against a Gram-Positive Bacterium

Preparations of boiled *M. oleifera* leaves, boiled ground *M. oleifera* seeds, and dry *M. oleifera* leaf powder were found to have a higher cfu/mL of *E. faecalis* than that of the Control after three days of follow up (see Figure 2). That said, small but steady reductions in cfu/mL were observed for the boiled seed mixture. The growth seen on TSA plates containing dilutions of the boiled leaf and dry leaf powder mixtures was confluent on both days of follow-up (hence the graphical representation for both preparations being the same).

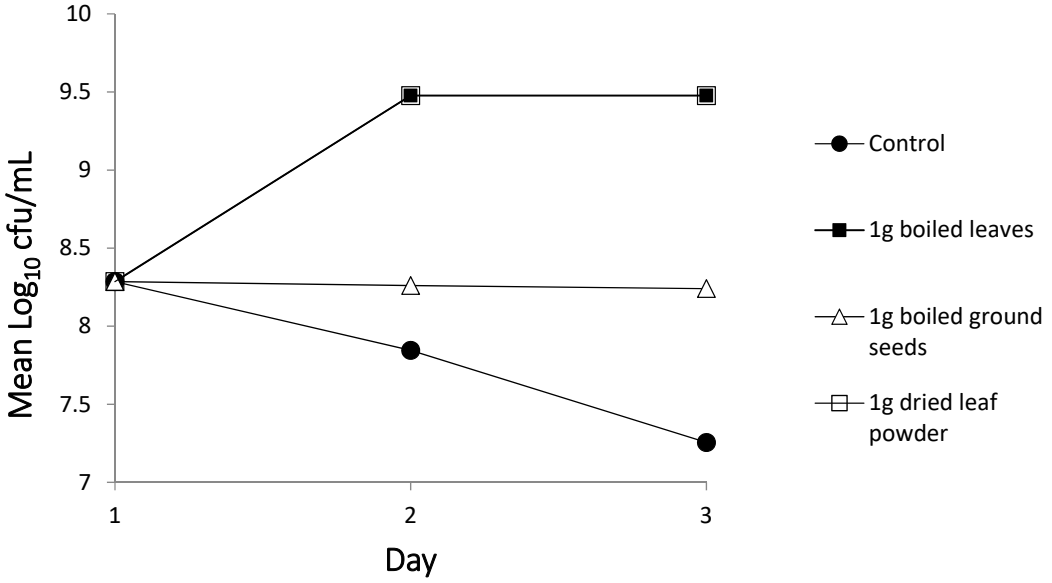

Figure 2. Mean $\log_{10}$ cfu/mL of *E. faecalis* in bottles containing different preparations of *M. oleifera* compared to Controls, Days 1–3.

3.2. Hand-Washing Trial

Hand-washing with 5 mL fresh boiled *M. oleifera* leaves and 4 g dry *M. oleifera* leaf powder resulted in a mean $\log_{10}$ reduction of 2.57 ± 0.26 cfu/mL and 2.02 ± 0.44 cfu/mL, respectively, as shown in Table 2. Both preparations were significantly less effective than regular, non-medicated soap which resulted in a mean $\log_{10}$ reduction of 3.37 ± 0.76 cfu/mL.

3.3. Rinse Water Collection

The $\log_{10}$ cfu/mL of each 250 mL rinse water sample, including regular, non-medicated soap rinse water, increased from Day 1 to Day 3, as shown in Figure 3. The $\log_{10}$ cfu/mL of regular, non-medicated soap rinse water increased by 1.338 ± 0.767 from Day 1 to Day 3. Increases were even more pronounced for boiled leaf rinse water and dry *M. oleifera* leaf powder rinse water which increased by 3.680 ± 1.060 and 4.511 ± 0.314 $\log_{10}$ cfu/mL from Day 1 to Day 3, respectively. The difference in mean $\log_{10}$ cfu/mL for boiled *M. oleifera* leaf rinse water and dry *M. oleifera* leaf powder compared to regular, non-medicated soap rinse water was significant (2.341 ± 1.060 cfu/mL (p-value = 0.012) and 3.173 ± 0.314 cfu/mL (p-value = 0.009) respectively). The mean $\log_{10}$ difference in cfu/mL between different rinse water samples between consecutive days, however, was not consistently found to be significant.

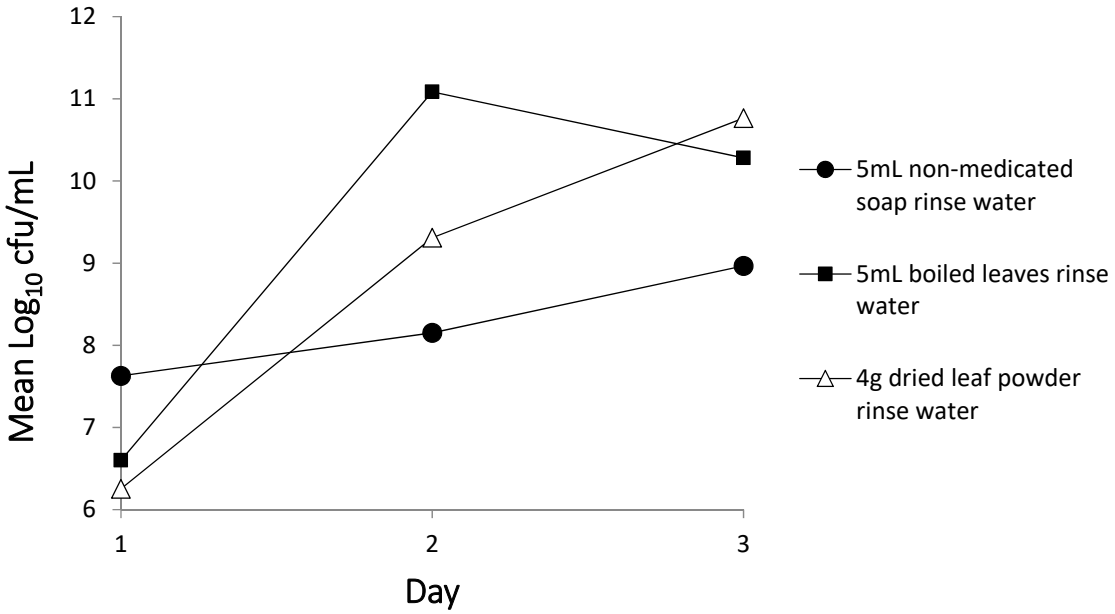

Figure 3. Mean $\log_{10}$ cfu/mL reduction of *E. coli* of bottles containing different rinse water samples including the control (regular, non-medicated soap), boiled *M. oleifera* leaves, and dry *M. oleifera* leaf powder, Days 1–3.

Table 1. Results of differential tests used for identification of bacterial species present on *M. oleifera* leaves.

Colour of Colonies	Gram Stain	TSI—Slope	TSI—Butt	TSI—H_2S	TSI—Gas	Citrate	Urea	Indole	Motility	Species Identified
Dark pink	Gram-negative rods	Yellow	Yellow	−	+	+	+	+	−	*Klebsiella oxytoca*
Light pink	Gram-negative rods	Yellow	Yellow	−	+	+	+	+	−	*Klebsiella oxytoca*
Orange	Gram-negative rods	Red	Yellow	−	−	+	−	−	+	*Serratia* spp.

Note: −—negative result for particular test; +—positive result for particular test.

Table 2. Log_{10} reduction of bacteria (cfu/mL) of different *M. oleifera* hand-washing treatments compared to Control.

Treatment	Mean Pre-Value (log_{10} cfu/mL)	Mean Post-Value (log_{10} cfu/mL)	Mean log_{10} cfu/mL Reduction	Standard Deviation	*p*-Value of Difference in Mean Compared to Control
Control (5 mL regular, non-medicated soap)	8.86	5.49	3.37	0.76	-
5 mL boiled *M. oleifera* leaves	8.90	6.32	2.57	0.26	0.005
4 g dry *M. oleifera* leaf powder	9.03	7.01	2.02	0.44	<0.001

4. Discussion

This study evaluated key antimicrobial properties of minimally processed *Moringa oleifera* in relation to its use as a hand-washing product in humanitarian and resource-limited settings. *M. oleifera* was found to be a significantly less effective hand-washing product than regular, non-medicated soap. *M. oleifera* also promoted the growth of *E. coli* and *E. faecalis* in both sterile and rinse water solutions, further suggesting its poor suitability as a hand-washing product. Moreover, the *Moringa* used in the study was found to be contaminated with *K. oxytoca* and *Serratia* spp., both of which can be pathogenic [45,46], which is especially concerning as individuals living in resource-poor settings are at a heightened risk of acquiring infections due to rampant malnutrition [47].

4.1. Bacterial Die-Off Studies: Antibacterial Properties of M. oleifera Against Faecal Indicators Bacteria in Solution

The findings of the die-off experiments suggest that fresh ground *M. oleifera* leaves, boiled ground *M. oleifera* seeds, and dry *M. oleifera* leaf powder are ineffective antimicrobial agents against the tested faecal indicator bacteria in aqueous solution. In fact, it seems that all three different preparations of the plant promote bacterial growth. The plant itself was also found to be contaminated with bacteria when arrival from the cultivation farm, reinforcing the idea that, in a minimally processed form, *M. oleifera* does not act as an antimicrobial.

While a dose–response relationship was seen for the fresh ground *M. oleifera* leaf mixture wherein bottles containing 50 g had a higher cfu/mL of *E. coli* than bottles containing 10 g, this pattern was not observed for the boiled seed and dry leaf powder mixtures. In the case of the boiled seed mixture, bottles containing greater amounts of the mixture had a lower cfu/mL at the end of five days compared to bottles containing lesser amounts of the mixture. This may be due to a cationic protein, known as *M. oleifera* cationic protein (MOCP), found in the seeds which causes membrane fusion of bacterial cells [14]. Because MOCPs have an overall positive charge, they bind to negatively charged particles, including bacteria [26,48]. Accordingly, this is one of the main reasons why *M. oleifera* seeds are believed to function as a flocculant [49]. That said, it has been suggested that, in the absence of turbidity, the flocculating properties of *M. oleifera* seeds do not function as well [26]. In fact, studies that have looked into the efficacy of *M. oleifera* as a method of water treatment have highlighted that for *M. oleifera*-treated water to be microbiologically safe to drink it should be filtered or sterilised further using sand water filters, solar sterilisation, chlorination, or boiling [13]. Studies that have evaluated *M. oleifera* seeds as point-of-use water purifiers also did not find the plant to be effective in reducing the number of thermotolerant coliforms in water [26]. It has also been noted that there is a risk of secondary infection if the process of water treatment is not followed exactly as prescribed [13]. Specifically, should the flocculation process take too long, bacteria may actually grow during flocculation [13]. Other studies have also discussed the need to properly prepare *M. oleifera* before use to ensure that all microbes are removed [12], again suggesting that it is known that the plant is host to bacteria to begin with.

These results support the idea that *M. oleifera* does indeed promote bacterial growth and are consistent with what was observed in the present study. While it was found that the bottles to which boiled ground *M. oleifera* seeds were added contained a fairly clear solution with much of the particulate matter having settled to the bottom, demonstrating the seed's flocculating properties, this process of sedimentation does not kill bacteria as has been suggested [50].

However, it is worth noting that in most studies non-boiled *M. oleifera* seeds have been used. As such, having boiled the seeds to kill residual bacteria may have changed or denatured the MOCP thus making it such that the seeds no longer had a noticeable antimicrobial effect. That said, the fact that the bacteria not only survived but grew suggests that the seeds themselves have nutrients that allow for the proliferation of bacteria. Further, despite the 50 g bottles having a lower cfu/mL than the 10 g bottles, the $\log_{10}$ reduction in cfu/mL from Day 1 to Day 5 was still significantly less than

that of the Controls, suggesting that the antimicrobial properties of boiled seeds are, at least using this amount and in this form, not very potent.

Similar to the boiled ground *M. oleifera* seeds, bottles containing greater amounts of dry *M. oleifera* leaf powder had a lower cfu/mL at the end of five days. A potential explanation is that the exponential growth phase took place during the 18–24 h of initial incubation followed by a sharp decrease in cfu/mL during the bacterial death phase. This would mean that by the time the Day 2 cfu/mL was assessed the exponential growth phase had already finished and bacterial die-off had begun. The feasibility of this explanation is reinforced by the fact that: (1) *M. oleifera* is highly nutritious and carbon-rich [51] meaning that it provides a good medium for bacterial growth; and (2) it has been found that dry *M. oleifera* leaf powder is more nutritious per gram compared to fresh *M. oleifera* leaves [51]. On average, 100 g of fresh *M. oleifera* leaves contain 86.6 kcal of energy, whereas 100 g of dry *M. oleifera* leaf powder contain 304 kcal of energy [51]. Moreover, given the abundant supply of nutrients available in bottles containing greater amounts of dry *M. oleifera* leaf powder, it is conceivable that the exponential growth phase took place over a shorter period, ultimately leading to a bloom in bacteria that went unnoticed due to the period in which the reassessment of cfu/mL took place (every 18–24 h). Accordingly, it may be that the exponential phase was not quite as steep for bottles containing 10 g of dry *M. oleifera* leaf powder as the cfu/mL of the such bottles was higher for the duration of follow-up with a gentler rise and fall of cfu/mL overall (see Figure 1).

The rate of bacterial die-off observed for the Controls is in accordance with findings elsewhere that suggest that die-off of *E. coli* does not occur at a constant rate [52]. Given that functions which affect the growth and survival of *E. coli* are more or less conserved amongst strains [39,53], the findings and implications of this study can be taken to apply to pathogenic strains of *E. coli* as well. That said, it would be concerning to promote the use of a hand-washing product that increases the number of bacteria in solution in water-scarce settings where water is commonly reused, especially as this can have serious implications for infections such as Enterohaemorrhagic *E. coli* (EHEC) which has a low infectious dose (~100 organisms) [39].

The trend observed for *E. coli* was relatively similar to that of *E. faecalis*. The fact that reductions, albeit small, were observed for the boiled seed mixture, suggests that boiled seeds may be more active against Gram-positive organisms as indicated by other studies [18].

4.2. Efficacy of M. oleifera as a Hand-Washing Product

While both forms of *Moringa* tested in the hand-washing trial were found to be less effective at removing bacteria from hands than regular, non-medicated soap, these findings stand in contrast to those obtained in a previous trial which evaluated dry and wet *M. oleifera* leaf powder as a hand-washing product [27]. Several factors may contribute to this difference in findings. Namely, the previous trial was conducted using commercially-sourced *Moringa* purchased from a European manufacturer. It may be that the process used for producing the leaf powder was more in accordance with European regulation for processed food or plant products. It is also unclear whether the *Moringa* used in the previous study was grown in Europe and under what conditions or if it was sourced from abroad and processed in Europe. Although the use of the *Moringa* products did result in a decrease in bacteria on hands, this decrease was significantly less than the reduction observed with regular, non-medicated soap.

Other traditional hand-washing products like soil, ash, and mud have been also used in many settings and have been shown to reduce bacterial counts on hands [20]. It has been suggested that the mechanical friction applied during hand-washing may be responsible for the removal of microorganisms. It is possible that *Moringa* could have similar properties which may explain why it was previously found that sterile *Moringa* was similarly efficacious to soap [27]. However, in this study, we have observed that in its minimally processed form, *Moringa* was host to potentially pathogenic organisms. Interestingly, this is also common of soil and ash which are frequently contaminated [20], suggesting that the use of all such traditional or directly-sourced, natural products may be dangerous

as they may promote the spread of infectious disease. Therefore, we need to be cautious when recommending the use of such products when sourced directly from nature, and more research is needed to determine the benefits and risks of such products before recommending their use.

4.3. Bacterial Die-Off in Rinse Water

Similar results to those obtained in the bacterial die-off experiments were observed in the rinse water die-off experiments as boiled *M. oleifera* leaves and dry *M. oleifera* leaf powder did not exhibit antimicrobial properties in rinse water solution, instead promoting the growth of *E. coli* to a greater extent than regular, non-medicated soap.

4.4. Contamination of M. oleifera

While it is unclear where exactly the contamination found on *M. oleifera* leaves, seeds, and powder came from, it is very likely that this was not contamination at all, instead being an amplification of natural plant or environmental flora as the species identified are known to be found in the environment and on plants [46,54–56]. It is also possible that the contamination came from the fertilizer or watering source used at the farm at which the *Moringa* was grown. This is feasible as *Klebsiella* species are common inhabitants of water environments and can multiply to high numbers if conditions are appropriate [39]. Recent studies in Accra have isolated *K. oxytoca* from *Cyperus esculentus* L. (tiger nuts) which were claimed to have been washed and which were being sold at local markets [57]. *K. oxytoca* has also been isolated from water used for hand-washing in preschools in the Accra, leading researchers to believe that the bacterium may be present in local water sources [29]. Moreover, studies in West Africa and Ghana have found faecal contamination of produce to be quite common [58], suggesting that the contamination observed in the present study should not be regarded as surprising.

Interestingly, both *K. oxytoca* and *Serratia* spp. have been found to thrive in salty environments [59,60] and thus the saline rinse used to remove bacteria from *Moringa* plant in the cultivation farm may have aided their survival and proliferation. This characteristic of being able to survive in salty environments is rather uncommon as salt can induce osmotic stress [61,62]. Accordingly, it may be that there were other bacteria present on *M. oleifera* plants which were removed by use of the saline rinse, with the two species identified being all that remained post-washing.

Given that the *M. oleifera* used for the study was sourced from a local *Moringa* farm based in Accra, the fresh leaves and seeds were often placed in aerated, plastic bags which were loosely knotted for purposes of transport. Thus, they often arrived warm or hot upon entry to the laboratory. This may in fact have accelerated the process of putrefaction, causing the heat generated in the bags, and accordingly increased the number of bacteria present on leaves and seeds by providing optimal conditions for growth. However, for the concentration of bacteria on the leaves and seeds to have increased, there must have been bacteria present on the leaves to begin with which still makes them unsuitable for consumption without boiling or proper preparation.

4.5. Limitations

While only two bacterial species were confirmed to have been present on the *Moringa* used for this study, it should be noted that Brilliance plates are selective and only allow for the growth of select Gram-negative coliforms. Accordingly, it may be that there are other species of bacteria present on *Moringa* that did not grow on the agars used, this being something that should be looked into further. Further, the *M. oleifera* used in the present study was sourced from only one producer and as such the results may not be representative for *Moringa* obtained elsewhere or produced differently. Going forward, it will be important to verify these results by testing *M. oleifera* sourced from elsewhere to note any similarities and/or differences in results. Moreover, while this study has assessed the efficacy of *Moringa* against commonly used faecal indicator bacteria (*E. coli* and *E. faecalis*) [39], the effect of *Moringa* against viral and parasitic causes of diarrhoea was not assessed and the results of such studies may prove different.

5. Conclusions

The results obtained in this study suggest that in a minimally processed form *M. oleifera* is a significantly less effective hand-washing product compared to regular, non-medicated soap. *M. oleifera* was also not found to be an effective antimicrobial against faecal indicator bacteria in aqueous and rinse water solution. Moreover, given that the *M. oleifera* used in the study was found to be contaminated with potentially pathogenic bacteria that may promote the spread of infectious disease, its use as a hand-washing product in resource-poor and humanitarian settings is not recommended.

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