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Antimicrobial Effect of Coagulant Protein Purified from Vigna Unguiculata Seed and Citrus Fruit Juice on River Water Bacteria

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Abstract:
Antibacterial effect of Vigna unguiculata coagulant proteins and Citrus fruit juice (CFJ) on bacterial strains isolated from Ruvu River at Mlandizi, in Tanzania was studied. Out of 126 isolated strains 60 were characterised by morphological, biochemical and 16s rRNA gene sequence analysis. A group of strains were identified either as Acinetobacter, Pseudomonas Vibrio, Aeromonas or Bacillus spp. Coagulant proteins of V unguiculata (VUP) seeds were purified by ion exchange chromatography. Flocculation studies showed that, VUP and CFJ aggregate both Gram-positive and Gram-negative bacteria. Growth studies revealed that minimum inhibitory dosages of VUP was between 0.38 and 0.75 mg/L while for CFJ was between 0.2 and 0.5%, and growth inhibition occurred within 2 to 4 hr of incubation. The VUP and CJF are suitable for simultaneous coagulation and disinfection of drinking water in poor communities.

Key words: *poor communities: drinking water: antimicrobial effect: Plant seed*

1. Introduction

Waterborne diseases are one of the major causes of mortality in the developing world where there is poor water supply systems [1]. Reappearance of cholera outbreaks in Tanzania is due to unreliable water supplies. Factors such as poor reliability of water supply, affordability and distance between a water source and the home have collectively led households to depend on unsafe sources, to reduce the volume of water used for hygiene purposes and to reduce spending on other essential items
[2, 3]. Since the provision of safe water infrastructure to the world's poor will take decades and require significant investment, the health of these people could be protected in the short to medium term by scaling up access to alternative water quality interventions that are effective and inexpensive. The appraisal of natural coagulation technology in the context of the present study is among such interventions, which could help a large proportion of the population in rural areas obtain clean drinking water.

A large population in Tanzania especially those in the rural areas, obtain water from small dams, rivers, ponds and hand dug wells for drinking and other domestic uses. These sources are turbid and grossly polluted by human and animal excretions. Rural women have been able to use turbid water fetched from polluted sources by clarifying them using locally available plant seeds that act as primary natural coagulants [4]. Investigations into Tanzanian natural coagulants have shown that active coagulating component in crude extract of *Vigna unguiculata* (VUCE) seed are water-soluble cationic peptides with molecular mass of about 6 kDa. Experiments to verify the coagulation potential of VUCE have revealed that their coagulation activities improved dramatically when the pH of raw water samples were regulated to acidic values using *Citrus* fruit juice (CFJ) [5]. Since the coagulation potential of crude extract and pure proteins of *V. unguiculata* seed and CFJ has been studied by the authors, additional investigation to ascertain the flocculent and antibacterial effect is necessary.

2. Objectives

The objectives of this study were to isolate bacterial strains from river water and investigate the flocculent and antibacterial potential of crude extract and purified protein of *V. unguiculata* seeds as well as CFJ on Gram-negative and Gram-positive bacterial isolates. The results from this study will provide useful information on how poor populations can use these natural materials for production of clean and safe water.

3. Methodology

3.1. Sampling of River Water

Sampling of water from Ruvu River located at Mlandizi area in the Coast region of Tanzania was in accordance with standard methods [6]. The water samples were transported to the Centre of Biotechnology at the Royal Institute of Technology (KTH), Sweden for analytical work.

3.2. Isolation and Identification Procedure of Bacteria

Tenfold serial dilutions of water sample were prepared in sterile distilled water and plated on nutrient and McConkey agar (Oxoid) plates incubated at different temperatures including 20°C, 30°C and 37°C aerobically and anaerobically for 24 - 48 hr. Isolated colonies were streaking repeatedly on sterile nutrient agar plates and incubated at 30°C for 24 – 48 hr until pure cultures of each bacterial strain was obtained.

Isolated bacterial strains were characterized by cultural and morphological characteristics as well as biochemical tests including Gram reactions, oxidase, catalase and triple sugar iron (TSI) agar tests, and growth on EMB medium [7]. Moreover, API 20E and API 50 CHB kits (bioMērieux, Lyon, France) were used according to the manufacturer's instructions. The API 20E software Version 4.1 identification database was used to interpret the results. Ten representative isolates were randomly selected from different groups for further identification. The selection of strains was based on representation of both Gram-negative and Gram-positive strains in antimicrobial studies.

The procedure for 16S rRNA gene analysis, which encompassed the total genomic DNA extraction from selected strains, Polymerase Chain Reaction (PCR) for amplification of 16S rRNA gene and sequencing reaction were done using the procedure reported earlier [8, 9].

3.3. Purification of Coagulant Proteins and Extraction of Citrus Fruit juice

Coagulating proteins were purified from crude seed extract (CSE) of *V. unguiculata* by cation exchange chromatography followed by elution of the bound cationic protein by 0.6 M NaCl and the protein purity was verified by SDS PAGE [5]. Purified protein fractions were pooled, concentrated and/or lyophilised and used for antibacterial experiments.

The CFJ was extracted manually from two ripe *Citrus* fruits. The final volume of juice after filtration through a fine fibre material ranged between 25 and 40 mL.

3.4. Bacterial Flocculation and Growth Inhibition

The ability of VUCE, purified proteins and CFJ to induce flocculation and inhibition of bacterial growth was analysed on four bacterial strains that belonged to different genera.

3.4.1. Bacteria Flocculation

Each strain was grown in 0.2 M phosphate buffered Lauryl Broth (LBB) at 30°C, 180 rpm overnight. Each bacterial culture was diluted ten times with diluted LBB to get an optical density (OD_{620}) of 0.3 – 0.4 nm. Different dosages of CSEs, purified proteins and CFJ were added to the culture suspension in sterile eppendolf tubes to make a total volume of 100 µL. The tubes were incubated at 30°C for 4 hr. Ten microlitre aliquots were pipetted from the culture tube onto the glass slide. The slides were observed for cell aggregations and other morphological changes under the phase contrast microscope (Olympus BX51 with AnalySIS) and images were captured using a Sony PC 120 camera [10].

3.4.2. Growth Inhibition

For bacteria growth curve studies, the overnight cultures were diluted in LBB to get an OD_{620} 0.1, and they were pipetted into 96-well microtitre plates (Greiner bio-one). A total of 10 µL of different diluted proteins and CFJ were added into the wells containing 90 µL bacterial cultures. Plates were incubated at 30ºC in FLUOstar* OPTIMA (LabVision, Sweden) spectrophotometer with shaking at 5 min interval and changes in the OD_{620} values were recorded [8].

Furthermore, growth inhibition was analysed directly on bacterial cells cultured overnight on nutrient agar. Different amounts of purified proteins ranging from 5 to 15 µL and CFJ (pH 4.8) were pipetted on different sterile paper discs placed on nutrient agar plates inoculated with bacteria inoculum that will result into confluent growth. The plates were incubated overnight and growth pattern and inhibition zones of bacteria were measured and compared to the control in which paper discs contained distilled water only.

4. Results and Discussion

4.1. Identification of Isolated Bacterial Strains

One hundred and twenty six strains were isolated from Ruvu river water in Tanzania. Sixty strains were further characterised by morphological, biochemical and identified to genus names by API identification system and 16S rRNA gene sequencing as shown Table 1. The results revealed that out of sixty strains isolated from Ruvu River twenty seven strains were identified as *Acinetobacter* spp., nine strains belonged to the genus *Pseudomonas*, thirteen strains belonged to the genus *Vibrio*, nine strains were identified as *Aeromonas* spp., and two strains belonged to genus *Bacillus.*

The results show that the Ruvu River is contaminated by different types of bacterial including the *Vibrio spp*., which is cause cholera infection, which claims the life many people annually due consumption of untreated or inadequately treated water along with poor

sanitation practices. Contamination of community domestic water supplies with high level of intestinal pathogens and fecal indicators has been reported to be common in rural areas of the Eastern Province, South Africa [11] and in potable water supply sources in Samothrace Island [12].

No. of strains	**Morphological and biochemical Characteristics of Isolated bacterial strains spp.**						**Identification by API 20E and API 50 CHB and 16S rRNA gene sequencing**
	Cell shape	Motility	Gram reaction	Growth condition	Catalase reaction	Oxidase reaction	
27	rod	-	-	aerobic	+	-	Acinetobacter spp.
9	rod	+	-	aerobic	+	+	Pseudomonas spp.
13	rod	+	-	facultative	+	+	Vibrio spp.
9	rod	-	-	facultative	+	-	Aeromonas spp.
2	rod	+	+	aerobic	+	+	Bacillus spp.

Table 1: Characteristics identification of the isolated strains

4.2. Antimicrobial Effect

The ability of VUP and CFJ to induce flocculation of bacterial cells of four strains that belong to *Pseudomonas, Aeromonas, Bacillus and Acinetobacter* was studied microscopically while the growth characteristics of the bacteria incubated different dosages of VUP and CFJ in 96-well microtitre plates.

4.3. Bacterial Flocculation Effect

The results of flocculation of *Pseudomonas, Aeromonas, Bacillus* and *Acinetobacter* cells after incubation after with minimum effective dosage of 0.47 mg VUP/mL and 0.5% CFJ are shown in Fig. 1a, 1b, 1c, 1d, respectively. It is clearly observed that both Gram-positive and Gram-negative bacteria cells could aggregate and form flocs upon incubation with VU and CFJ. Moreover, the results revealed that, bacterial flocculation was accompanied by immobilization of individual cells as opposed to the control 1culture in which the cells were actively moving and did not aggregate (Fig. 1a (i), b (i), 1c (i). The results in Fig. 1d showed that *Acinetobacter* cells could form flocs even in the absence of coagulant protein. The ability of bacteria to aggregate enhances the settlement and the clarification of turbid water treated with coagulants.

Analogous studies have revealed that, coagulant proteins extracted from *Parkinsonia aculeata* seed and CFJ also have the ability to induce flocculation of bacterial cells [8]

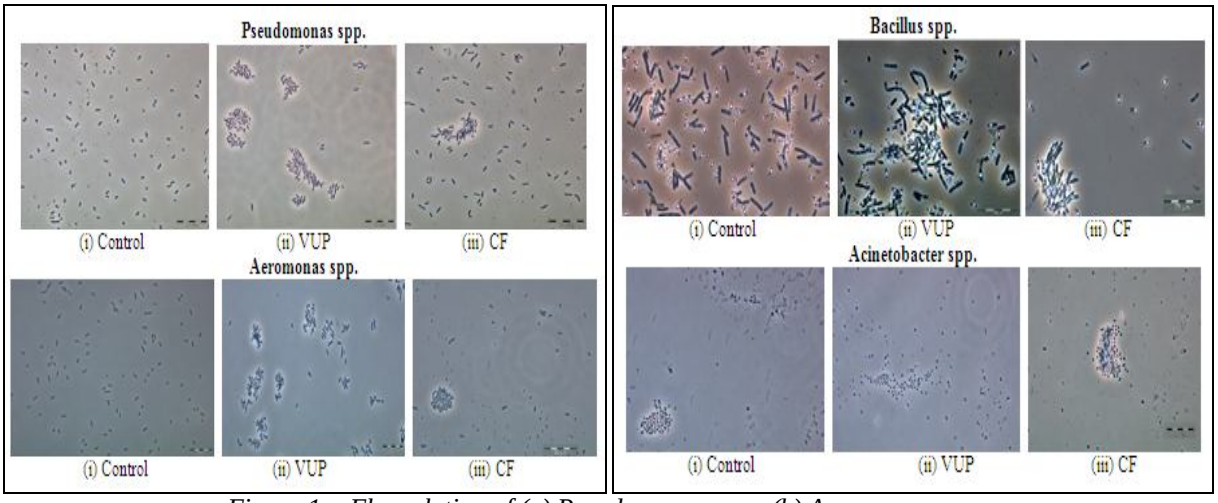

Figure 1: Flocculation of (a) Pseudomonas spp.; (b) Aeromonas spp.; (c) Bacillus spp. and (d) Acinetobacter spp. by minimum effective dosages of 0.47 mg/mL, VUP and 0.5%, CF

4.4. Inhibition of Bacteria Growth

Growth characteristics of *Pseudomonas, Aeromonas and Bacillus* spp. are presented in Fig. 2a, 2b, 2c, respectively. The results showed that, VUP and at the minimum effective dosages of The results showed that, VUP at the minimum dosages of 0.38 mg/L; 0.75 mg/L and 0.38 mg/L inhibited the growth of *Pseudomonas, Aeromonas* and *Bacillus* spp. respectively, after 2 to 4 hr of incubation. However, the level of inhibition varied among the bacteria, in which, the inhibition was higher for *Bacillus* spp. Those that observed for *Aeromonas* and *Pseudomonas* spp. However, *Bacillus* culture showed resumption of growth after 4 hr of incubation possibly due

formation of endospores which are resistant to peptides. Also, antimicrobial activity of recombinant polypeptide of *Moringa oleifera* has been reported to occur against bacteria including human pathogens [13].

Also, the results showed that, the growth of bacterial cells was inhibited remarkably after incubation with CFJ at the minimum dosage of 0.5% for *Pseudomonas, Aeromonas* and *Acinetobacter* spp. The minimum dosage of CFJ for inhibition of *Bacillus* was only 0.2%. This shows that the CFJ is potential natural biocide disinfection of water treated with natural coagulants before drinking in rural areas where safe water is lacking.

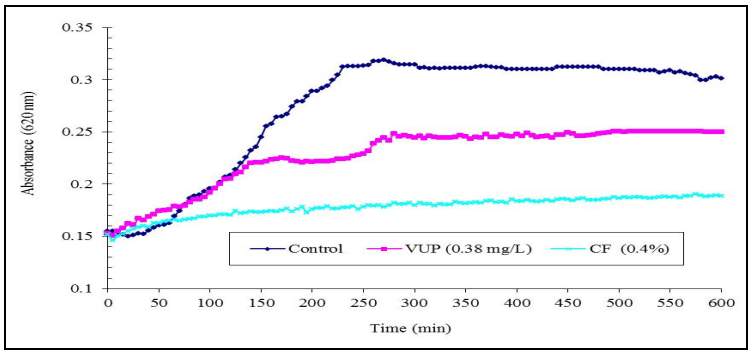

Figure 2a: Growth pattern of Pseudomonas strain incubated with purified protein of V. unguiculata seed (VUP) and Citrus Fruit juice (CFJ)

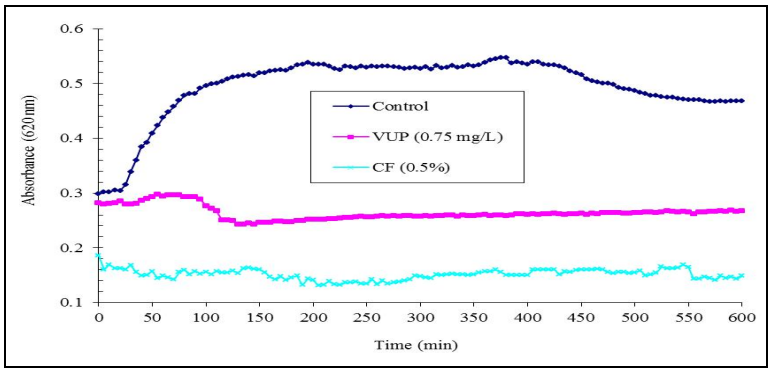

Figure 2b: Growth pattern of Aeromonas strain incubated with purified protein of V. unguiculata seed (VUP) and Citrus Fruit juice (CFJ)

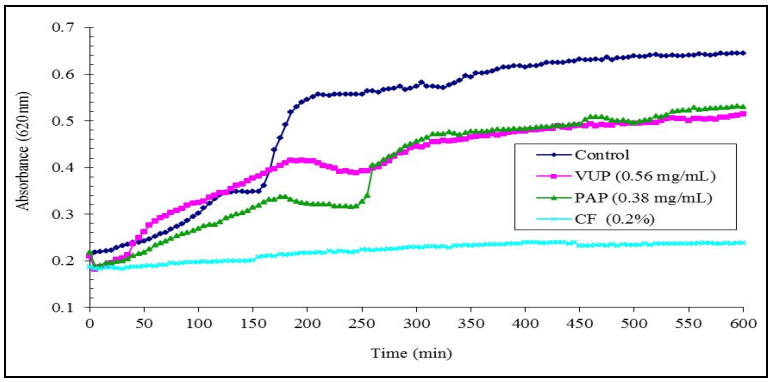

Figure 2c: Growth pattern of Bacillus strain incubated with purified protein of V. unguiculata seed (VUP) and Citrus Fruit juice (CFJ)

4.5 Growth Inhibition Zone of Bacterial Cells

Inhibition of bacterial cell growth cultured on nutrient agar plates in the presence of paper discs containing different amounts of VUP and CFJ are presented in Fig. 3. The results revealed that both the *Pseudomonas* and *Bacillus* strains were unable to grow around CFJ because of their sensitivity to antibacterial effect of CFJ possibly due the acidic nature of CFJ. However, the bacteria were not sensitive to VUP as the bacteria grew well even on CFJ containing discs. Similar results were observed for *Aeromonas* and *Acinetobacter* strains cultured in the presence of VUP and CFJ (results not shown). These results are in agreement with the results reported earlier that clarification of turbid water using crude seed extracts and purified proteins is mainly due to flocculation of

suspended matter and settlement or along with the bacteria flocs [8]. The inhibition zone of the four bacteria strains by CFJ ranged between 10 and 20 mm.

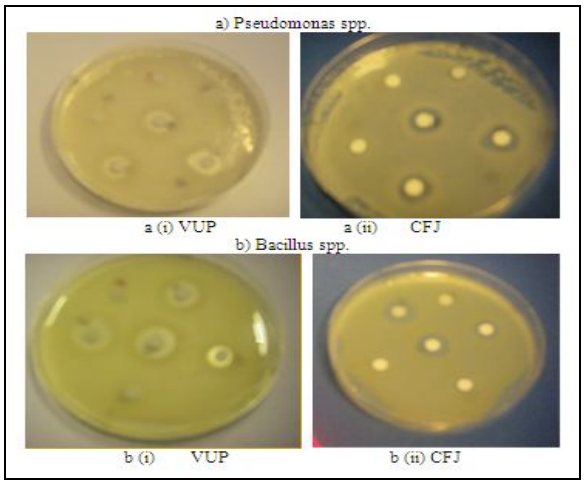

Figure 3: Inhibition zone pattern of bacterial cells of (a) Pseudomonas spp.; (b) Bacillus spp. in the presence of different quantities of VUP and CFJ.

5. Conclusion

Bacteria isolated from Ruvu river in Tanzania were characterized by morphological, biochemical, API and 16S rRNA gene sequence analyses. The results showed that the river is polluted by different groups of bacteria including *Vibrio, Aeromonas, Pseudomonas, Acinetobacter* and *Bacillus spp.* Further antimicrobial studies showed that, purified proteins of *V.unguiculata* seed can aggregate and inhibit the growth of bacterial cells at the minimum inhibitory dosages that ranged from 0.32 mg/L to 0.88 mg/L. *Citrus* fruit juice also showed high flocculent activity and inhibited the growth of bacteria at the minimum dosage of 0.2 to 0.5%. The CFJ at the pH of 4.8 produced clear growth inhibition zone with diameter ranging between 16 and 22 mm. The simple and rapid method used for purification of proteins together with high antimicrobial and coagulation activity of proteins, makes proteins suitable for simultaneous coagulation and disinfection of polluted drinking water sources in developing countries. The *Citrus* fruit juice is a natural biocide, which is suitable for post disinfection of water prior drinking in rural households.

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