Inactivation of Waterborne Pathogens Using Medicinal Plants

Report to the
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EXECUTIVE SUMMARY

Worldwide, pathogenic organisms are a serious public health concern because of their prevalence in the environment. They are shed in high numbers through the faeces of infected individuals and are transmitted via the faecal-oral route. The effectiveness of wastewater treatment in removing such pathogens and the possible health hazards posed by their discharge into receiving water bodies are all critical issues related to the environment. Enteric viruses among other pathogenic organisms have been noted to survive for an extended period in the environment under a wide range of pH and temperatures. They are mostly responsible for gastroenteritis or stomach flu. Enteric viruses as the smallest of the enteric pathogens have a low infective dose, robust and very difficult to detect. For instance, COVID 19 pandemic outbreak caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a severe problem to environmental scientists. There is controversy over the shedding of the viruses from faeces into sewage by infected patients, which could lead to survival of environmental media if the sewage is not treated correctly. Similarly, Norovirus has been reported in different water bodies including sewages, rivers, recreational waters, municipal water, and groundwater. To rule out the notion that surface water and wastewater facilities could be a major or potential transmission route of SARS-CoV-2 genetic material and Noroviruses, it is necessary to implement nationwide environmental surveillance to inform health authorities and decision-makers on environmental health.

Hence, this study investigates the prevalence of SARS-CoV-2 RNA and Noroviruses (I and II) in raw and treated wastewater from four wastewater treatment plants (WWTPs) as well as four rivers within eThekwini, Durban, KwaZulu-Natal, South Africa. The study is a wastewater-based epidemiology (WBE) investigation, an emerging area in the environmental field that aids in providing an early warning signs of waterborne enteric viruses (WBV) within the environment. Their identification is critical for preventing infection and responding to an outbreak. The study further evaluates the phytochemical composition, antimicrobial and antioxidant activities of aqueous, methanolic and ethanolic extracts of Ocimum gratissimum, Moringa oleifera, Azadirachta indica (Neem) and sesame plants. It further determines the ability of Moringa oleifera's young stem ethanol (MYSE) and Moringa oleifera's young stem aqueous (MYSA) extracts in the inactivation of bacterial pathogens during water treatment.

The occurrence of these viruses in surface water and wastewater samples was determined using Reverse Transcription quantitative PCR (RT-qPCR) assay. While aqueous, methanol and ethanol were used as solvents for extraction. In all, extracts from ten different plant parts were generated for analysis. The presence of phytochemicals such as phenols, flavonoids, tannins and alkaloids in the extracts were determined by phytochemical testing using standard methods. Then, the antimicrobial activities of all the extracts under investigation were tested against both Gram-positive and negative bacterial as well as fungal isolates using the disk diffusion methods. A concentration of 5 mg/mL was employed for each extract and the zones of inhibition were observed for antimicrobial susceptibility. Lastly, the antioxidant assay for each extract was evaluated by scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical while the percentage inhibition of each extract was equally calculated.

For analysis of viruses in water samples, a total of 12 samples, including seven influent and five effluent samples were positive for SARS-CoV-2_N2. Kingsburgh WWTP is the most efficient with 89.12% removal efficiency of SARS-CoV-2_N2 viral load. Isipingo was the least efficient with 49.87% removal efficiency. Similarly, the five analyzed river water samples (n = 16) were within the quantifiable concentration with 31.25% of the river water samples being positive while 68.75% were below the detectable limit. The effects of treated wastewater on surface water were also demonstrated during the monitoring period on the Kingsburg and Isipingo rivers, which had the highest virus concentrations. For noroviruses, the Ct values below 40 were considered positive and above 40 were considered negative for the virus. A total of 33 samples (74.33%) were positive for NoV GI while 32 samples (71.11%) were positive for NoV GII. Out of the 33 samples that were

positive for NoV GI, nineteen were influent samples (76%) while the remaining fourteen (70%) were effluent samples. In the same vein, for NoV GII, nineteen were influent samples (76%) and the remaining thirteen (65%) were effluent samples. In September 2020, Amanzimtoti WWTP was fully efficient because the influent samples were positive for both NoV GI and GII but were not detected in the effluent samples.

Conversely, out of twenty river water samples analyzed, NoV GI and GII were detected in 14 samples (70%). The results of this study would be the first to quantify SARS-CoV-2 and determine the efficiency of a wastewater treatment plant that affects river health in eThekwini Durban in KwaZulu-Natal, South Africa. As observed in this study, viral load in both wastewater (influent and treated wastewater) and rivers have some public health implications. Therefore, further epidemiological investigations of the study area need to be carried out which could serve as a potential early warning system for public health to prevent future outbreaks or reinfection of SARS-CoV-2. However, the lack of vaccine and antiviral drugs for the prevention and treatment of noroviral infection respectively made the viral surveillance of NoVs in environmental media a critical tool towards preventing future outbreaks of NoVs.

Phytochemical results revealed the presence of various compounds in each extract. These extracts showed positive results for different bioactive compounds. All the three solvents, aqueous, methanolic and ethanolic extracts showed varied antimicrobial activities against the tested pathogens. A similar result was observed for the estimated antioxidant assay for all the extracts at different concentration levels. Application of aqueous and ethanol crude extracts of Moringa oleifera young stem (MYSA and MYSE, respectively) in water treatment showed that small concentration of extract used per litre has high treatment potential when compared with the efficiency of alum. The capacity of the MYSE and MYSA extracts in turbidity and bacterial removal heightens with increase in settling time throughout the experiment with high bacterial removal efficiency between 99-100%. The highest calculated turbidity removal recorded for ethanol extract was at 0.31 mg/L (93.4%) followed by 0.93 mg/L (90.30%) and 0.62 mg/L (86.4%) within the settling period. However, aqueous extract (MYSA) showed less removal efficiency between 81-92% within 60 mins of contact and settling time across the treatment dosage. This strongly indicates that the plant extracts could be used for various medicinal and biotechnological purposes especially their application in the water sector to inactivate or prevent waterborne transmission diseases. In view of the results and challenges encountered during the study, specific recommendations were made for ease of undertaking similar scientific investigation by other researchers in this field.

ACKNOWLEDGEMENTS

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS & ABBREVIATIONS

CA	Cetrimide agar
DBPs	Disinfection by-products
DPPH	1,1-diphenyl-2-picrylhydrazyl
HPLC	High Performance Liquid Chromatography
MBA	Eosin Methylene Blue agar
MYSA	aqueous *Moringa oleifera* young stem
MYSE	Ethanol *Moringa oleifera* young stem
RT-qPCR	Reverse Transcription quantitative PCR
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
WBE	Wastewater-based epidemiology
WBV	Waterborne enteric viruses
WHO	World Health Organisation
WWTPs	Wastewater treatment plants

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Today, no doubt developing countries are confronted with several challenges in meeting rising demands for clean water as the available supplies of freshwater are in short supply due to: (i) extended droughts; (ii) population growth; (iii) more stringent health-based systems and; (iv) competing demands from a variety of users (US Bureau). Due to these factors, water protection against possible biological and chemical contaminants is becoming a critical issue in water resources management. For instance, enteric viruses are some of the smallest emerging enteric pathogens responsible for diseases in such countries, especially in developing countries. Viruses are obligate intracellular parasites containing bundles of gene strands of either RNA or DNA as a core nucleic acid surrounded by a protective coat called protein capsid (Jassim and Naji, 2003). Sometimes the capsid protein is surrounded by an additional spikey coat called the "enveloped viruses [or] the nucleocapsid [that] is surrounded by a lipid bilayer derived from the modified host cell membrane and studded with an outer layer of virus envelope glycoproteins." (Gelderblom, 1996). Based on genome composition, viruses are divided into four groups: single stranded DNA (ssDNA), double stranded DNA (dsDNA), single stranded RNA (ssRNA) and double stranded RNA (ssRNA).

Unlike bacterial cells, which are free-living entities, viruses utilize the host cell environment to propagate new viruses. Studies have shown that viruses can latch onto host cells as can be seen in the current coronavirus (COVID-19) pandemic situation caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Ghinai et al., 2020, Tang et al., 2020). One of the causes of different diseases in the world is due to exposure to low viral concentration which has led to the continuous spread of viruses and diseases. Thus, human enteric viruses are increasingly recognized as significant causes of human diseases, and prevalence of different viruses have been reported in water and wastewater (WW) due to their ability to survive in environmental media (Osuolale and Okoh, 2017, Rutjes et al., 2009, Atabakhsh et al., 2020, Aw and Gin, 2010, Bačnik et al., 2020, Ahmed et al., 2020a, Katayama et al., 2008).

There are several instances where they are detected in environmental water that is deemed compliant for bacterial indicator, resulting into cases of waterborne diseases (Rames et al., 2016) . The prevalence and diversity of enteric viruses in seawater, recreational water, rivers, domestic water and potable water, sewage influent, and effluent have been reported (Wang et al., 2018, Saïd et al., 2014, Love et al., 2014, Mans et al., 2014). Hepatitis A, adenovirus (AdV), rotaviruses (RV) and enteroviruses (EV), astroviruses (ASV), noroviruses (NoV), and bacteriophages were detected in surface waters (Shaheen et al., 2019, Altintas et al., 2015); wastewater treatment plants (WWTPs) (Gonzales-Gustavson et al., 2019); dams and treated drinking water sources (Sibanda and Okoh, 2012, Prevost et al., 2016) while, the detection in post chlorinated water are not exempted in SA (Osuolale and Okoh, 2017). In the recent severe acute respiratory syndrome-associated coronavirus (SARS-CoV) outbreak, scientists discovered that the viruses that causes the disease – SARS-CoV-2 – is shed in faeces and detected in the sewage system and treated effluent (Kitajima et al., 2020, Wu et al., 2020, Collivignarelli et al., 2020).

Another route of enteric virus transmission is through food products due to the use of polluted irrigation water (Sánchez and Bosch, 2016b, Purpari et al. 2020). Hence, finding solutions to reduce this problem has become an issue of keen interest (WHO/UNICEF, 2014). This process could start by establishing a baseline to understand the range of enteric viruses in water and WW in different regions of the world especially in sub-Saharan Africa (SSA) to establish viruses that could be used as indicators for faecal contamination as well as provision of efficient natural disinfectants that are environmentally-friendly. Thus, there is a need for more real-time surveillance of enteric viruses circulating in environmental samples in SSA.

South Africa (SA) which occupies the southern tip of Africa with a long coastline of 2 500 km and classified as semi-arid, is not insulated from this global problem. It is instructive to note that the country is undergoing rapid urbanization with immigrants from inlands occupying the crowded peri-urban and impoverished townships, which have resulted in the demand for tonnes of litres of water that results in daily wastewater generation. Additionally, SA has no navigable rivers, and the combined flow of all its rivers is less than half of that of the Zambezi River. The local geology of hard rock worsens the situation. There are few exploitable aquifers, and the country's numerous artificial dams (lakes) are subject to a high evaporation rate.

Also, poor operational state and inadequate maintenance (inappropriate design, overloaded capacity, faulty equipment, and machinery) of many wastewater treatment plants (WWTPs) in South Africa have resulted in increased discharge of the pathogens and pollutants to the receiving water bodies. For example, there have been reports about the detection of enteric viruses in effluents discharged from WWTPs and river water in different parts of South Africa (Adefisoye et al., 2016, Osuolale and Okoh, 2017). A performance audit of WWTPs also revealed that more than 90% of the WWTPs are working under suboptimal conditions (Mema, 2010a).

The application of plant-based treatment technology is a useful biodegradable and environmentally friendly treatment that could be employed for bio-coagulation and disinfection of contaminated turbid water. This is one of the ancient undocumented water treatment methods. Currently, the application of plant materials is attracting much attention among researchers worldwide in matters related to environmental contamination due to their merits and the quest for green treatment technology by the water sector (Varkey, 2020). Some of the benefits of using this technique include simple design and operation, low to no initial costs for chemicals usage, adsorption, and inhibition of spectrum microorganisms with high decontamination rate and no disinfectant by-products (Baptista et al., 2017). It is believed that the use of plant-based treatment technology is expected to play a crucial role in water purification as research into them is still at the infant stage with regards to the removal of waterborne pathogens especially, in South Africa. Hence, the primary goal of this report is to briefly give background details of four selected plants for this study, namely; *Ocimum gratissimum L*; *Azadirachta indica* commonly known as *Neem tree; Moringa oleifera* seeds, old and new stem; lastly, sesame plant.

At present, there are few studies on the antibacterial and antifungal properties of plant extracts, thus this study screens the ability of the selected plant materials for antioxidant and antimicrobial activities. *Ocimum gratissimum, A. indica* and *M. oleifera* (MO) plants are known for their potential antibacterial and antifungal properties, however, there is still little study on medicinal plants as an alternative material for inactivating waterborne pathogens such as bacteria, fungal and viruses. Given to the number of medicinal benefits of these plants, their applications as possible raw material for water purification are worth investigating. To this end, this report examines some conventional treatment methods and detection techniques used for monitoring waterborne viruses. Some of the mechanisms involved in using medicinal plants for coagulation and disinfection activities were also postulated based on previous studies. Lastly, the social, environmental and health impacts of using natural polymers for decontamination, especially in the water sector, was briefly reviewed.

1.2 AIMS OF THE STUDY

1. To monitor the occurrence of enteric viruses and SARS-COV-2 in different wastewater treatment plants and receiving water bodies in KwaZulu-Natal, South Africa.
2. To extract crude medicinal active portions from four selected plants using solvents of different polarities, determine their antioxidant, antifungal and antibacterial activities.
3. To investigate the efficiency of plant extracts in activating waterborne and clinical pathogens in water.

1.3 OUTCOMES AND EXPECTED IMPACTS

1. Folami A.M. & Swalaha F.M. Epidemiology of human enteric Viruses in Water and Wastewater sources (To be submitted to Heliyon**).**
2. Folami A.M. & Swalaha F.M. Social, environmental-health nexus of using *Moringa oleifera* plant as low-cost technology to remove contaminants during water purification (To be submitted to Journal of Herbmed Pharmacology**).**
3. Folami A.M. Lanrewaju A.O. & Swalaha F.M. Prevalence and efficiency of wastewater treatment plants in the reduction of SARS-COV-2 viral load: a temporal case study within eThekwini Durban, KwaZulu-Natal, South Africa
4. Human capacity development: Training of doctoral, Masters, B. Tech, and three undergraduate students (Third-year students)

CHAPTER 2: EPIDEMIOLOGY OF HUMAN ENTERIC VIRUSES IN WATER AND WASTEWATER SOURCES

2.1 INTRODUCTION

A sustainable supply of adequate clean water is a life-supporting natural resource and a fundamental element of any country's public health, environmental, and economic development (Krishnakumar et al., 2017, Bashir, 2005). Although water occupies more than 70% of the world's space with less available freshwater annually, the record has shown that more than 60 billion m^3 of freshwater is required for use due to humans' growing demand. Population growth is rampant in developing countries, implying a surge in demand for quality water and more wastewater generation. Sadly, contaminants have been shown to cause global economic challenges that render water sources unsafe for direct human consumption. This has denied about one billion people worldwide access to safe drinking water, leaving roughly 6 million others (of which 2 million are children) to die of diarrhoea annually (Enitan et al., 2018, Virk et al., 2019). Records have shown that developing countries (Africa, Asia, and Latin America) have the highest population globally, with approximately 80% of the present world population and 99% of the global growth (Population Reference Bureau, June 1, 2000). Therefore, about 80% of all diseases in developing countries have been attributed to waterborne cases due to the consumption and use of poor water quality that is high in chemical and microbial contaminants (Singh et al., 2019). The adulteration and turbidity of water sources due to negatively charged suspended particles caused by anthropogenic activities through human interaction or biological activities have worsened the water sources. Such particles repel each other, which makes them unable to aggregate and settle. These particles are carriers of unwanted contaminants such as clay, silt, toxins, colour and odour-causing particles.

In contrast, others include pathogenic organisms such as bacteria, viruses, protozoa, helminths, fungi, natural organic matter (NOM), which become dissolved and suspended solids. Studies have shown that patients suffering from gastroenteritis may excrete about 10^5 to 10^{11} virus particles per gram of stool per day (Clemente et al., 2012). Others are often shed from sputum, urine, hand washing, vomit, and respiratory secretions of the host that could end up in the water environment through the discharge of foul sewerage or partially treated wastewater and thus poses public health risk (Chattopadhyay and Taft, 2018, Han and Yang, 2020). Common asymptomatic respiratory viruses that cause clinical diseases are being circulated within the community and environmental media.

2.2 ENTERIC VIRUSES IN WATER AND PUBLIC HEALTH IMPLICATIONS

In this review, the literature focused on enteric viruses in water and their public health implications when exposed to humans. Literature from scientific databases such as Google Scholar, Science Direct, PubMed, and Scopus published up to 2021 were searched for this study. The search strategy was based on the use of keywords which include waterborne enteric viruses, discharge of wastewater, water treatment techniques, sewage, public health, viral diseases, waterborne viral infections, and wastewater epidemiology. The reference lists of selected publications were manually examined to find other relevant articles that were not included in the databases.

2.2.1 Occurrence of enteric viruses in water and wastewater

Viral pathogens have been suggested as one of the most promising tools to determine the sources of faecal contaminants in environmental water and may be used in conjunction with bacterial indicators to assess water quality and improve public health surveillance (Saxena et al., 2015). Compared with bacteria and protozoa, viruses are smaller, between 20 and 160 nm. Table 2.1 shows some waterborne viruses and their appropriate dimension (Farkas et al., 2020, Radin, 2014, El-Senousy et al., 2014, Gonzales-Gustavson et al., 2019). Both enveloped (e.g. coronaviruses) and non-enveloped viruses (Figure 2.1) have also been found in treated and untreated wastewater (Farkas et al., 2020, Fernandez-Cassi et al., 2018, OPERE, 2019).

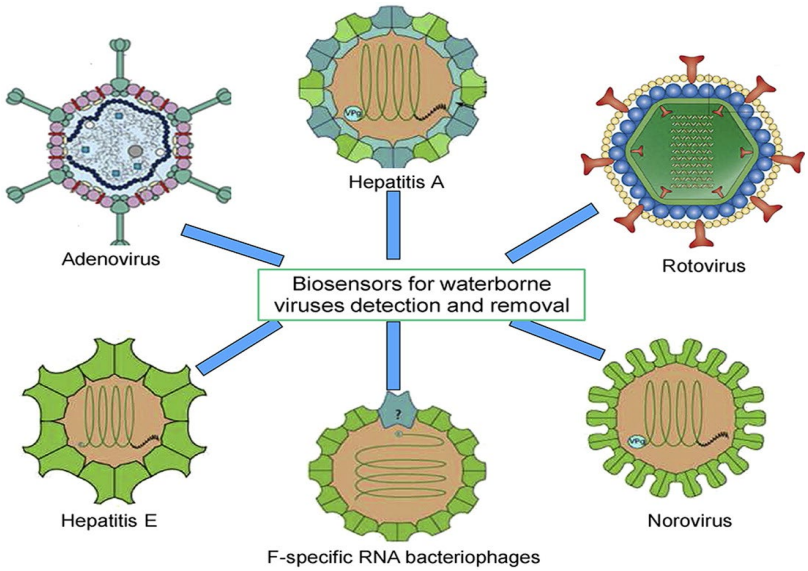

Figure 2.1: Typical structure of viruses.

Table 2.1: Dimension of some of the common selected human viruses found in a water environment

Virus Family	Genera	Species	Nucleic acid	Diameter/genome size
Picornaviridae	Enterovirus	Poliovirus	ssRNA	32nm/ 7.3-7.4 kb
		Enterovirus	ssRNA	22-30 nm
		Coxsackievirus A Coxsackievirus B	ssRNA	33nm
		Echovirus	ssRNA	32nm
	Hepatovirus	Hepatitis A virus	ssRNA	27 nm/7.5kb
Hepeviridae	Hepevirus	Hepatitis E virus	dsDNA	27-30 nm
Adenoviridae	Mastadenovirus	Adenovirus	dsDNA	90-100 nm
Caliciviridae	Norovirus Calicivirus	Norovirus	ssRNA	7.5 kilobases (kb), 23-40 nm
		Calicivirus	ssRNA	41 nm
	Astrovirus	Astrovirus	ssRNA	27-30 nm
Reoviridae	Reovirus	Reovirus	dsRNA	75 nm
	Rotavirus	Rotavirus	dsRNA	50 nm core with a 80 nm envelope
Coronaviridae	Alphacoronavirus	Human coronavirus 229E/NL63	ssRNA	25 to 32 kb

Virus Family	Genera	Species	Nucleic acid	Diameter/genome size
	Betacoronavirus	Human coronavirus OC34 & HKU1		
		SAR coronavirus		

2.2.2 Noroviruses

Human noroviruses (HuNoV), previously known as Norwalk virus, are positive sense non-enveloped and single-stranded RNA belonging to the family of *Caliciviridae* (Chigor and Okoh, 2012). They are subdivided into seven genogroups (GI-GVII) based on the complete capsid gene nucleotide sequences (Gonzales-Gustavson et al., 2019). The HuNoV GI and GII are further divided into many genotypes (Genotypes, GI.1 to 14 and GII.1-17). They are 27 nm to 32 nm in diameter with approximately 7.5 kilobases (kb) encoding a large polyprotein that are cleaved into six small non-structural proteins (NS1/2 to NS 7) and major structural protein (VP1) of about 58 to 60 kDa. The genus Norovirus is categorized as a member of the Caliciviridae family and is classified into ten genogroups (GI-GX) with different genotypes (Chhabra et al., 2019). The GI and GII are the most common causes of human NoV infections, with GII.4 variant being the most predominant cause in the last ten years (Siebenga et al., 2009, Vinjé, 2015, Mans et al., 2016). The NoV genome is divided into three open reading frames (ORF1-3) that encode a sizeable non-structural polyprotein (separated into six proteins), the primary VP1 capsid protein, and the lesser VP2 capsid protein (de Graaf et al., 2016, Upfold et al., 2021).

The outbreak of noroviruses has become an increasing public health concern due to various transmission pathways, from person-to-person contact, contaminated food, waterborne droplets of infected vomits and drinking water (El-Senousy et al., 2014, Sibanda and Okoh, 2012, de Graaf et al., 2015). Globally, human NoV is the primary pathogen that causes acute non-bacterial gastroenteritis in children with a significant impact on public health (Seo et al., 2014, Barrios et al., 2018, Brown et al., 2016). In particular, GI, GII, and G1V amongst the five genogroups of NoVs are recognized as the primary cause of epidemic and sporadic gastroenteritis infections in humans with an average incubation time of 12-48 hours (de Graaf et al., 2015, Vinjé, 2015, Goddard, 2017). Since noroviruses are highly transmissible, they have been associated with several outbreaks of diarrhea and vomiting in closed spaces such as schools, cruises ships, and healthcare institutions with limited morbidity associated with immune-compromised persons (Brown et al., 2016, Bouseettine et al., 2020).

Between 2 and 3 years, GII.4 genetic and antigenic revolve as new variants to replace the previously predominant strains worldwide with at least nine distinct GII.4 variants since 1995. Six variants have been linked with NoV-associated gastroenteritis pandemics (Prasad et al., 2016, Mabasa et al., 2018). During the 2014/15 gastroenteritis season in Asia, NoV GII.4 was replaced by the novel GII.17 Kawasaki 2014 strain as the predominant genotype causing gastroenteritis outbreaks (de Graaf et al., 2015). These strains have also been identified in other parts of the world, especially in sporadic cases (Mabasa et al., 2018, Medici et al., 2015). New recombinant pandemic strains are named as follows: GII.P4 New Orleans 2009 and GII.4 Sydney 2012 emerged in Asia, Canada, Denmark, Europe, South Africa (SA), and the United States of America (USA) between 2012 and 2013 (Hasing et al., 2013, Martella et al., 2013, Mans et al., 2016). A similar strain was detected in Australia at a lower rate in late 2015 and mid-2016. The emergence of a novel variant known as Melbourne 2016 with different genetic compositions was equally reported. This strain is believed to be a possible emerging pandemic variant (Bruggink et al., 2016).

A significantly high level of NoV contamination in treated sewage effluent and river water was reported Mans et al. (2016). Genotype GI is frequently found in environmental water. Rezaeinejad et al. (2014) reported a

high prevalence of norovirus with a mean concentration of 3.7 x 10^2 gene copies of HuNoV GII per litre as the dominant genotype among the targeted enteric viruses. In Korea, Lee et al. (2018) reported that genetically diverse HuNoV serotypes were detected in groundwater samples. In contrast, 100% of the sewage and secondary effluent samples in the Far East, Singapore, tested positive for both HuNoV GI and GII (Vergara et al., 2016). The prevalence and diversity of NoVs were reported in African environmental media (surface water, sewage influent, and effluent and shellfish) with significantly high levels of contamination in treated effluent and river water (Mans et al., 2016). Recently, astrovirus and norovirus were detected in the Rosetta branch of the River Nile, and the El-Rahawy drain Egypt (Shaheen and Elmahdy, 2019).

The ten most prevalent Africa's clinical NoV genotypes were detected in the rivers Mans et al. (2016). In a similar study by Kiulia et al. (2014), 9 (90%) of samples collected from urban rivers and streams in Kenya were positive for these genotypes, while 1 (8.3%) and 3 (25%) of the 12 samples collected from a rural river were reported positive for NoV GI in and GII respectively. In South Africa, the prevalence of NoV GI, GII, GIV, and sapovirus was reported in three polluted sewage rivers from Gauteng and wastewater samples from different communities in five provinces between 2008 and 2011 (Murray et al., 2013, Du Plessis et al., 2015, Page et al., 2017, Mabasa et al., 2018). About 16 genotypes and novel recombinants were observed in circulation within the investigated samples, with eight similar variants to NoV genotypes found in hospitalised pediatric patients (Mans et al., 2014). Though the first documented NoV outbreaks in South Africa was around 1993, there appears to be insufficient report or data on the occurrence of NoVs and circulating genotypes in South African water environments despite the outbreaks. Seasonal variation of NoV during winter was associated with waterborne outbreaks due to the contaminated portable, surface, and recreational waters (Kiulia et al., 2014, Kishida et al., 2012b). Noroviruses are highly infectious, with a single viral particle having the probability of causing infection at about 49% infection rate (Rusiñol et al., 2015, Teunis et al., 2008).

2.2.3 Rotavirus

Rotavirus (RoV), belonging to Reoviridae's family, is listed by the World Health Organisation (WHO) as a relevant waterborne pathogen. A double-stranded RNA virus is categorised into seven serogroups such as A to H (Desselberger, 2014). The genome of dsRNA has 19-32 kb pairs encoded with VP1-4, VP6, and VP7 structural proteins, while others are non-structural proteins NSP (1-5) (Figure 2.2) (Bouseettine et al., 2020). Rotavirus can be transmitted through person-to-person contact or contaminated food and water (Seo et al., 2014). Serogroups A, B, and C cause gastroenteritis in humans, while group A causes severe diarrhoea in young children (Bouseettine et al., 2020). This virus is the most common cause of gastrointestinal disease in children under five, with severe outcomes such as hospitalisation and death (Prez et al., 2015). Rotavirus has been found more frequently in surface waters as compared to norovirus. It is considered a winter virus as it is the most detected during the winter months. However, it has been reported all year round in most cases worldwide (Osuolale and Okoh, 2016). Rotaviruses in surface water and wastewater have been registered in different parts of the world, especially in developing countries (Katayama et al., 2008, Rezaeinejad et al., 2014). Rotavirus has been suggested as a faecal indicator. However, there is little to no correlation between FIB and rotavirus concentrations; hence, its use as an indicator organism is not feasible (He et al., 2008).

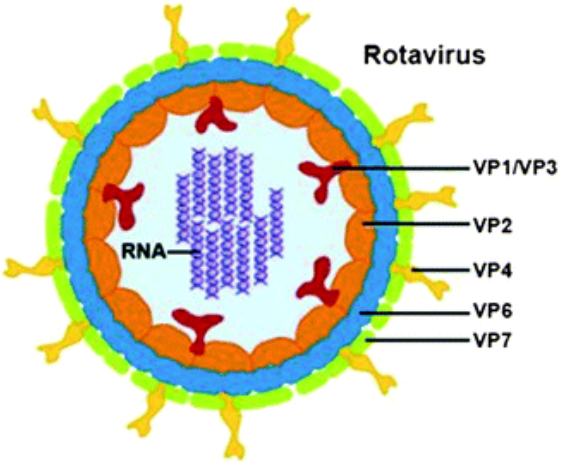

Figure 2.2: Structure showing the major structural protein in rotavirus genome

2.2.4 Hepatitis A virus

Enterovirus, rhinovirus sp. (RVA, B, C species) and hepatovirus form part of the *Picornaviridae* family. The genus hepatovirus only comprises one member, the hepatitis A virus (HAV). This virus is one of the most characterised human viral pathogens found in water (Fuster et al., 2016). The non-enveloped and single-stranded RNA HAV particles of the icosahedral capsid's positive polarity are stable in the environment compared to other picornaviruses. They have a genome size of 7.5 kb (Chou and Williams-Hill, 2018). Based on the sequence function of VP1-2, seven genotypes of HAV exist, with genotype I, II, III, and VII infecting humans while the monkey is infected by serotype IV-VI (Bouseettine et al., 2020). The genotypes I-III are further divided into sub-genotypes A and B, while former genotypes II and VII are regrouped into subgenotypes IIA and IIB, respectively (Costa-Mattioli et al., 2002).

The Hepatitis A virus is considered stable in environmental media due to its non-enveloped nature. It can retain infectivity for 4 weeks at room temperature and between 3 to 10 months in water and up to 9 days as aerosols. They are known to be epidemiologically significant due to their ability to resist free chlorine, temperature, and low pH, particularly in cases associated with high organic matter (Cormier and Janes, 2016, Osuolale and Okoh, 2017, La Rosa et al., 2017). HAV has been found in surface river water and dam (impoundment) water used for domestic, irrigation, and recreational purposes in South Africa (Sibanda and Okoh, 2012, Chigor and Okoh, 2012). Across the world, human enteric HAV is one of the main causative agents of hepatitis infection associated with waterborne outbreaks transmitted via faecal-oral route, person-to-person contact, or food and water consumption. The prevalence of HAV has been closely related to economic development (Sooryanarain et al., 2020), while the Hepatitis A virus exhibits a significant health risk, which causes substantial morbidity and economic loss.

There are approximately 1.4 million cases of HAV occurring worldwide each year, with the estimated death of about 7 134 in 2016 accounting for 0.5% of the mortality due to viral hepatitis) (O'Neil, 2018). If as low as 10-100 viral particles are ingested, it could cause an infection in humans within 15 to 50 days of incubation period (Mandli et al., 2017). Viral replication occurs in the liver resulting in hepatic injury. Multiple clinical symptoms are displayed when infected with HAV, including anorexia, cholestatic jaundice, liver enlargement, vomiting, abdominal pain, and fever. The frequency of symptoms is directly related to the infected person's age, with jaundice being the most prominent symptom. Those who show symptoms are considered most infective throughout the 14 days when the virus is at its highest concentration in the patient’s stool. Approximately 70% of children under six are asymptomatic and shed HAV for almost ten weeks longer than adults. Thus, they are considered a high-risk spreader of infection (O'Neil, 2018, Munoz-Martinez et al., 2018). Although, the incidence of HAV infection has decreased in developing countries due to better access

to safe drinking water (Manzano et al., 2018, Koroglu et al., 2017). However, early viral detection with suitable sensitive methods is still highly needed to detect low virus concentration in a sample before an outbreak (Mandli et al., 2017).

2.2.5 Hepatitis E virus (HEV)

Hepatitis E virus (HEV) forms part of the *Hepeviridae* family genus Hepevirus. HEV is divided into four genotypes that affect humans. It is also a non-enveloped single-stranded positive-sense RNA virus that ranges between 27-34 nm in diameter icosahedral capsid with approximately 7.2 kb genome size. It causes acute hepatitis and in some cases, it may lead to chronic hepatitis. Genotypes 1 and 2 are known only to infect humans and have been related to waterborne outbreaks in low-income countries (Randazzo et al., 2018). Infected persons excrete up to 10^{11} genome copies per gram of feces before showing symptoms at an average incubation period for HEV infection of between 15 and 60 days (Ishida et al., 2018). The incubation time makes early detection and transmission of HEV via fecal-oral route by direct contact with infected persons or by consuming contaminated water or food unnoticeable and rugged. Based on the WHO report, about 20 million estimated Hepatitis E infections with over 3 million symptomatic cases and 56,600 HEV-related deaths each year have been reported (Tseng et al., 2020, López-Santaella et al., 2020). The incidence of HEV within a particular population is affected by interactions between various dynamics such as living conditions, behavioural habits, and host immune status. Environmental factors also affect the persistence of HEV in different areas and viral aspects, which impact genetic diversity and the host range of the virus. This species causes acute hepatitis through faecal contaminated water (Seo et al., 2014). Research has shown that polluted environmental waters are the primary vector for transmitting HEV in endemic regions (Randazzo et al., 2018). There have been numerous HEV outbreaks related to faecal contaminated drinking water (Givens et al., 2016, Tarantino et al., 2016). Hepatitis E virus has been found in environmental samples such as wastewater effluents and soil (Parashar et al., 2011, Vaidya et al., 2003).

2.2.6 Enterovirus

Waterborne enterovirus (EV) is a spherical non-enveloped virus containing a 7,500-nucleotide positive-sense single-stranded RNA genome. They are 7.3-7.4 kb in length and protected by an icosahedral capsid (OPERE, 2019). The EV sizes are very small, ranging from 22 to 30 nm in diameter. The genus enterovirus belongs to the family of Picornaviridae with over 100 serotypes and eight groups of species identified (Wylie et al., 2015). These species include poliovirus, coxsackievirus A and B, Human enterovirus A to D, Echoviruses, bovine enterovirus and Porcine enterovirus A and B (OPERE, 2019, Wylie et al., 2015). Polioviruses, coxsackieviruses, and echoviruses belonging to this large group are associated with human infections (Table 2.2) (Munivenkatappa et al., 2018).

Table 2.2: Species of Human Enteroviruses and associated pathologies

Species	Serotypes	Associated pathologies
Enteroviruses	68-71	Encephalitis, conjunctivitis, meningitis, paralysis
Echovirus	1-9, 11-21, 24, 29-34	Encephalitis, conjunctivitis, meningitis, paralysis, gastroenteritis
Coxsackievirus A	1-22, 24	Encephalitis, fever, meningitis, paralysis
Coxsackievirus B	1-6	Encephalitis, gastroenteritis, myalgia, meningitis, paralysis, pericarditis
Poliovirus	1-3	Encephalitis, gastroenteritis, pericarditis

Studies have shown that enteroviruses are resistant to common preservatives such as chlorine which is more sensitive to ultraviolet light (UV) and methanol than adenovirus (Gerba et al., 2002, OPERE, 2019). Carriers of enterovirus include raw sewage, sewage sediments, rivers receiving sewage, and treated wastewater and sewage (Tiwari and Dhole, 2018, Potgieter et al., 2020, Kocwa-Haluch, 2001). The sources of enteroviruses may be groundwaters, river waters, coastal marine waters, aerosols emitted from sewage treatment plants and solid waste landfills, soils and insufficiently treated drinking water. Humans are the only known reservoir of enteroviruses and can survive in the faeces for a long time. They contaminate hands, utensils, food, water and cause gastrointestinal infections transmitted through the faecal-oral route. They can generate a wide range of diseases like meningitis, respiratory diseases, myocarditis, rash and neurological disorder (Table 2.0.2). Infections caused by EV in humans are known to increase during summer and early autumn months, which correlates with increased recreational water activities and bathing. Enterovirus is known to replicate in cell culture and has been considered an indicator of infectious diseases in environmental waters (Aslan et al., 2011, Prez et al., 2015). Van Zyl et al. (2019) also reported the recovery of enteroviruses and pepper mild mottle virus (PMMoV) in wastewater and wastewater-packed surface waters. In South Africa, the presence of adenovirus (AdV), rotaviruses (RV) and enteroviruses (EV) were detected in all the water samples collected from Umgeni River, Durban, South Africa, with AdV and EV particles the highest detected groups (Lin and Singh, 2015).

2.2.7 Human Adenovirus

Human adenoviruses (HAdV) are icosahedral capsid structure, non-segmented, non-enveloped and double-stranded DNA viruses with a diameter ranging between 90-100 nm (Figure 2.3) (OPERE, 2019). They belong to the family of *Adenoviridae* that consists of five genera of viruses. These include Aviadenovirus, Ichtadenovirus, Masterdenoviridae, Mastadenovirus and Siadenovirus (Arnold and MacMahon, 2017). Human adenovirus is a DNA virus with a lower mutation rate than other RNA viruses because portions of this viral DNA persist in host cells after viral replication has stopped. They can survive either as circular extra chromosomes or integration into the host DNA (Tang et al., 2020). Adenovirus is a non-linear envelope single-stranded DNA in the Adenovirdae family and genus Mastadenoviruses. HAdV is divided into six subgroups (A-F) comprising of 51 serotypes. Species F of HAdV containing two fastidious enteric serotypes, 40 and 41, are called enteric adenovirus; HAdv species cause a wide range of respiratory diseases and enteric infections. Many adenoviruses are shed in high concentrations with $>10^{11}$ particles per gram of faces (Hewitt et al., 2013).

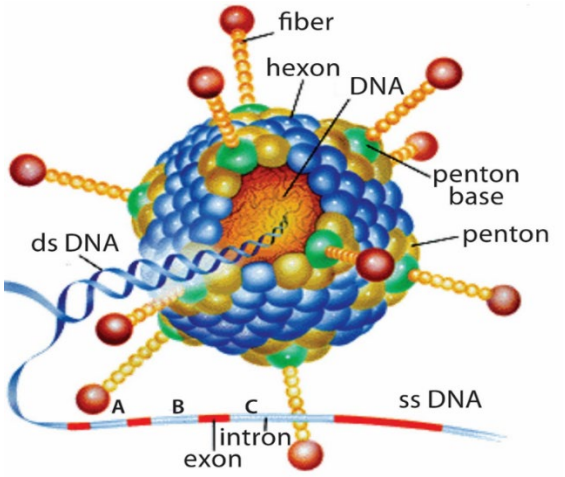

Figure 2.3: Stylised structure of adenovirus (Allard and Vantarakis, 2017).

Human adenoviruses are the most crucial virus indicator in drinking water and wastewater since they are thermally stable (Sidhu et al., 2013). They are reasonably resistant to disinfection, including UV disinfection and chlorination (Calgua et al., 2014). Furthermore, their stability contributes to their survival for long periods in water, increasing the chances of humans encountering viruses, especially in recreational waters. Adenoviruses are important etiological agents, and their exposure to humans through partially treated wastewater poses a risk of infection by causing a range of diseases, including gastroenteritis in children (Huang et al., 2016), pneumonia, hepatitis, conjunctivitis, poliomyelitis, meningitis, and myocarditis diseases among others however, adenovirus is problematic to diagnose due to its symptoms (Leifels, 2017). The signs and symptoms could persist for up to 3 weeks and transmissible up to 14 days after infection. Therefore, patients are advised to follow acceptable hygiene practices to prevent the condition. Adenoviruses rarely cause fatal diseases. However, infants and immune-compromised individuals are at risk of developing severe illnesses that cause morbidity and mortality in weak people, especially children (Gonzales-Gustavson et al., 2019, Prevost et al., 2016). Serotypes HAdV subgroup F 40 and 41 are among the leading causes of childhood diarrhea; even older children and adults may also be infected (Banerjee et al., 2017, Holly and Smith, 2018).

Globally, HAdv has been reported in the urban catchment and surface water despite strict treatment conditions for enteric viruses (Aw and Gin, 2010). Approximately 23 river water samples (44%) out of 52 samples tested positive for HAdv in Tone River water in Tokyo metropolitan area, Japan (Kishida et al., 2012a) and in Nile water (Elmahdy et al., 2020); in Germany (97%) and Southern California; USA (16%) and South Africa 13%. (Van Heerden et al., 2005, Choi and Jiang, 2005, Hamza et al., 2009). Human adenovirus serotype 41 is the most frequent group in sewage and surface waters. It can remain stable and infectious for more than 70 days at 4°C and 20°C. This species is the second most prevalent pathogen causing gastrointestinal infection in children worldwide (Leifels et al., 2016). Adenovirus serotypes 8, 19, and 37 are most often associated with epidemic keratoconjunctivitis while types 4 and 7 are major causes of acute respiratory diseases. About 62.5 % (30/48) HAdV were detected in the effluents collected from wastewater treatment plants at Eastern Cape, South Africa (Adefisoye et al., 2016). About 86.7 % (26/30) of HAdV-B (serotype 2) and 6.7 % (2/30) of HAdV-F (serotype 41) were detected in the HAdV-positive samples (Adefisoye et al., 2016). Other studies have also reported the detection of human enteric viruses in chlorinated treated water and wastewater within SA (Potgieter et al., 2020, Osuolale and Okoh, 2017, Van Abel et al., 2017).

2.2.8 Coronavirus

The recent outbreak of the COVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has become a significant concern to environmental scientists. It has become the object of several research since the latter part of 2019 due to the unimaginable spread of coronavirus disease worldwide (Lu et al., 2020, Amoah et al., 2021). The coronavirus virion is typically spherical in shape (Figure 2.4), with a diameter of about 60-140 nm, and is enclosed by an outer viral envelope covered by projections (9-12nm) (Zhu et al., 2020). The virion is organized in a distinctive exterior structure that resembles a crown (corona in Latin), where the family name emanates from (Foladori et al., 2020). Coronaviridae (coronavirus) belongs to a family of positive-sense single-stranded RNA viruses that cause a variety of common-cold-like and severe respiratory diseases (Yeo et al., 2020, Qu et al., 2020). Coronaviruses are classified into four groups: Alphacoronavirus (Alpha-CoV), Betacoronavirus (Beta-CoV), Gammacoronavirus (Gamma-CoV), and Deltacoronavirus (Delta-CoV) (Delta-CoV) (Qu et al., 2020). The SARS-CoV-2 is phylogenetically related to the subgenus Sarbecovirus of the genus Betacoronavirus, which is one of four genera of CoVs in the Coronavirinae subfamily (Foladori et al., 2020).

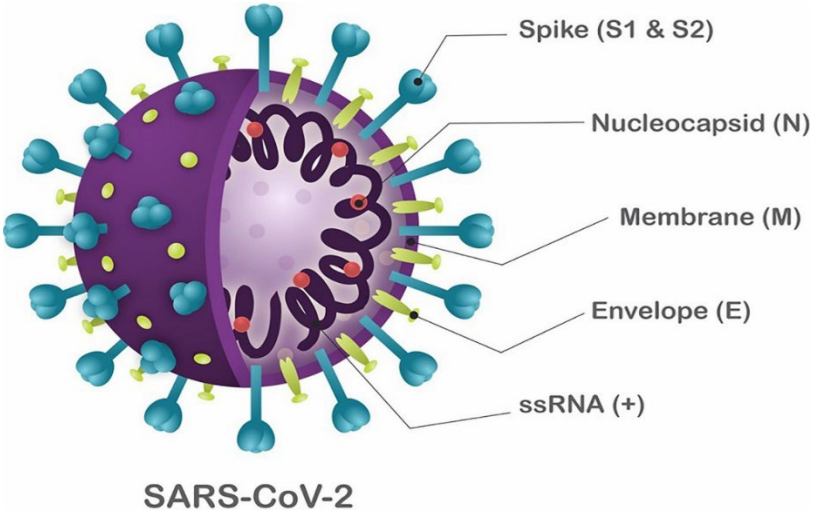

Figure 2.4: Structural representation of Severe Acute Respiratory Syndrome Coronavirus 2 (Santos et al., 2020).

The transmission of SARS-CoV-2 occurs majorly through surface contact and the inhalation of viral droplets and aerosols (Deng et al., 2020). However, there is a controversy of infected patients shedding the viruses through faeces into wastewater, resulting in environmental media survival [128-131], thereby raising concern about its presence in water and wastewater.

2.3 PROPOSED INDICATOR ORGANISMS USED IN MONITORING VIRUS IN WATER AND WASTEWATER

2.3.1 Faecal coliform bacteria and *Escherichia coli*

The prevalence of human enteric viruses indicates the need for real-time surveillance of viruses circulating in environmental samples (South Africa) and the development of an efficient removal technology. Water quality management and evaluation of human health risks through microbial assessments require effective detection of the pathogen. Faecal coliform bacteria and *Escherichia coli* are used as bacterial indicators of faecal contamination by predicting the possible presence of pathogens (Barrios et al., 2018). However, viruses and protozoa are widespread and more persistent in water as much as bacteria. Faecal coliform bacteria do not distinguish between faecal contamination sources and are less resistant to treatments than protozoa and viruses (Barrios et al., 2018). There have been cases where samples were positive for viruses in the absence of coliform bacteria and *E. coli* (Blaschke et al., 2016). The lack of correlation between viruses and bacteria is influenced by their small physical size, limited host range, and great resistance to treatment compared to bacteria (Qiu et al., 2015). Moreover, contamination by viruses is more likely to be disregarded due to the large volumes of water required for concentration and the high cost of analysis.

2.3.2 Bacteriophages

On the other hand, the potential application of bacteriophages has received significant attention. Bacteriophages are the most abundant organisms on earth, especially in water. They are used as an indicator organism for enteric virus contamination because they are easily controlled in the laboratory environment

compared to harmful pathogenic viruses (Ho et al., 2016b). Bacteriophages possess viral properties (Table 2.3) and have the structure, composition and size comparable with enteric viruses. They replicate in the same way as human enteric viruses. Furthermore, the bacteriophage is either positively corresponding with enteric viruses in wastewater or is removed more conventionally than human enteric viruses. Due to bacteriophages' resemblance with viruses, they have been used to assess enteric viruses. They have also been useful as alternatives to other microbiological and chemical tracers in surface water due to their non-toxic nature (Wu et al., 2017).

Table 2.3: Structure, composition and size of different bacteriophages

Bacteriophage strain	Shape	Size (Diameter)	Isoelectric point
ΦX174	Icosahedral capsid (T*=1)	27 nm	6.6
MS2	Icosahedral capsid (T*=3)	27 nm	3.5
PRD1	Pseudo lattice (T = 25)	63 nm	3-4
PM2	Icosahedral capsid (T*=12)	60 nm	7.3
Qβ	Icosahedral capsid (T*=3)	24-26 nm	5.3
*T is the triangulation number of the protein capsid, which is equal to the number of protein subunits in the unit of symmetry of protein capsid			

Bacteriophages that are used as indicators of viral faecal contamination are somatic coliphages, male-specific RNA coliphages and phages that infect *Bacteriodes fragilis.* Coliphages are viruses that infect *E. coli*. They have been proposed as indicators of viral faecal contamination because of their structure, size, environmental survival, and nature like that of viruses (Barrios et al., 2018). Moreover, coliphages are considered a microbial source tracker for faecal contamination and can be used to differentiate between human and animal sources in terms of faecal contamination (Barrios et al., 2018, Vergara et al., 2016). Coliphages are more abundant than pathogenic viruses and are quantifiable using simple plaque assays. They correlate better with enteric viruses than bacterial indicators (Vergara et al., 2016). Besides, the presence and concentration of coliphages and viruses differ according to the geological site (Rezaeinejad et al., 2014).

Male-specific RNA coliphages (also called F-RNA bacteriophages) are a broad group of coliphages that are associated with both animal (FRNA GI and GIV) and human (FRNA GII and GIII) faecal sources (Vergara et al., 2016). They infect Gram-negative bacteria and contain a plasmid coding F or sex pilus. F-RNA phages possess impaired replication in environmental conditions with similar morphology and behave to human RNA viruses such as hepatitis A, E, enteroviruses, astroviruses, and caliciviruses, giving them a preference for their own use as viral indicators (Barrios et al., 2018). Indicators used for the prediction of viral faecal contamination do not always correlate with the presence of enteric viruses in water sufficiently. There are no regulatory standards for removing viruses in wastewater treatment, and no viral indicators have been approved in the wastewater treatment. The occurrence and persistence of these viruses in wastewater may vary depending on the geological location and climate.

2.4 FACTORS AFFECTING THE SURVIVAL OF VIRUSES IN WASTEWATER

Due to some of the viruses' resistance to chemical disinfection during treatment may be persistent in high concentrations on treated water despite the disinfection process, which might end up in the environmental media. Therefore, the survival of these viruses depends on different factors such as the following:

2.4.1 Environmental conditions

Human enteric viruses are common emerging water contaminants that reach the surface water through other routes. It is imperative to consider them in water quality studies because of their incidence as causal agents for a diarrheal disease and their ability to survive for an extended time in the environment for long periods and tolerate changing environmental conditions. They could grow in the host cells at different pH and temperature or attaches with particles in the environment to shield themselves (Pang et al., 2019). Though enteric viruses cannot replicate outside the cells of its host, they could remain viable in the environment under favourable conditions. This makes the removal of enteric viruses during water treatment processes a necessity.

2.4.2 Viral Composition

The primary source of pathogens in the surface water is the discharge of raw and treated sewage and manure runoff into the environment (Jurzik et al., 2015). The survival of viruses also depends on the viruses' viral size and strains and environmental conditions. The cellular and molecular structures of viruses make them survive in favourable ecological conditions and influence their resistance to current disinfectants. Hence, they find their ways into water bodies via different routes (Girardi et al., 2019). They have a simple nucleic acid structure (either DNA or RNA) surrounded by a protein capsid with an additional protein envelope for some viruses. Based on the virus strain, the protein capsid usually has some chemical groups that can charge at different pH values to give the virus a surface charge that is typically negative at neutral pH.

2.4.3 Wastewater Composition

Viruses are excreted in high concentrations through infected patients' faeces and transmitted through the faecal-oral route (Haramoto et al., 2018). Raw sewage contains more infectious viruses due to direct excretion with faeces. Although the survival of viruses is influenced by environmental factors and structural composition, the composition of wastewater, such as surrounding microbial contents and adsorption to or resuspension affinity of viruses in the sediments, also affect their survival (Pang et al., 2019). This is further aided by organic debris of the clinical matrix whereby the virus is shed, for example, in the form of faeces or vomit. Therefore, it forms aggregation with the virus that protects the virus into a new human host (Rusinol and Girones, 2017).

2.5 TREATMENT OF WATER AND WASTEWATER

2.5.1 Overview

Most treatment processes rarely consider viruses as contaminants that need to be removed during water treatment. Therefore, for removing turbidity, colour, organic matter, adequate water treatment and sanitation based on the coagulation and flocculation, sedimentation, filtration and disinfection processes must be readily available for conventional water treatment pathogens, especially viruses (Ndabigengesere et al., 1995, Choy et al., 2015). This is necessary to alleviate the stress on the freshwater bodies and avoid future projected water scarcity, especially in developing countries, to minimize the health-threatening potentials due to waterborne-related outbreaks (Choy et al., 2014, Edokpayi et al., 2018, Enitan et al., 2018). Coagulation/flocculation is one of the most used techniques for efficient sludge-liquid separation in wastewater treatment (Awaleh and Soubaneh, 2014). Small suspended colloids in water are destabilized during flocculation and diminish their surface charges by adding coagulants with opposite charges. The

destabilized particles then settle down (Awaleh and Soubaneh, 2014). Broadly, inorganic synthetic or natural coagulants are added to the wastewater to destabilize colloidal material and cause the aggregation of small particles into more extensive and more easily removable flocks (Jayalakshmi et al., 2017). Aluminum sulfate (alum), ferric salt, and poly aluminum chloride are commonly used as conventional inorganic synthetic chemicals used for coagulation and flocculation, owing to their easy use and availability in the developed world. The efficiency of coagulation/ flocculation is determined by selected coagulants/flocculants. Each coagulant/flocculant has different coagulation/flocculation performance because of their unique structural characteristics: charge characteristics, ionic properties, functional groups and molecular weight (Wei et al., 2018).

2.5.2 Common disinfection methods for water and wastewater

Natural or synthetic methods are used to remove viruses from water and wastewater. Chlorination and photocatalytic techniques are employed for the disinfection of pathogens, especially bacteria.

2.5.2.1 Chlorination

Chlorine has been widely used for the disinfection of water. Chlorine disinfection enables the inactivation of pathogens while maintaining the residual disinfectant during the distribution of the treated waters. Chlorine is the most widely used disinfectant in potable water because of its low cost and accessibility. Chlorine can react with natural organic matter found in the water source and form harmful disinfection by-products. Therefore, to maintain the disinfection residual in most post-chlorination treated water is used (Mao et al., 2018). Studies have revealed that reoviruses are the most sensitive to chlorine (Folkins et al., 2020, Betancourt and Gerba, 2016).

2.5.2.2 Disinfection using photocatalytic methods

Photocatalytic disinfection is a practical approach to reducing waterborne pathogens (Zhang et al., 2019). These include solar radiation (sunlight), UV, radiation, photocatalytic ozonation, photocatalytic oxidation, single ozonation, H_2O_2 and ozone (Figure 2.5).

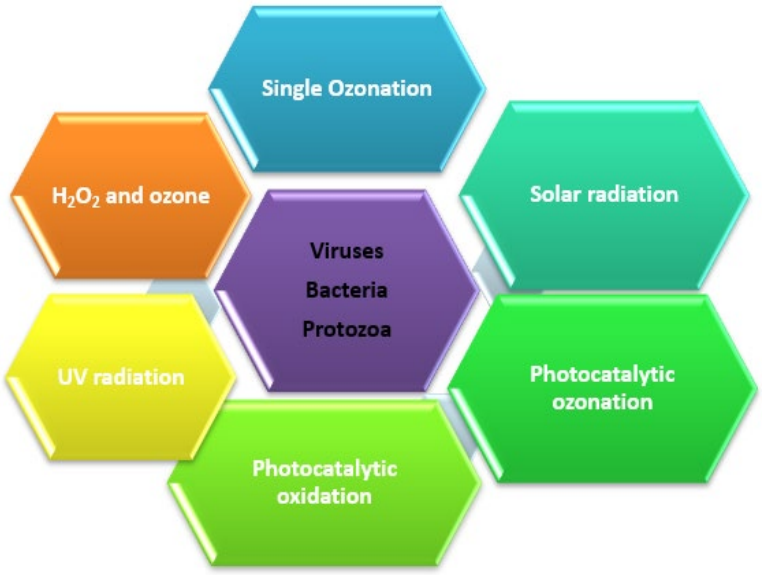

Figure 2.5: Photocatalytic methods used for disinfection of water.

2.5.2.3 Ozonation

Ozone is primarily employed to decolorize wastewater. It is a highly selective oxidant and allows the degradation of organic compounds with double bonds such as activated aromatic rings, deprotonated amines, and reduced sulphur groups in water (Pavithra and Jaikumar, 2019). Ozonation removes chemical oxygen demand (COD) and total organic carbon (TOC), and the degradation depends on the dosage of ozone used. However, COD, inorganic oxidant demand, influent and bacterial effluent concentrations influence the concentration of ozone that will be used for treatment (Castro et al., 2017). Moreover, ozone reacts with other water constituents to form hydroxyl radicals. It is a less selective oxidant and can oxidise all organic types found in water. As ozone is transferred to water, it reacts with organic and inorganic compounds present in wastewater. The radicals split as ozone decomposes oxygen. Ozone has less operational costs once the initial installation begins. Other advantages of ozone use are significant degradation with no other waste or sludge generation. The disadvantages associated with ozonation include the initial capital cost; the end by-products formed during the reaction may harm human health. It also requires energy in the form of electricity throughout the process (Pavithra and Jaikumar, 2019).

2.5.2.4 Sunlight

Viruses could be removed via sedimentation or viral attachment to large particles during water treatment. Sunlight is regarded as an effective method of reducing viral infectivity. It is employed in natural wastewater treatment systems such as waste stabilization ponds—the process where a solar system or photo-inactivation is used for the disinfection of waters. Mechanisms of virus removal in WWTPs by photocatalytic methods and viral particle interaction involve viral adsorption particles to suspended particles such as biological or chemical flocs, colloidal materials and dissolved natural organic matters (NOM). The inactivation of viruses by sunlight involves three mechanisms: direct and indirect exogenous as well as indirect endogenous inactivation mechanisms.

Direct light-mediated mechanism: This involves the absorption of photons by viruses through effective radiation with wavelength ranges between 100-280 nm (UVC), 280-315 nm (UVB), 315-400 nm (UVA), and 400-500 nm (visible) (Verbyla and Mihelcic, 2015). Increasing the wavelength is an efficient direct method for the inactivation of virus concentration depending on genome type and damages on the photons' capsid proteins or viral genome. Virus genome deformation by the absorbance of photons is by pyrimidine dimers formation, especially thymine dimers that prevent virus replication. It has been argued that "most of the RNA viruses are more resistant to direct light-mediated inactivation than DNA viruses" because of the absence of thymine dimers according to studies on the survival of viruses in water and wastewater after exposure to sunlight (Bosshard et al., 2013). According to Lytle and Sagripanti (Lytle and Sagripanti, 2005), larger genome viruses are more vulnerable to direct sunlight damage than smaller genomic viruses. Double-stranded DNA viruses are the most resistant, followed by this order of magnitude, dsRNA, ssRNA and ssDNA viruses. Similarly, higher inactivation rate of double-stranded RNA porcine, rotavirus compared to MS2 coliphages (ssRNA) when exposed to direct sunlight has been reported. Damages to adenovirus protein due to direct UVB radiation has been reported (Eischeid and Linden, 2011).

Indirect sunlight inactivation: Virus inactivation mechanism through indirect exposure of water and wastewater to sunlight could be endogenous or exogenous photosensitizers. This involves the absorbance of photons by sensitizer molecules (Verbyla and Mihelcic, 2015). Indirect endogenous inactivation is mediated by virus internal chromophores that occur indirectly. It is initiated by light absorption via sensitizers associated with microorganisms of interest. For endogenous, virus photosensitizers such as the amino acid (tryptophan, cysteine, histidine, phenylalanine and tyrosine) in the virus capsids protein area are an active disinfectant. The energy or electron transfer from excited photosensitizers to dissolved oxygen (DO) leads to the formation of reactive oxygen species (ROS) that damages the internal targets like proteins and nucleic acid, etc. in the virus during the treatment process (Schuch and Menck, 2010). This means that chromophores such as nucleic acid or aromatic amino acids in the virus capsid protein absorb solar light and

transfer energy to dissolved oxygen or other solution constituents. As light is excited, the chromophores oxidize the surrounding virus constituents, rendering the virus inactive through energy transfer to ROS, which helps in the degradation of nucleic acid or virus capsid proteins.

For indirect exogenous virus photosensitizers, the photo-oxidative ROS formed by common exogenous photosensitizer (EPS) in WW (NOM), algae, and particulates) causes damage to microorganisms. Although direct and indirect endogenous inactivation occurs concurrently, inactivation of viruses by direct sunlight is more efficient. However, the total exogenous inactivation process's influence depends on the target virus. For instance, exogenous inactivation contributed to the photoinactivation of human adenovirus (Silverman et al., 2013), rotavirus (Romero-Maraccini et al., 2013), MS2 phages (Silverman et al., 2013) and native F-RNA phages have been reported (Park et al., 2021).

2.5.2.5 Ultraviolet light treatment

Ultraviolet light treatment has also become another alternative environmental-friendly treatment method. Many treatment plants have adopted ultraviolent lamps' disinfection of water and treated wastewater using the UV radiation method. Still, its efficiency in virus removal depends on radiation's transmittance at a specific wavelength through the water column. The principle of ultraviolet light disinfection relies on UV light's ability to damage nucleic acid, therefore inhibiting important cellular processes such as replication, which results in the inactivation of microorganisms. Photons are absorbed by DNA nucleotides thymine and cytosine during UV treatment, thus leading to DNA damage (Park et al., 2021). The formation of dimers by adjacent nucleotides during UV irradiation and induced strand breakdown prevents polymerases' function, thereby causing damages to the nucleic acids (Rastogi and Sani, 2011). This could only occur on medium pressure UV lamps when UV light absorption damages viral structures by proteins if broader wavelengths are emitted (Ho et al., 2016a). Wang et al. (Wang et al., 2018) reported that conventional methods are efficient in inactivating bacteria and protozoa, but not effective for most enteric viruses, including adenoviruses. Studies have shown that adenoviruses are resistant to UV light disinfection; thus, they require a much higher wavelength to be inactivated (Zhang et al., 2019).
Low-pressure lamps have higher wavelength energy; for instance, adenoviruses are more susceptible to UV treatment by medium-pressure lights (Linden et al., 2008). They have been known to emit a broader spectrum of radiation, thus susceptible, leading to damages to viral protein capsid (Eischeid and Linden, 2011). Adenoviruses' resistance to UV irradiation is due to the double-stranded DNA (Alberts et al., 2002). The rate of inactivation of viruses by indirect sunlight is affected by the level of light penetration and dissolved oxygen in the treatment system. Based on the rate of solar inactivation, an indirect mechanism for dsDNA HAdv and MS2 coliphages has been shown to be slightly less efficient than direct mechanisms. Not all enteric viruses are resistant to indirect sunlight. Based on host cell binding and viral infectivity, fiber and hexon protein contents in human adenoviruses are destroyed in the presence of photosensitizer on exposure to sunlight without UVB portion (Bosshard et al., 2013). Hence, virus genome to reactive species, adsorption of viruses, and vulnerability of capsids proteins surfaces and mechanism determine the inactivation during water treatments.

2.6 MECHANISMS OF VIRUS REMOVAL BY VIRUS-PARTICLE INTERACTION DURING WASTEWATER TREATMENT

One of the main mechanisms of virus removal from the wastewater treatment plant is sedimentation via viral-particle interaction. In this case, the virus is attached to larger particles present in wastewater. Electrostatic and hydrophobic interactions are two significant interactions between particle surfaces and virus capsids. Electrostatic interaction between virus and particle is highly influenced by the surface charge of the virus capsid and particle surfaces (Templeton et al., 2008). The amines and carboxylic groups in viral capsid ionize

at different pH values, thereby giving the virus an electrical charge based on the capsid surface (Verbyla and Mihelcic, 2015). Virus adsorptive behaviour also depends significantly upon different surface charges on the virion capsid coat. The genetic composition of viruses determines the isoelectric point (IP) of a virus, which is the overall net charge of viruses. A virus will have a net positive charge at pH levels below IP, while at pH above IP, the virus will have net negative charges. Most waterborne viruses generally have IP between 3.5 and 7 (Michen and Graule, 2010), anticoagulants, and disinfectants at IP and pH that could trigger electrostatic interaction. In general, high pH favors free virus, and low pH favors adsorbed virus, the isoelectric points of both the virus and surface play roles in this interaction (Gerba, 1984). So, during water treatment, suspended solids and dissolved solids in wastewater (e.g. humid materials bioflocs, NOM, etc.) usually have net negative charge typically at neutral pH, normal WWTP running condition. Hence, the overall net charges of viruses play an essential role during water treatment.

Apart from electrostatic, hydrophobic interaction contribute to virus interaction with hydrophobic surfaces based on the hydrophobic amino acids present in the virus capsid protein (Templeton et al., 2008). These amino acids contain weakly acidic and basic groups (i.e. carboxyl and amino groups), which give the viral capsid an electrical charge. Dissolved natural organic matters in wastewater have more hydrophilic and less humid materials than the quantity in natural aquatic systems, thus providing a high quantum yield of reactive oxygen species that can inactivate viruses (Mostafa and Rosario-Ortiz, 2013). The role of hydrophobicity in the clumping and particle-association of bacterial spores was described by Mamane-Gravetz and Linden (Mamane-Gravetz and Linden, 2005) to force hydrophobic groups together by water molecules-based van der Waals bonding interaction. The potential for hydrophobic interactions is supported by the finding of nonpolar groups on virus particles (Rao et al., 1986). The nature of hydrophobic groups on suspended particles is unknown. Still, it is consistent with the observation that soluble proteins and other natural substances bind to suspended matter and contain hydrophobic residues.

Empirical observations further explain this interaction and adsorption behaviour of viruses, and the strains, virus, and particle type determine the virus adsorption (Templeton et al., 2008). In the study conducted by Meschke and Sobsey (Meschke and Sobsey, 1998), the authors detected that the adsorption of poliovirus 1, Norwalk virus, and MS2 coliphage differs for six different soil types suspended in wastewater (bentonite, coarse sand, clay-loam, kaolinite, organic muck, and sand-loam). Virus particle interaction is also influenced by microbial communities in the wastewater or other water sources, especially by reversible viral adsorption to suspended bacteria and algae depending on the level of oxygen demand of environmental media (Verbyla and Mihelcic, 2015).

2.7 FACTORS AFFECTING INFECTIVITY, INACTIVATION OF VIRUSES AND SHORTCOMINGS OF CURRENT TREATMENT METHODS

2.7.1 Overview

Successful reduction of microbial communities depends on various factors such as pathogen type, pH, DO, TOC, temperature, suspended particles, ionic strength, and the concentration of the disinfectant. Different disinfectants have different sites of action on the pathogen. Moreover, the dose of the disinfectant and its type of intrinsic factors and behaviour of the viruses contributes to the disinfection process (Gall et al., 2015). Several reports have shown that removing viruses in water is by sedimentation using adsorption depending on the virus type, the surrounding environment, and the surface properties (Hassard et al., 2016, Templeton et al., 2008), and virus predation by higher tropic-level organisms. Disinfection and contact time are considered during water and wastewater treatment to remove microbial load in treated water efficiently. Disinfectants target specific proteins in the capsid or the nucleic acid, which contributes to the effectiveness of the disinfection process (Marti-Carvajal et al., 2017). Moreover, different disinfectant concentrations will

exert different modes of action against the target virus (Gerba and Betancourt, 2019). Viruses also vary in terms of their resistance to the disinfectants and other chemicals or organic matter that affects the efficacy of the disinfectant.

Interestingly, there is an ongoing debate over the worrying impacts of chemical-based coagulants and disinfectants on human health. For instance, many people are at risk due to shortcomings and health-related issues linked to chemical-based coagulants and disinfectants used for water purification. Among the problems are overdosing of alum in water and wastewater treatment leading to a high residual aluminum concentration, high cost of coagulants and disinfectant in developing countries, training of personnel, and availability of chemicals to local communities with importation cost (Foster and Greiner, 2019, Okpara et al., 2011, Bodlund et al., 2014). The coagulation process has drawbacks as it may result in small flocculants (micro-flocs) as the coagulation process occurs or cause the production of fragile flocculants that break up when physical forces are applied (Suopajärvi et al., 2013). Chlorination as a means of disinfection is also a major concern globally due to the generation of harmful disinfection by-products during water treatments and their discharge into the water bodies, which poses risks to public health. Likewise, some particles (~0.3µm) can shield viruses from chlorine disinfection (Templeton et al., 2005).

Furthermore, the virus's infectivity is compromised by several environmental stressors, including temperature variation, exposure to sunlight, and predation by other microorganisms during water treatment. Other important factors that need consideration during solar inactivation include; the strength of solar radiation/intensity of light penetration, physicochemical characteristics of water and wastewater (water harness), genome size, and type of viruses (Verbyla and Mihelcic, 2015). Despite the benefits of photocatalytic disinfection technique, biofouling of UV lamps due to algae suspended solids and resistance of some viruses at specific wavelength limit the application of UV disinfection for water treatment. Viruses may be observed in suspended solids, algae, colloidal material, bacteria, and chemical flocs. They can be entangled within bioflocs, thereby protecting or shielding them from being inactivated. Association of particles as small as 7µm could protect viruses from UV exposure and reduce their susceptibility to direct sunlight. Viruses that have been inactivated can still be infectious to cells through multiple reactivations (MR). This process involves two viruses with damaged nucleic acids infecting the same host cell and replicating the whole genome. It requires cell infection by the cooperative effort of two or more UV and Gamma irradiated damaged viruses that cannot function independently without the help of another. This has been demonstrated in organized host cells like; adenovirus, reovirus, poliovirus, influenza, and vaccinia viruses after exposure to UV light (Gerba and Betancourt, 2019).

2.7.2 Potential risks of infection cause by waterborne enteric viruses

The occurrence of viral particles in water and wastewater is a major health concern to people directly in contact with wastewater and the aerosols generated during the treatment process.

Workers risks: Transmission of infection through direct contacts of workers to viral particles in sewerage or partially treated effluents or inhalation of aerosols formed by the viruses during aeration of wastewater is a major health risk factor. Because most workers are unknowingly exposed to aerosols and raw sewerage, they can be infected via different infection routes. There are currently fewer studies or investigations regarding the potential health risks and transmission of viruses through treated wastewater or workers' exposure to aerosols; therefore, it is essential to monitor air quality around wastewater treatment plants. Due to its nature, it is necessary to determine the microbial load in the atmosphere, especially the viral load. Long-time exposure to air pollution could lead to life-threatening diseases.

Wastewater reuse: Attention is now shifted towards recycling of treated wastewater to supplement water demand and supply for irrigation, recreation and potable purposes due to an increased population,

urbanisation and other ecological factors such as emission of greenhouse gases that affect global climate change that causes water shortages (Gonzales-Gustavson et al., 2019). Alternative water sources such as wastewater reuse are being considered to enhance conventional water sources. There are microbial pathogens contained in domestic wastewater that are capable of causing a variety of diseases. Viruses are present in numbers with great infectivity, posing more human health risks than bacteria and parasitic protozoa (Gerba and Betancourt, 2019, Yeo et al., 2020). Also, the discharge of treated or raw sewage or wastewater to the environment results in the constant input of enteric viruses in surface water, causing major environmental pollution (Haramoto et al., 2018).

Harvesting drinking water of high quality from almost any water source, including treated wastewater, may not successfully be microbiologically safe for consumption for reuse leading to a higher risk than the conventional water sources (Haramoto et al., 2018, Pype et al., 2016). Achieving a complete elimination of the virus is challenging with traditional wastewater treatment processes. As a result, many communities lack interest in accepting water that has been reclaimed from the municipality treated effluent because waterborne pathogens such as viruses are present in low concentrations in water with a low infectious dosage (Pype et al., 2016) and they belief that there could be a transfer of pathogens such as viruses into farm produce (Jurzik et al., 2015).

However, many studies have reported the detection of human enteric viruses in various water matrices, including reclaimed treated wastewater (Haramoto et al., 2020, Gerba and Betancourt, 2019). Molecular methods for detecting viruses in wastewater and recycled water suggest that other viruses could be present in concentrations of 10^7 to 10^9 genome copies per litre above the recommendation or standard limits (Eftim et al., 2017). However, the relative proportions of infectious to non-infectious viruses are still unknown. Therefore, care should be taken to avoid any outbreaks because enteric viruses are the major etiological agents of waterborne diseases (Barrios et al., 2018) and are associated with outbreaks from recreational waters (Sinclair et al., 2009). Gastroenteritis has been associated with human rotavirus, adenovirus, and norovirus worldwide. Due to the nature of viruses and their ability to escape disinfection agents during the wastewater treatment process (Haramoto et al., 2018), research focus has shifted worldwide towards the identification of emerging pathogens that could survive and grow rapidly under favourable conditions in WWTPs and their receiving water bodies (Kumaraswamy et al., 2014b, Wurtzer et al., 2020b, Osuolale and Okoh, 2017, Farkas et al., 2020). Extensive research on the persistence and high concentration of viruses in the aquatic environment, wastewater, and freshwater bodies as well as their transmission via the faecal-oral route should therefore be undertaken (Sibanda and Okoh, 2012, Lin and Singh, 2015, OPERE, 2019, Ahmed et al., 2020c).

2.8 DETECTION OF ENTERIC VIRUSES IN ENVIRONMENTAL MEDIA

Culture and molecular methods are being used for the detection of a large amount of pathogens present in the receiving water bodies (Zhu et al., 2018a, Bofill-Mas and Rusiñol, 2020). One of the challenges of the current detection methods for human enteric viruses is that human enteric viruses are present in relatively low water concentrations. As a result, viruses need to be concentrated from large water into smaller sample volumes to enhance the detection assay. The development and application of different techniques for concentrated viruses have contributed to the detection of various viruses by molecular-based or culture-based assays (Haramoto et al., 2018). More reliable and specific molecular techniques such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) have been reported to detect pathogenic viruses in wastewater. Droplet digital PCR (ddPCR) and next-generation sequencing (NGS) as the most recent techniques have been applied for accurate detection, determination of viral diversity, and quantification in environmental media (Wéry et al., 2008).

2.8.1 Cell culture

The concentration of viruses in wastewater was estimated using cell culture before molecular assays were developed. These methods do not detect every virus found in wastewater and miscalculate the true value of infectious viruses present in the sample. Cell culture is used as sensitive method for detecting potential infectious viruses, especially enteric viruses. Different cell lines have a wide range of values for the same type of virus, and the cell's susceptibility to a particular virus may vary in the laboratory over time. Moreover, cultivating naturally occurring viruses in wastewater varies from developing laboratory-adapted strains specifically selected for rapid growth (Gerba and Betancourt, 2019). Enteroviruses were the first viruses grown in animal cell culture, and since then, a number of methods for virus detection in cell culture have been developed. However, no single cell culture system has detected all enteric viruses simultaneously in the same assay. One of the shortcomings of using this technique is the use of specialised laboratory and equipment as well as personnel training.

2.8.2 Molecular assays

Molecular methods such as PCR and reverse transcription PCR (RT-PCR) are used to directly detect and quantify human enteric viruses in water and wastewater (Teixeira et al., 2020, Fout et al., 2015, Wang et al., 2020). Molecular methods are more sensitive, specific, and rapid than cell culture. However, they are more expensive and complicated. The dissemination of viruses in cell culture followed by polymerase chain reaction (PCR) provides a better procedure for monitoring infectious viruses that do not prompt cytopathic effects or plaques in cell cultures. This method reduces the time for virus detection and increases sensitivity. The advancement of molecular techniques such as quantitative PCR and ddPCR enables quantifying viral genomes occurring in water in low concentrations. qPCR provides more accurate results. Moreover, results obtained via qPCR are limited due to uncertainties caused by many environmental factors and the variability of the method. The concentration of viruses in water is influenced by various factors (Gerba et al., 2018).
Factors that cause variability of viruses in a specific sample are as follows (Gerba et al., 2018):

- Sample matrix: Inhibitory substances are inherent in environmental samples. These substances include humic and fulvic acids and heavy metals. Each sample has its target DNA/RNA and depends on the sample matrix.
- Sample processing: Sample concentration methods used present considerable variability in the recovery efficiency of viruses and the amount of inhibitory substances. Some virus concentration methods involve elution buffers that demonstrate inhibitory effects on molecular detection. The extraction of DNA/RNA is a critical step in detecting and quantifying viruses by qPCR.
- Molecular detection sensitivity is the measurement error or viability caused by PCR inhibition. Parameters that contribute to variability include the number of replicates, design of the qPCR assay, primer and probe used, and the efficiency of amplification assay. A variety of qPCR assays may not be equally subjected to inhibitory effects by substances extracted together with viral nucleic acids (Gerba et al., 2018). While qPCR has been used extensively in wastewater analysis to detect and quantify the many specific groups within the wastewater system, it is still limited by the reliance on standard reference material for quantification. Therefore, the reliability and consistency of the utilised standards greatly affect the accuracy of qPCR quantification of the unknown. Studies have shown the degree of variation between commercial standards in that the standard material was responsible for approximately half a log difference in results between vendors (Cao et al., 2013) and 2-fold between batches within a single vendor (Sivaganesan et al., 2011). Lack of access to reliable and consistent standard material has been identified as the biggest obstacle to qPCR for water monitoring (Cao et al., 2013).
- Other limitations of qPCR are also problematic for environmental applications. Many microbiological targets are present in environmental waters only at very low concentrations; thus, it is often difficult to detect the target molecules through qPCR alone, as they may fall well below the detection limits

of conventional methods. Furthermore, qPCR is susceptible to inhibition from common constituents found in environmental samples, which are complex and often contain substances that interfere with PCR amplification (Cao et al., 2013).

Most molecular methods do not give much information about the infectivity of the detected virus. However, cell culture methods have been used in conjunction with real-time PCR to define the potential infectivity of the virus by the use of intercalating dyes such as propidium monoazide (PMA) (Gerba and Betancourt, 2019, Leifels et al., 2016). Such photoactive fluorescent dyes enter cells with damaged membranes and intercalate with nucleic acids. The azido group binds with the DNA strands due to exposure to blue light (Prevost et al., 2016). However, UV light reduces the infectivity in some viruses such as adenoviruses and can use host cell enzymes to repair the damages DNA (Ho et al., 2016a). Studies were done on *E. coli* (Rudi et al., 2010) and calicivirus (Xu et al., 2015) showed that longer amplicons in qPCR increase the probability of detecting DNA that has been damaged by UV treatment. Similarly, *Ho et al. (2016a)* successfully used a two-step PCR with long amplicons to detect the damage done by UV treatment in adenovirus. While this method has been used mainly for bacteria, study has shown that it can be used for enteric viruses (Fittipaldi et al., 2010).

Although qPCR is currently the molecular quantification benchmark, ddPCR was developed to detect the very minute concentration of pathogens in a sample to leverage qPCR's shortcomings, among other significant advantages. These include a higher degree of sensitivity that does not rely on a user-generated calibration curve for sample target quantification, nor does it require any reference standards or endogenous controls. Both qPCR and ddPCR are used to amplify, detect, and count individual nucleic acid molecules; however, ddPCR is more precise, making it better for quantifying rare genetic mutations, deletions and duplications in DNA. For example, with ddPCR, it is possible to distinguish samples containing 10 copies of a gene from those with 11 copies, while in contrast with qPCR, it is difficult to distinguish even two copies from three copies.

Being a relatively new and more expensive technology, ddPCR has not yet been widely applied in quantifying microbiomes in environmental samples, especially for the quantification of virus concentration (Martinez-Hernandez et al., 2019). Since digital PCR counts the frequency of positives in small volume partitions, digital droplet PCR is less affected by PCR inhibitors, reducing the amplification efficiency and, therefore, more robust against inhibition (Huggett et al., 2013). Since only a small amount of target or non-target DNA is present in each partition, PCR interference between DNA molecules and substrate competition during amplification of different DNA targets is minimized. This feature could enable cost-saving strategies such as multiplexing to simultaneous quantify multiple targets (Morisset et al., 2013), which may be particularly advantageous for environmental monitoring applications. However, the reagents and machines for ddPCR are more expensive than qPCR budgets.

2.8.3 Transmission Electron Microscopy

The application of transmission electron microscopy (TEM) as a basic type of quantification involves counting virus particles with latex particles of a known concentration. In some cases, it is laborious, requires a skilled technician to quantify a single sample and quantification of high concentrations is not practical due to its high detection limit. However, TEM quantification is faster than infectious titration and allows a qualitative observation of the virion’s morphology. The TEM is more advantageous for a broad spectrum of viruses maintained in the laboratory. A single protocol can be used for various viruses. TEM determines the number of virions, while infectious titration determines the efficacy of a virus that infects the cell line and depends on many infectious particles. Non-infectious particles are defective because their genomes have deletions (Malenovska, 2013).

CHAPTER 3: APPLICATION OF PLANT-BASED MATERIALS FOR WATER PURIFICATION: A REVIEW

3.1 INTRODUCTION

Concerns about the use of inorganic compounds and other disinfection methods mentioned in Chapter two have led to an increasing search for natural, safer, greener, and locally sourced cost-efficient technologies for water treatment in developing countries (Choy, 2015). It is noteworthy that attention has shifted from chemical-based to biodegradable materials through plant-based natural polyelectrolytes to treat water due to many advantages. Table A1 in Appendix 1 provides some of the advantages and disadvantages of conventional and natural coagulants/disinfectants for water treatment. Likewise, the medicinal plant application for bioremediation of water and wastewater, for removal of water pathogen, has attracted more attention in the water sector as a major raw material and one of the most uncommon methods that people rarely use. Yongabi (2010) reviewed some of the available indigenous plants used as natural coagulants and disinfectants for water purification by the rural Africans. These include *Citruss aurontifolia, Jatropha curcas, Strynos potatorium, Abelmoschus esculentus (L.) Moench, previously known as Hibiscus esculentus (L.), Moringa oleifera, Pleurotus tuberregium, Nirmali seeds, Dolichos biflorus,* Guar gum, *Coccinia indica* fruit and isolated tannins extracts (Yin, 2010, Choy et al., 2014, Vijayaraghavan et al., 2011, Pritchard et al., 2009). Hence, this chapter reviewed three different medicinal plants that could be investigated for the presence of important phytochemical compounds and microbial activities.

3.2 USE OF PLANT BASED COAGULANTS AND DISINFECTANTS FOR WATER PURIFICATION

3.2.1 Literature search strategy, eligibility criteria for data generation and results

To find appropriate and pertinent data for our survey, we searched for scientific items such as Google Scholar, Hindawi, PubMed and Sciences Direct. Only free and downloadable papers were considered. The keywords *moringa* tree, *M. oleifera*, neem tree or Azadirachta *indica*, *Ocimum gratissimum* and *Sesame* plant were used on each search site to find the relevant papers. This keyword was often associated with terms referring to the biological activities of this plant. Advanced key search includes natural polymers for antiviral properties, mechanisms for water purification, the significance of plant for biocoagulation and disinfection of water. To be selected, the year of the item's publication had to be between 1995 and 2021. Articles published before 1995 were systematically eliminated, regardless of the relevance of their information. Then, only literature that focused on at least one biological activity or chemical composition, water purification as well as health and environmental benefits of *A. indica*, moringa tree/*M. oleifera*, *Ocimum gratissimum* and *Sesame* plant were selected. Finally, the selected papers were divided and classified into different subheadings.

An ancient natural coagulant and disinfectant from medicinal plants have been explored as a substitute for chemicals used for water purification. In the past, biological control was used as a water purifier by adding whole or plant extracts based on a vast array of different types of bioactive compounds produced by the plants. Choy et al. (2015) stated that ‘one of the possible solutions might be the use of natural coagulants of plant origin’ because they have a huge array of natural bioactive compounds that can be extracted or used as a whole plant for water clarification without any environmental-health risks (Ahmed et al., 2018). Several studies have shown the performance of integrated natural plant materials in water and wastewater treatment

technologies in communities with limited access to clean water in developing countries (Bhuptawat et al., 2007, Sarah et al., 2008). Various plant materials as a source of natural coagulant and disinfectant were reported to compete favourably with their synthetic counterpart like alum and chlorine (Table 3.1) (Boothe et al., 2010, Megersa et al., 2014b, Miller et al., 2008, Odiyo et al., 2017, Yongabi et al., 2011). Reports on many plants' bioactive compounds have shown that natural polymers could act as effective natural coagulants and disinfectants with robust and powerful antibacterial and antifungal effects (Table 3.0.2) (Abd El-Hack et al., 2018, Fahey et al., 2001). The use of natural coagulants produces more compact and denser flakes, which reduce the requirement for inorganic coagulant dosage and produces smaller quantities of sludge (Fatombi et al., 2013, Antov et al., 2012, Yin, 2010). Some plants can reduce or kill the spectrum of microorganisms remove colour, turbidity, chemicals, and heavy metals without forming any derivatives or by-products – a function played by many inorganic and synthetic chemicals used in water purification (Mnisi and Ndibewu, 2017, Baptista et al., 2017). However, there are few available reports on water treatment antiviral studies (Samineni et al., 2019).

Table 3.1: Plant-based coagulant and antimicrobial agents for water treatment

Tree	Part	Application	Reference
Mangifera Indica. L	Fruit	Coagulation	(Qureshi et al., 2011)
Vigna unguiculate (L)	Seed	Coagulation and disinfection	(Marobhe and Gunaratna, 2012)
Parkinsonia aculeata L.	Seed	Coagulation and disinfection	(Marobhe and Gunaratna, 2012)
Trigonella foenum-graecum (L)	Seed	Coagulation	(Ramamurthy et al., 2012)
Strychnos potatorum	Seed	Coagulation and disinfection	(Ramamurthy et al., 2012)
Cyamopsis tetragono	Seed	Coagulation and disinfection	(Pritchard et al., 2009)
Abelmoschus	Gum	Coagulation	(Binayke and Jadhav, 2013)
Mannihot esculenta crantz	Root	Coagulation and disinfection	(Vara, 2012)
Dicerocaryum eriocarpum	leaves	Coagulation	(Odiyo et al., 2017)
Dicerocaryum Eriocarpum	leaves	Bioabsorption	(Jones et al., 2016)
Moringa oleifera	Seeds	Coagulation and disinfection	(Bhuptawat et al., 2007, Egbuikwem and Sangodoyin, 2013, Virk et al., 2019)
Moringa oleifera	Seeds	Colour removal	(Pecora et al., 2018, Prasad, 2009)
Moringa stenopetala	Seeds	Coagulation	(Megersa et al., 2017)
Mammea suriga	Root bark	Disinfection	(Poojary et al., 2015)
Jatropha curcas	Seeds	Coagulation	(Yongabi, 2004)
Pleurotus tuberregium	Mushroom	Coagulation and disinfection	(Yongabi, 2004)
Parkia biglobossa	Seeds	Coagulation and disinfection	(Sofowora, 1984)
Lactuca sativa	Seeds	Colour and recalcitrant removal	(Moraes and Bidoia, 2015)
Eruca sativa	Seeds	Colour and recalcitrant removal	(Moraes and Bidoia, 2015)
Cucumis sativus	Seeds	Colour and recalcitrant removal	(Moraes and Bidoia, 2015)

3.2.2 *Ocimum gratissimum* L.

Ocimum gratissmum L.*,* known as African basil and clove basil, is an herbaceous plant belonging to the Lamiaceae family. *O. gratissimum* L. originates from tropical regions mainly, West Africa and India. It is cultivated in the South Sea Islands, Ceylon, Bengal, Nepal and Chittagong. The plant is known by different terms around the world. In India, the natives have given the plant many vernacular names, the most common being Ram Tulsi (Hindi), Vriddhutulsi (Sanskrit) and Nimma Tulasi (Kannada). In Nigeria, there are also many different names given to it and these include "effinrin-nla" by the Yoruba-speaking nation in the South Western region and "Daidoya" by the Hausas in the Northern region (Prabhu, 2009). *Ocimum gratissimum* L. is extensively used in many countries as a traditional source of medicine. In Brazil, the plant is used for culinary and condiment purposes besides its medicinal usefulness. In Nigeria's coastal regions, the plant is used to treat epileptic seizures and high fever; and in the Savannah areas, it is used to treat some mental illnesses. Ethnic groups in Nigeria also use the leaf extract as an anti-diarrheal and cold leaf infusions are used to treat stomach aches and hemorrhoids.

In Kenya and sub-Saharan Africa, the plant has been used to treat blocked noses, abdominal pains, ear infections, regulation of menstruation and convulsions (Prabhu et al., 2009). Still, in India, the leaves of the plant are used to treat chicken pox and mumps by turning the leaves into a paste and subsequently lathering the affected area with the substance. The plant is also used in religious ceremonies and rituals in the Hindu religion. In Sumatra, Indonesia, tea is made using the leaves, while the eugenol *O. gratissimum* L. is used for ceremonial washing of corpses and planted in graveyards (Ukoroije et al., 2018). Phytochemical compounds in this plant have bioactive components that can provide necessary health benefits and normal nutritional contents that help reduce the risk of major chronic illnesses (Ghoshal, 2018). *O. gratissimum* L. has a wide range of bioactive compounds such as polyphenols, flavonoids and volatile compounds. These compounds allow the plant to be used for medical purposes due to its vasorelaxation, antimycotoxicogenic, anti-inflammatory and anti-oxidant activities (Venuprasad et al., 2014).

3.2.3 Azadirachta indica

Azadirachta indica, commonly known as *Neem*, originated in Burma and Assam of South Asia. However, it may have also originated in Pakistan, Sri Lanka, Malaysia and Thailand. *Neem* is a member of the Meliaceae family and is commonly found in tropical and semi-tropical areas worldwide, including India, Pakistan, Nepal, and Bangladesh. Neem is a fast-growing tree with widespread branches that is evergreen. Under severe drought, however, it may shed nearly all or most of its leaves. For thousands of years, the beneficial properties of *A. indica* have been recognized in Indian tradition (Murthy et al., 2017). In India, different names are given, such as, 'Divine Tree,' "Heal All" and "Village Dispensary." Each part of the Neem tree, the bark, leaves and branches have some medicinal properties (Maithani et al., 2011, Yadav et al., 2016). Neem ingredients are known to be applied in Ayurveda, Unani, homeopathy, and modern medicine to treat many infectious and metabolic diseases. The plant possesses antimicrobial, anti-cariogenic, antidiabetic, antioxidant, antiviral, cytotoxic, and anti-inflammatory activities. Traditional Ayurvedic use of neem include the treatment of fever, diabetics, leprosy, malaria, ophthalmia and tuberculosis (Haider and Zhong, 2014, Bhalla, 2020, Adki et al., 2020). Some people who have access to the tree do use the twigs for brushing their teeth; the juices as remedies for skin disorders and spread the leaves throughout their homes to keep away insects. *Azardirachta indica* is one of the most useful traditional medicinal plants (Adki et al., 2020). Every part of the tree can be used for various treatments. The fruits, leaves, roots, seeds, and bark have proven to be a virtue that possesses antiseptic, antiviral, antipyretic, anti-inflammatory, anti-ulcer and antifungal properties (Mistry et al., 2014). Neem has antibacterial properties and it can be used to control airborne bacterial contamination. The seeds are used to treat infections with the eyes and the leaves can also be used to treat other bacterial illnesses like diarrhea, malaria and gastrointestinal diseases. *A. indica* is proven to have antimicrobial activity against

bacteria such as *Bacillus pumillus, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Owing to the tree's richness in antioxidants and antimicrobial activities (Ezeigwe et al., 2020).

3.2.4 "The tree of life" moringa tree

Moringa is one of the fast-growing tropical and subtropical trees with its origin from India and Pakistan's sub-Himalayan tract. This tree spreads from South India to Southeast and West Asia, sub-Saharan Africa, Peninsula, and Central America. Out of 14 cultivars of moringa in the family of *Moringaceae* that grows in Arabia, Africa, India and Madagascar (Shindano and Kasase, 2009), 6 species have natural coagulant property with only *M. oleifera* as the most widely cultivated; used and documented species among *Moringaceae* family in the tropical and subtropical regions of the world due to its quick growing ability (Oluduro et al., 2010). During the colonial era, *M. oleifera* spread to sub-Saharan Africa through Sudan for ornamental purposes. It is also widely distributed in Burma, Cuba, Jamaica, Malaysia, Pakistan, India, Thailand, Sir-Lanka, Singapore, and the Philippines. It is a fast-growing perennial tree with variants such as a horseradish or drum stick tree that requires less water and nutrient to grow. The tree has capsulated and dehiscent fruits and flowers. It starts flowering within the first six months after planting and could bear fruits within the first year of growth when planted through stem cuttings. The tree can grow straight to 2 meters in height and grow up to 5-12 meters high with 30-120 cm pods long (Paliwal et al., 2011) as soon as it begins to branch.

The tree can adapt quickly and grow in lowland with loamy or sandy soils in arid and semi-arid conditions (Raman et al., 2018). It can survive in areas with a rainfall of up to 3000 mm and a pH of 4.5-9 at the optimal temperature of 25-35℃, but cannot tolerate waterlogged conditions at high saline conditions (Camacho et al., 2017, Okuda and Ali, 2019, Raman et al., 2018). Every part of the moringa tree has beneficial properties, making it a multipurpose tree that can be used as fertilizer, food, forage, nectar for bees, herbal medicine, natural coagulants, and spices (Walia et al., 2019). For instance, the leaves and the pods contain high concentrations of vitamins consumed by animals and humans as vegetables. The wood pulp of the moringa tree is used for paper production and fuel or natural windbreaks. Different parts of this tree are employed in traditional medicine to treat epilepsy and diarrhea, among other diseases (Bakre et al., 2013, Fahey, 2005, Gopalakrishnan et al., 2016, Sharma et al., 2013). Moringa seeds contain about 30-40% oil that has been used for soaps, cosmetics and cooking. In contrast, the press cake obtained during oil production can be used as soil fertilizer, natural antimicrobial, and turbidity removal during water purification processes.

3.2.4.1 Natural polymer in moringa plant for coagulation and disinfection

There are several phytochemicals in moringa plant that are responsible for both coagulation and antimicrobial activities. Functional groups (O-H, N-H, C-H, and C-O) like carbohydrates, lignin, lipids alkaloids, saponins and flavonoids present in cell walls of *M. oleifera* tree do not only enhance its efficiency as a coagulant, clarifiers, disinfectant, and adsorbents agents but helps in the removal of heavy metals, dyes, perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS), surfactants and other pollutants (Omojate Godstime et al., 2014, Beltrán-Heredia et al., 2011b). According to (Yaméogo et al., 2011), the tree's bark contains secondary metabolites such as saponins, tannins, and alkaloids. The roots are rich in antibiotics and secrete an antidiarrheal, diuretic, and febrifuge resin. Alkaloids possess antiviral and bactericidal properties (Okwu, 2004, Omojate Godstime et al., 2014). Grubben and Denton (Grubben and Denton, 2004) reported moringine and moringinine as the two main alkaloids in moringa root bark, as cited in (Abd El-Hack et al., 2018). Flavonoids are part of the polyphenolic compounds found in moringa for anti-inflammatory, anticancer, antimicrobial antioxidant, and anti-allergic properties (Aiyelaagbe and Osamudiamen, 2009). The flavonoid pigments found in moringa flowers include isoquercitrin, kaempferol,

kaempferitrin, quercetin, and rhamnetin (Bennett et al., 2003). The anthelmintic activity of bioactive compounds in MO flowers, leaves, and seeds has also been demonstrated (Cabardo and Portugaliza, 2017, Tayo et al., 2014).

Terpenoids like diterpenes, sesquiterpenes and triterpenes are also known to have antiviral, antibiotics, antiseptic, anthelmintic and insecticidal properties (Cabardo and Portugaliza, 2017). The antibacterial and antiviral activities of different parts of moringa are attributed to niazimicin, niazirin, niazirinin, niaziminin A and B, glucosinolates isothiocyanate and rhamnose (i.e. simple sugar). Others include (Figure 3.1); 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, 4-(4-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate, benzyl glucosinolates (Abd El-Hack et al., 2018), 3-caffeoylquinic and 5-caffeoylquinic acid, kaempferol-3-O-glucoside and quercetin-3-O-(600-malonyl-glucoside), kaempferol-3-O-(600-malonyl-glucoside), caffeic acid, gallic acid, myricetin, ferulic acid (Magaji et al., 2020, Muhammad et al., 2016, Moulin et al., 2019). Other water-soluble natural polysaccharides for destabilization of colloidal suspensions by flocculating small particles and reducing turbulent drag in water are L-galacturonic acid, L-rhamnose and D-galactose (Warr et al., 2003, Agarwal et al., 2001). Anthonine and spirochin found in the root are bioactive compounds that inhibit bacterial growth. Moringa seeds have been shown to contain pterygospermin (originally found in *Moringa pterygosperma*), a powerful antibacterial and antiviral agent, with moulds and fungicidal effects (Fahey, 2005, Pandey et al., 2012, Yongabi, 2010). Undoubtedly, *M. oleifera* is a good source of proteins like globulin, albumin, glutein and prolamin with different amino acids such as Ile, Arg, His, Leu, Lys, Thr, Trp, Met, Phe, Val and soluble proteins (Gopalakrishnan et al., 2016, Teixeira et al., 2014).

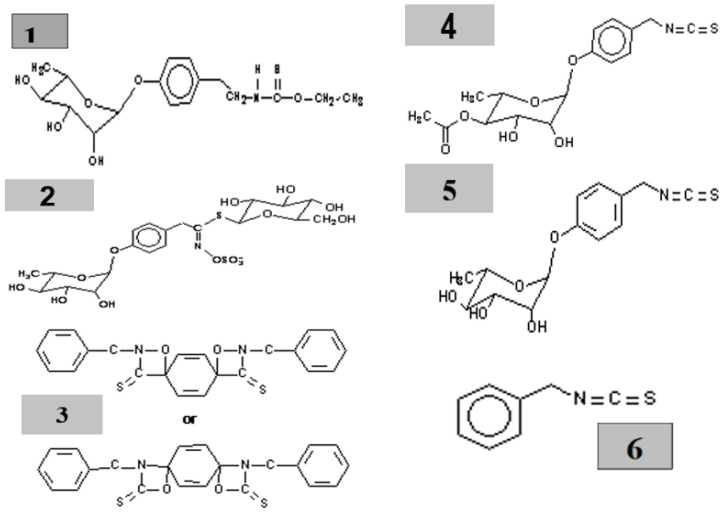

Figure 3.1: Structures of selected phytochemicals from Moringa spp.: (1) niazimicin, (2) 4-(a-L-rhamnopyranosyloxy)benzyl glucosinolate, (3) pterygospermin, (4) 4-(4′-*O*-acetyl-a-L-rhamnopyranosyloxy) benzyl isothiocyanate, (5) 4-(-L-rhamnopyranosyloxy)benzyl isothiocyanate, and (6) benzyl isothiocyanate.

Antiviral properties of different plants, herbs, essential oils, and their compounds have a broad antiviral spectrum against the reproduction of various types of RNA and DNA viruses. Antiviral property of plants is due to different bioactive compounds such as alkaloids, flavonoids (e. g quercetin), polyphenolic, coumarins, tannins, polyphenol, phenolic acid, glucosinolates, gallic acid, isocyanate, glucoside (like niaziminin and pterygospermin) kaempferol, lectins, procyanidin, terpenoids, isoquinoline,

isobavachaldone, polysaccharides proteins and peptides among many others. These secondary metabolites have been tested against wide range of viruses (Verbyla and Mihelcic, 2015).

3.2.4.2 Mechanisms involve in using Moringa oleifera polyelectrolytes for water purification

Water and wastewater pollution caused by colloids and suspended particles characterised by negative surface charges have been shown to be a good basis for electrostatic repulsion between particles. It thereby prevents their sedimentation and removal (Bouseettine et al., 2020, Beltrán-Heredia et al., 2011a). However, such particles are destabilised when a cationic substance such as a natural flocculant is added to coagulate the particles during water purification. Using plant material for water treatment aims to treat fine colloidal particles to create larger aggregates or flocs, which settle rapidly and are easily removable by optional secondary processes such as filtration and thickening. Polyelectrolytes perform this process of aggregation of suspended particles. The moringa plant's ability to synthesize aromatic substances, mostly cationic protein (CP), phenols and other oxygen-substituted derivatives, provide the defence mechanism against microorganisms, insects, and herbivores and its ability to act as a natural coagulant during water treatment. The coagulant and antimicrobial properties of the moringa plant have been attributed to phytochemical components, including positively charged and water-soluble soluble cationic protein (MOCP) that act as natural cationic polyelectrolytes. *Moringa oleifera* seeds contain soluble cationic protein (MOCP) such as low molecular weight cationic proteins, chitin-binding protein isoforms (Mo-CBP3), mabinlins, napins, lectins, and other seed proteins extracted from MO seeds for efficient potable water production and removal of hardness due to their potent antimicrobial and coagulant properties (Kansal and Kumari, 2014). These proteins possess antibacterial, antiviral, anticancer, and hypotensive activity (Anwar et al., 2007, Kumar et al., 2012).

Moringa seeds are suspected of having different sizes of MOCP that possess coagulant and disinfectant capacities that are yet to be identified and isolated. Therefore, attention should also be given to the detection, isolation and characterisation of flocculating cationic protein in *M. oleifera* seeds as natural polyelectrolytes that can be used for water treatment. Research on identification, isolation, purification, and characterisation of secondary metabolites as the active agents in moringa mucilage has been identified as one way to overcome some of the challenges of using moringa trees to produce potable water using different techniques (Oluduro et al., 2010). These techniques include dialysis, ultrafiltration, lyophilisation, ion-exchange, chemical precipitation, liquid chromatography, X-ray diffraction, mass spectrometry, neutron reflection, SDS-PAGE and electrophoresis (Moulin et al., 2019, Oluduro et al., 2010, Ndabigengesere et al., 1995).

Based on the reported studies, the exact form and size of MOCP as an active cationic polyelectrolyte has a molecular weight (MW) between 3 to 60K Dalton (kDa) (Ghebremichael et al., 2006, Ndabigengesere et al., 1995, Gassenschmidt et al., 1995). Foidl et al. (Foidl et al., 2001) identified the molecular weights of MOCP to be between 7-, while other studies reported the MOCP in the aqueous extracts of *M. oleifera* to have dimeric cationic proteins with a molecular weight of about 13 kDa protein at pH value of 10-11 (Ndabigengesere et al., 1995) and 6.5 kDa at an isoelectric point above pI 10 (Ghebremichael et al., 2006, Gassenschmidt et al., 1995). Using protein profile analysis, Bodlund (2013) further confirmed that the major protein bands had molecular weights of around 6.5 and 9.0 kDa, respectively. The *M. oleifera* chitin-binding protein (CBP_3) and *Mo*-CBP_3 isolated from *M. oleifera* seeds with water treatment capacity are 14-kDa thermostable chitin-binding protein that inhibit the germination and mycelial growth of phytopathogenic fungi and viruses (Gifoni et al., 2012, Freire et al., 2015). Recently, Alves et al. (2017) reported globulin and albumin to be the highest protein fractions in *M. oleifera* seeds with 53 and 44%, respectively.

During the process of coagulation or destabilisation, a collision of particles may occur through four types of mechanisms: adsorption and bridging, double-layer compression; charge neutralisation; and sweep

coagulation (Freitas et al., 2015). However, the mechanism varies depending on the type of contaminants (Table 3.2). The coagulation mechanism of *M. oleifera* was reported to be through charge neutralization (Ndabigengesere et al., 1995, Brilhante et al., 2017) while positive protein(s) bind with negatively charged soluble particles to bring about the colloidal particles, which clump together, leading to large flocs or sludge to settle at the bottom of water which are then removed by filtration (Figure 3.2). These seed cakes also remove dirt, solid particles, certain viruses, bacteria, fungi, heavy metals, PFOS and PFOA, etc. (Bina et al., 2010, Eman et al., 2014, Pandey et al., 2012). Besides being a flocculation agent, *moringa* seed extracts show antimicrobial activity (Horwath and Benin, 2011).

Table 3.2: Mechanisms involved in different flocculants reported in the literature

Category of flocculants	Type of flocculant	Flocculation mechanism
Chemical coagulant	Inorganic metal salts	Charge neutralisation
	Polyelectolytes with low MW and low CD	Charge neutralisation
Chemical flocculant	Polyelectolytes with high MW and low CD	Bridging
	Polyelectolytes with low MW and high CD	Electrostatic patch
	Polyelectolytes with high MW and high CD	Electrostatic patch + Bridging
Bio/flocculant	Cationic	Charge neutralisation + Bridging
	Anionic	Bridging
	Anionic/Neutral plant-based flocculants	Bridging
Grafted flocculant	Amphoteric/Cationic/Anionic graft polymer	Charge neutralisation + Bridging/ bridging only

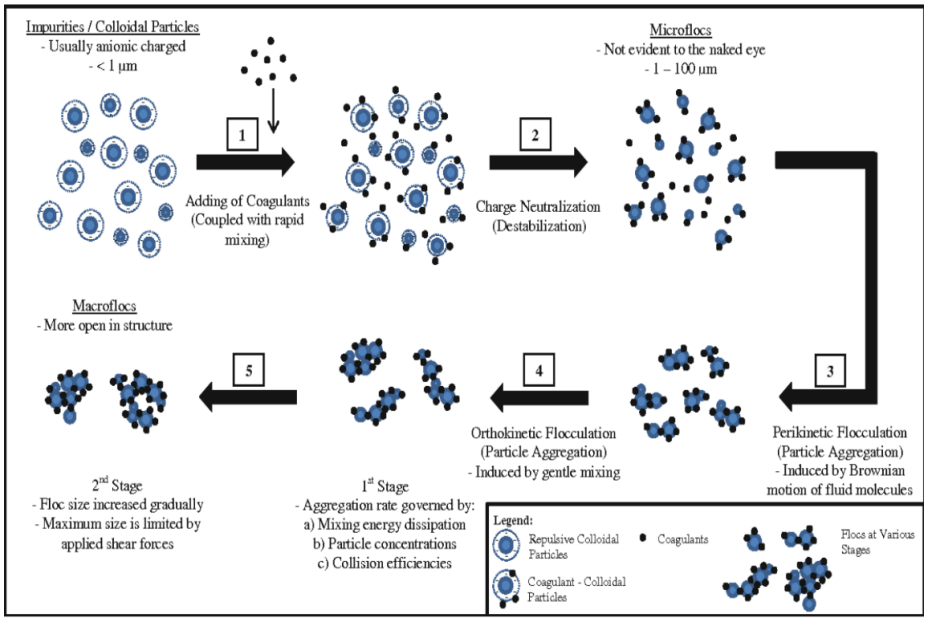

Figure 3.2: Coagulation via charge neutralization and flocculation mechanism of the colloidal particles present in water (Choy et al., 2015).

The mechanism of using MOCP as antimicrobial agents involves membrane fusion (Shebek et al., 2015). The plant's phytochemical components form complex extracellular and soluble proteins that lyse the bacterial cell wall and disintegrate the cell membrane (Omojate Godstime et al., 2014). The antimicrobial

properties of MOCP in water treatment are caused by a helix−loop−helix motif that causes the fusion of inner and outer cell membranes of microorganisms when in direct contact with the plant extract (Shebek et al., 2015, Suarez et al., 2005). The extract constituent with the phospholipid bilayer of the cell membrane causes one of the following: (i) enhanced ion permeability; (ii) leakage of vital intracellular components, or; (iii) the impairment of viral capsid protein or bacterial enzyme systems (Zhao et al., 2001). Apart from MOCP, purified pterygospermin from synthesized *Moringa* seeds form active derivative compounds known as benzyl isothiocyanate and 1,4-benzoquinone are the potential antimicrobial agent that helps in the disinfection of contaminated water (Figure 3.0.3). For instance, benzyl-isothiocyanate is a plant-derived peptide that inhibits bacterial growth by disrupting the mechanisms of membrane and enzyme synthesis (Suarez et al., 2003, Brilhante et al., 2017). For more details on the theoretical investigation of pterygospermin as an essential antimicrobial agent, see Horwath and Benin Horwath and Benin (2011) and Yongabi (Yongabi, 2010). Tannins can inactivate vital microbial adhesion enzymes and cell envelopment proteins and thus resist microbial development or replication (Omojate Godstime et al., 2014).

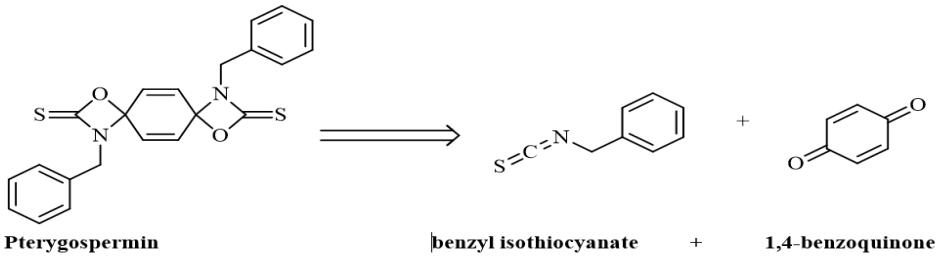

Figure 3.3: Structure of pterygospermin from seeds of Moringa oleifera breaking down into two derivatives (Yongabi, 2010).

“Although the mechanism of action of – and many other chitin-binding proteins is not fully understood as an antifungal agent; however, the antifungal activity of these CBPs was suspected to be as a result of protein binding to nascent fungal cell wall chitin’’(Freire et al., 2015). Recently, Mo-CBP4 was isolated and purified as the new chitin-binding protein from M. oleifera seeds with potential antifungal applications (Lopes et al., 2020). With this, MoCBP adsorbed on sand is responsible for virus removal activity. Recently, Mo-CBP4 was isolated and purified as the new chitin-binding protein from M. oleifera seeds with potential antifungal and antiviral applications (Lopes et al., 2020, Samineni et al., 2019). The carboxylic and amine group in the extracts, especially MO seeds, may cause charge neutralization when virus-plant interaction occurs during water treatment. As earlier discussed pH, ionic strength, water hardness, and virus electrical charges based on capsid may cause the interaction and inactivation of viruses during treatment (Verbyla and Mihelcic, 2015). Apart from turbidity and microbial contamination, heavy metals are some of the most important pollutants that affect water quality. Biosorption of metal by moringa tree during water purification with different functional groups' adsorption mechanisms have been reported (Abd El-Hack et al., 2018, Mnisi and Ndibewu, 2017, Sharma et al., 2006b). These absorption mechanisms include chemisorption, complexation, microprecipitation, ion exchange, and adsorption-surface complexation (Meneghel et al., 2013, Shan et al., 2017, Sharma et al., 2006a). For example, lignin, cellulose, and hemicellulose groups in the secondary cell wall aid in the absorption of metals and the metal ions that bind to the two hydroxyl groups present in the cellulose/lignin unit and form insoluble complexes with heavy metals. This removes metal ions in the treated water through ion exchange and complex formation (Abd El-Hack et al., 2018).

3.2.4.3 Industrial significance of using M. oleifera for biocoagulation and disinfection of water

For sustainable economic-environmental health nexus, the benefits of moringa as an essential component in water purification is well documented for its turbidity and microbial removing properties (Ghebremichael et al., 2006, Ghebremichael et al., 2005, Megersa et al., 2017, Shan et al., 2017). The use of a whole or part of moringa tree as natural coagulants for water and wastewater clarification has been in existence dating back to more than 2000 years in India, Africa (Sudan), and China (Asrafuzzaman et al., 2011). The ability of the seeds of the tree to serve as a natural coagulant for water treatment in Africa was first discovered in the 20th century by women in Sudan (Bergman and Arnoldsson, 2008). The women used swirling powdered seeds in cloth bags for a few minutes with water to flocculate contaminants. After that, they then allowed it to settle for an hour before filtration and used it to purify drinking water (Bergman and Arnoldsson, 2008). The coagulant in *moringa* seeds was later scientifically confirmed by German scientist. Since then, the seeds have proven to be very useful for high turbidity water with similar coagulation and disinfectant effects better than alum and chlorine (Abubakar et al., 2017, Muyibi and Evison, 1995, Shebek et al., 2015). Various agencies and researchers have recommended different procedures to use *M. oleifera* seeds in turbid water clarification and disinfection for safe domestic activities and consumption (Lea, 2014, Muthuraman and Sasikala, 2014). Studies have revealed that MO's seeds do not produce any toxic effects when used for water and wastewater treatment. Therefore, it is generally considered safe for human health (Adeniran et al., 2017, Bina et al., 2010, Jayalakshmi et al., 2017, Mangale Sapana et al., 2012). *Moringa oleifera* as a tropical plant is degradable and biomass produced during the water and wastewater treatment, which can also be used as soil fertilizer due to its non-toxicity.

3.2.4.4 Application of Moringa oleifera as natural clarifying agents in water treatment

The potential of natural materials such as *Moringa oleifera* in water treatment has been found to be a good coagulant and disinfectant suitable for softening high-density water. However, several reports on the use of Moringa stenopetala and Moringa peregrina indicated both to be natural coagulants Asrafuzzaman et al. (2011) and Megersa et al. (2017). The WHO's several years of investigations on the use of MO as a low-cost water purification supplement in countries that are less developed worldwide (Virk et al., 2019) have added impetus to the effectiveness of its seeds in treating potable water for safe human consumption. For example, *Moringa oleifera* seed was one of the most widely researched natural plants by the health organization, which was considered the most effective in treating surface and groundwater (Pritchard et al., 2010b). Performance of *M. oleifera* as an alternative coagulant for removal of dissolved organic carbon (DOC), alkalinity, turbidity, hardness and humic acid from raw water have been explored and shown that the seeds can act as natural buffering agent that can handle moderately high to high alkaline surface and groundwater (Abubakar et al., 2017, Baptista et al., 2017, Omodamiro et al., 2014). However, the coagulation efficiency of about 92% to 99% could be achieved using MO extracts, depending on the initial turbidity of water (Muyibi and Evison, 1995).

Moringa seeds have been successfully used to treat domestic effluent (Adeniran et al., 2017), batik effluent (Dotto et al., 2019, Effendi et al., 2015), dairy industry waste (Pallavi and Mahesh, 2013), and palm oil mill effluent waste (Bhatia et al., 2007). In a study conducted in Malawi, the coagulation removal efficiency of *M. oleifera* was 100% using the optimum dose of 250 mg L^{-1} for treating shallow well water (Pritchard et al., 2009). Scientists from Uppsala University also reported the efficiency of seed material from *M. oleifera* in water purification (Hellsing et al., 2014). Drastic removal of turbidity, water hardness, alkalinity, dissolved oxygen, pH value, total solid, dissolved solids biological oxygen demand and chemical oxygen demand in domestic sewage by MO seed were reported based on the optimum treatment efficiency of the dosage used during water purification (Adeniran et al., 2017). Other reports recommended the use of moringa seed extracts as a coagulant in water and wastewater treatment (Okuda and Ali, 2019) and the removal of other different pollutants such as dyes (Agarwal et al., 2019, Agbahoungbata et al., 2016, Pecora et al., 2018).

Prasad (2009) carried out colour reduction studies on distillery wash using moringa seeds in which optimum colour reduction was found to be between 56% and 67% using KCl and NaCl salts, respectively. Pecora et al. (2018) evaluated the coagulation capacity of supernatant and powder of *Moringa oleifera* seeds on the colour removal efficiency of three different textile dyes. The authors reported that each tested dye interacted differently with the *M. oleifera* powder and supernatant, while dye removal was rated above 70% at varying aqueous pH. This confirmed that the coagulating protein of *M. oleifera* could also be responsible for dye removal depending on the dye molecule. Other studies conducted using river water showed that moringa seed powder could remove over 90% colour and turbidity as well as the total hardness in water (James and Zikankuba, 2017, Pritchard et al., 2010b). Further discussion on the efficiency of *M. oleifera* and other species such as *Moringa stenopetala* seeds treatment compared to conventional synthetic materials used for water treatment can be found in Baptista et al. (2017) and Megersa et al. (2017) based on their compositional properties.

3.2.4.5 Antimicrobial effect of Moringa oleifera in water and wastewater purification

Several studies on medicinal plants as antimicrobial agents have been reported (Abd El-Hack et al., 2018, Baptista et al., 2017, Egbuikwem and Sangodoyin, 2013). A survey [289] on the importance of *M. oleifera* as natural biocoagulants to reduce bacteria demonstrated the suspended solids and turbidity in polluted water. Apart from using MO's whole seeds and the by-product produced from the oil extraction process, the "seed cake residue" can be used for water treatment (Eman et al., 2014). The bioactive components in MO seed extract were used to inactivate and prevent pathogens' growth in water (Eman et al., 2014, Bergman and Arnoldsson, 2008, Bina et al., 2010). Moringa seeds' aqueous extract was reported to have more than 95% *Escherichia coli* removal rate; reduced the concentration of *Staphylococcus aureus and Bacillus subtili*s fecal coliforms in rivers, streams, and well water (Abd El-Hack et al., 2018, Egbuikwem and Sangodoyin, 2013). *M. oleifera* eliminated 80-99% microbial load in contaminated water (Bodlund, 2013, Omojate Godstime et al., 2014, Pritchard et al., 2009, Virk et al., 2019). Aruna and Srilatha (2012) further demonstrated the antibacterial properties of moringa seed powder in water purification and clarification of fish ponds.

According to Shebek et al. (Shebek et al., 2015), MOCP from the seeds inhibits bacterial growth by fusing the inner and outer membranes of *E. coli* cells with a disruptive effect on the membrane and enzyme synthesis (Brilhante et al., 2017). Moringa seed sand filters (f~sand) as sustainable water purification could remove particles and *Escherichia coli* from contaminated water based on the attractive electrostatic interactions (Xiong et al., 2017). The cationic antimicrobial proteins in *Moringa oleifera* seeds have also been utilised as natural coagulants for turbidity and microbial removal. New strategies on retaining *M. oleifera* cationic proteins (MOCP) present in the seed for efficient potable water production with less to no residual organic matter during water treatment have been developed (Jerri et al., 2011, Xiong et al., 2017). One of the advanced methods employed to improve secondary metabolites in moringa seeds is functionalisation. This is one of the techniques employed to develop a reusable plant-based sand filter (f-sand) for water treatment to reduce bacteria count and, at the same time, clarify dirty water (Xiong et al., 2017).

The method uses the reverse charge principle of sand particles using the cationic and antimicrobial proteins/peptides in moringa seeds. Briefly, the cationic protein in MO is dissolved, absorbed, and immobilized onto a sand granules surface, then packed into a filter (f~sand), as shown in Figure 3.4. The filter is rinsed before treatment to prevent bacteria regrowth and excess organic matter in the treated water. Using MOCP-f~sand enhances pathogens and colloidal removal in water, rendering the bacteria non-viable and reducing water turbidity. The process absorbed solid particles, cleared the water, and removed up to 90-99.9% of the bacteria from water (Virk et al., 2019, Clasen et al., 2007, Xiong et al., 2017). The presence of 1% flocculent proteins/polypeptides in moringa seed oil cakes binds mineral particles and organics

during the treatment of drinking water (Suarez et al., 2005). This study proved the importance of using the synthetic chemical-free method to treat drinking water by using the moringa seed incorporated f-sand filter. Moringa seed extracts at 5.6 g of seeds/m^2 of sand were used to reverse the charge of f~sand to 10 mV. The authors reported approximately 4 log removal of 1 µm polystyrene particles and more than 8 log *E. coli* removal as compared to < 0.1 log removal for plain sand used (Xiong et al., 2017). In another study, MO seeds proved to inhibit the development of total bacteria and coliform in raw water, with about 90-99% of bacteria removal (Lea, 2014). For full protocol on using MO mature fruit pods or press cake for water purification, see Lea (Lea, 2014).

Figure 3.4: Moringa seed extract-functionalized sand filter (f-sand) to enhance colloidal and pathogen removal for potable water production with less to no residual organic matter production (Xiong et al., 2017).

In another study, Virk et al. (2019) adopted another method to develop a water purification kit using seeds of *M. oleifera*. They developed a portable water purification kit in the form of a dip bag enclosing MO seed powder that could reduce up to 99.9% microbial load in contaminated water. The authors discovered that 100 mg of dip bag enclosing MO seed powder could kill about 99.9% of the microbial load in 1L of water within 5 minutes. Other studies have shown MO's capacity to reduce *Cryptosporidium parvum* oocysts (Petersen et al., 2016) and control helminths such as *Schistosoma mansoni* in different water and wastewater sources (Mangale Sapana et al., 2012, Rocha-Filho et al., 2015). In the study by Nwosu and Okafor (1995), anthonine was reported to show strong inhibitory activity against *Vibrio cholerae*. The inhibition activity of *Vibrio* spp could be attributed to gallic acid and tannins (Shebek et al., 2015, Brilhante et al., 2017). Other secondary antibiotic metabolites like 2,4-diacetyl phyloroglucinol and carboxylic acid were produced during water treatment when *M. oleifera* seed solutions were reported to show antifungal potential (Omodamiro et al., 2014).

Furthermore, the discharge of viruses via treated effluent-containing viruses into freshwater bodies has been reported to transmit enteric viruses to food products due to polluted irrigation water (Figure 3.5). Despite the different studies on antimicrobial agents based on bacterial and fungal inhibition, there is less report on natural disinfectants for viral removal during water treatment. Therefore, it is crucial to find biological antiviral agents that are environmentally friendly, safe, and inexpensive in the water sector. Due to the different benefits of using natural products as an alternative to chemical or synthesized reagents, research is shifting towards an ancient plant application method for water treatments, which is rarely used in the water sector. Plants are rich sources of biological compounds that are long recognized for varied health benefits. In the medical field, the absence of a vaccine for some viruses has resulted in plant extracts

as anticancer, antiparasitic, antibacterial, and antioxidant. Similarly, applying plant cells or extracts as treatment material to augment the disadvantages of current inorganic coagulants and disinfectants used in the water sector to remove residual enteric viruses is likewise necessary. However, there are still limited reports or studies on using the antiviral potential of plant materials or phytochemical compounds for water treatment.

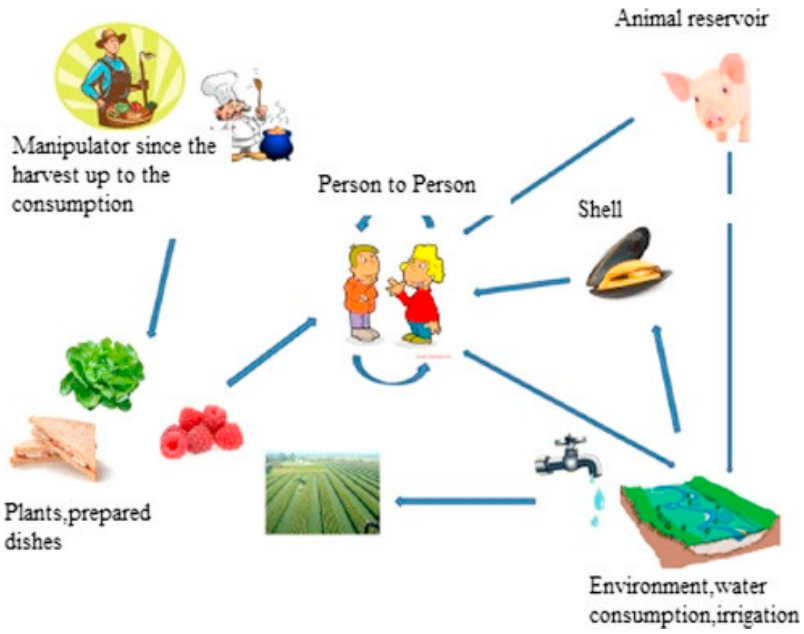

Figure 3.5: Route of transmission of the pathogen from water to food.

Many compounds are present in the plant extracts that can be used as antiviral agents in the water sector. Moringa contains secondary metabolites that can bind free enzymes and reversibly to carboxylic groups of virus capsids. For instance, *Moringa oleifera* extracts against human viruses have been reported for single-stranded RNA respiratory syncytial virus RSV (Onah et al., 2017). Aqueous MO seeds extract against Newcastle disease viruses NDV ((Chollom et al., 2012), Herpex simplex virus (HSV-1 and HSV-2) (Nasr-Eldin et al., 2017) and Hepatitis B virus, Adenovirus (Fabres et al., 2017). *Anogeissus shimperi* against Herpex simplex virus, *Bauhinia thonningii* against (Kudi and Myint, 1999). The antiviral potential of ethanol extract of *M. oleifera* seeds was reported against human herpesvirus-4, called the herpes simplex virus type 1 (Lipipun et al., 2003) and Epstein-Barr virus (Guevara et al., 1999). Also, hydroalcoholic leaf extracts inhibit hepatitis B virus replication (Waiyaput et al., 2012), and silver nanoparticles synthesized using *M. oleifera* seed extract as reducing and stabilizing agents have inhibitory activity against dengue virus type 2 (Sujitha et al., 2015). As mentioned in Guevara et al. (1999) report, the significant biocompounds in moringa that are associated with antiviral activity include niaziminin and isocyanate. Despite these reports; there are still few studies on the antimicrobial potential of *M. oleifera* for water treatment, especially antiviral potential; hence more studies are needed to search for natural biomaterials that can be used in water treatment.

3.2.4.6 Parameters that affect the capacity of polymers present in moringa extracts for water clarification

Contact time, dosage, pH and temperature are some parameters that must be controlled for the best coagulation efficiency of *M. oleifera* tree extracts in water treatment (Abd El-Hack et al., 2018). The pH of the solution exerts a strong influence on coagulation and antimicrobial activities, as earlier discussed. The

initial aqueous pH is an important parameter determining the coagulating capacity of moringa treatment because it affects adsorbate's degree of ionization and solubility (Abd El-Hack et al., 2018, Pritchard et al., 2010b). It has been observed that moringa seeds have more stable activity in varied pH ranges as compared to aluminium sulphate (Brilhante et al., 2017, Yuliastri et al., 2016). The coagulation process using moringa seed powder during water treatment worked better in alkaline conditions at pH 6.5-9 (Abd El-Hack et al., 2018, Thakur and Sonal, 2014). Temperatures below 15℃ negatively affect the coagulating power of moringa seed, while high temperature enhances the coagulation effects during the treatment process (Pritchard et al., 2010b).

3.3 SOCIAL, ENVIRONMENTAL HEALTH NEXUS OF *M. OLEIFERA* FOR WATER PURIFICATION

Today, global concern about the social, environmental, and health implications of water shortages, long-term droughts, population growth, water pollution, and associated problems of the current chemical-based water-treatment methods is increasing. Most countries face formidable challenges in meeting the rising demands for clean and safe water as the available supplies of freshwater are depleting. Most developing countries are undergoing rapid urbanization, with immigrants from inlands occupying the crowded peri-urban and poor townships, which have resulted in the demand for tons of litres of water daily. These demands are already causing different environmental pollution due to the discharge of untreated domestic, industrial, and municipal wastes (Chaurasia et al., 2012). Sadly, water or wastewater treatment has become a problem in developed and developing countries due to poor operational conditions and inadequate maintenance (i.e. inappropriate design, overloaded capacity, faulty equipment, and machinery) of many water and wastewater treatment plants (WWTPs). Therefore, there is an increase in the discharge of pathogens and pollutants to the receiving water bodies, such as exploitable aquifers, numerous artificial dams (lakes), and rivers that are being utilised for domestic activities (Okeyo et al., 2018, Gómez-Duarte et al., 2009). The release of untreated or inadequately treated municipal wastewater effluents could pose a public health risk due to the discharge of deadly and dangerous emerging pathogens such as bacteria, viruses, protozoa, helminths, fungi, and sometimes resistant genes into the freshwater bodies.

Waterborne pathogens represent major contributors to disease outbreaks worldwide, with attendant negative impacts on people's health. For example, this is the cause of an average death rate of 25 000 people every day in developing countries. Millions of others also suffer from severe health-related hazards reported by health experts (Virk et al., 2019, Chen et al., 2018). The presence of waterborne pathogens such as *Shigella, Salmonella, Vibrio cholera,* and enteric viruses that cause severe diarrhea or sometimes human mortality have been isolated in the receiving water bodies (Okeyo et al., 2018, Gómez-Duarte et al., 2009). Similarly, the prevalence of disinfectant, antimicrobial-resistant bacterial, and several water pollution incidents due to partial treatment of sewage discharges from sewage treatment plants and resistant pathogens have also been reported (Bradshaw et al., 2005, Samie et al., 2009, Osuolale and Okoh, 2017). Besides, protozoan parasites are commonly found in different freshwater sources due to the discharge of partially treated wastewater from the treatment plants (Omarova et al., 2018, Petersen et al., 2016). Another significant health risk associated with this is *Cryptosporidium* oocysts in farm products due to contaminated irrigation water (Figure 3.0.5) (Adegoke et al., 2018, Domenech et al., 2018).

In addition to these, some disinfectants generate different types of disinfection by-products, thereby negatively impacting humans (Vivar et al., 2019, Wang et al., 2018). Disinfection by-products are of great health concern because they could cause cancer and other chronic health effects such as cardiac anomalies, stillbirth, miscarriage, low birth weight, and pre-term delivery. To circumvent the pollution of freshwater bodies, many national, regional, local and private agencies have begun to find natural materials that could be integrated into water treatment to address the current challenges in the water sector.

According to the WHO, about 70-80% of the world's population depends on medicinal plants as primary health care sources for treating diseases (Muhammad et al., 2016). However, attention has shifted from nutritional and therapeutic benefits to a readily available low-cost water purification supplement or disinfectant that can be used as a substitute for chemical-based disinfectant. This involves using a whole plant or an isolated compound from medicinal plants such as the moringa tree for water treatment.

It is important to note that some people living in the deep rural communities still lack access to clean and fresh municipality water. Nonetheless, with this method, such communities can treat their water before consumption using locally available plant materials that are cost-effective without any side effects on their health. The application will alleviate chemical-based metal salts and disinfectants during water purification. It can also provide useful biodegradable and environment-friendly treatment technology for bio-coagulation and disinfection of contaminated turbid water (Omodamiro et al., 2014). In addition, the application of plants in the rural communities will create a sustainable development initiative and empower the local communities to grow more trees in the rural area to treat drinking water (Yin, 2010).

Besides medical, nutritional and social applications (Brilhante et al., 2017), the use of natural medicinal plants will further provide an attractive water sanitation option for resource-poor settings worldwide, especially for people in rural areas. The use of the plant as biodegradable, eco-friendly materials for water clarification has been shown to be lucrative, affordable, harmless, competent, sustainable, non-toxic, low cost, efficient and straightforward method to make water free of contaminants without any harmful disinfectant by-products (Alegbeleye, 2018, Ferreira et al., 2014, Ntila et al., 2018, Yin, 2010). It is interesting to note that the application of *M. oleifera* in portable water production is a sustainable method, especially for socio-economically disadvantaged communities, because it reduces health-related risks and deficiency of present chemical-based water treatment methods. Across the world, *M. oleifera* tree seeds and their isolated cationic proteins are paving new ways for green indigenous technology for water treatment (Abiyu et al., 2018, Garcia-Fayos et al., 2016).

Several studies have shown the capacity of *M. oleifera* to remove pathogens in different water and wastewater sources, as earlier discussed. Therefore, it may serve as an alternative to synthetic or inorganic treatment agents that are environmentally friendly and non-biodegradable. It will reduce the money spent on chemicals by the developing countries and encourage local trades by cash crop farmers. Therefore, this plant and other selected plants for this study are promising and socially relevant water treatment resources globally with environmental, public health, and economic sustainability. It will help improve the quality of life in socially neglected communities because of its multifunctional social, economic, environmental-health impacts in battling malnourishment, hunger, water purification, and its possession of therapeutic properties.

CHAPTER 4: DETECTION AND QUANTIFICATION OF SARS-COV-2 VIRAL LOAD IN WASTEWATER TREATMENT PLANTS AND SURFACE WATER: A TEMPORAL CASE STUDY WITHIN ETHEKWINI DURBAN, KWAZULU-NATAL, SOUTH AFRICA

4.1 INTRODUCTION

Worldwide, a high amount of wastewater is being generated due to rapid urbanization, population growth, and economic development. A wastewater treatment plant (WWTP) system is developed to reduce a load of pollutants before it's released into the environment or receiving water bodies. However, in some countries, wastewater infrastructure is inefficient in removing viruses (Edokpayi et al., 2015, Van Abel and Taylor, 2018). Therefore, pollution from poor sewage and wastewater treatment infrastructure directly impacts human health and the environment. In South Africa, several water quality studies have shown that the discharge of pathogens (such as; bacteria, viruses, and protozoa) contributes to the pollution of water resources used by most rural communities (Mema, 2010b). These pathogens can last for hours and even days in wastewater (Murphy, 2017). They can lead to major global health concerns such as low infectious dose, rapid increase in viral-associated infections due to exposure to contaminated water and wastewater, high mortality, and common medications to treat viral infections. Therefore, it is essential to prevent the outbreak of new viruses by monitoring and detecting an emerging virus in environmental media water. This action helps prevent potential outbreaks caused by viral pathogens and provides an epidemiological database for infection control (McCall et al., 2020). Thus, the detection of enteric viruses in the treated effluent before its discharge is an important criterion to consider in assessing the local environmental and public health (Osuolale and Okoh, 2017).

The recent outbreak of the COVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has become a significant concern for environmental scientists. Due to the unimaginable spread of coronavirus disease, it has been the subject of several studies worldwide since the end of 2019 (Lu et al., 2020, Amoah et al., 2021). Various studies have detected SARS-CoV-2 RNA in untreated wastewater samples worldwide (Ahmed et al., 2020b, Sherchan et al., 2020). The presence of RNA of this virus in untreated wastewater has been reported in Australia (Ahmed et al., 2020c), France (Wurtzer et al., 2020a), Italy (La Rosa et al., 2020), Netherlands (Medema et al., 2020), South Africa (Pillay et al., 2021b) and the United States (Sherchan et al., 2020). As a result, detecting SARS-CoV-2 RNA in municipal wastewater is becoming more and more common to monitor changes in COVID-19 cases in a community (Ahmed et al., 2020b, Kocamemi et al., 2020, Peccia et al., 2020, Randazzo et al., 2020a, Randazzo et al., 2020b). However, limited studies have reported the occurrence of SARS-CoV-2 in treated wastewaters (Randazzo et al., 2020b, Haramoto et al., 2020). Unfortunately, we are still dealing with the COVID-19 pandemic because the virus is constantly mutating, resulting in the appearance of new variants with higher transmissibility and pathogenicity (Jmii et al., 2021). Therefore, it is vital to confirm the efficiency of wastewater treatment plants to reduce the SARS-CoV-2 viral load and determine its detection in river water to prevent its spread in water bodies within the province. Hence, the focus of this study was to confirm the efficiency of WWTP in reducing SARS-CoV-2 viral load and its detection in River water within eThekwini Durban, KwaZulu-Natal province, South Africa.

4.2 MATERIALS AND METHODS

4.2.1 Sampling Sites

In this study, surface water (i.e. river) raw and treated municipal wastewater was tested for SARS-CoV-2 viral RNA. To this end, several field surveys were carried out between the end of October and the middle of November 2020 and four wastewater treatments plants (WWTPs) were selected for this study. These include Isipingo WWTP, Amanzimtoti WWTP receiving 50% domestic and 50% industrial influent, Kingsburgh and Shallcross WWTP receiving 100% domestic influent, and Marrianridge receiving 65% industrial influent. Based on our research finding, it is interesting to note that Shallcross WWTP is the plant treating Marrianridge influent (Figure 4.0.1). All sample sites are located in Durban, KwaZulu-Natal, South Africa. These WWTPs use the activated sludge system as a biological treatment followed by chlorination of the final aqueous effluent before discharge into the receiving environments.

4.2.2 Collection of Water Samples

The grab sampling method was used for sample collection. In total, sixty-one samples were collected in autoclaved polypropylene plastic bottles between September 2020 and October 2021. Briefly, forty-five wastewater (WW) samples consisting of primary influent (n = 25) and final /tertiary effluent (n = 20) were collected from the four WWTPs during the sampling period for analysis. Sixteen water samples from Isipingo, Kingsburgh Shallcross, and Amanzimtoti rivers, where the WWTPs are discharging their treated effluents, were further surveyed. Then, the collected samples were transported on ice to the Department of Biotechnology and Food Science, the Durban University of Technology for concentration and further analysis. Figure 4.1 shows the concept used in this study.

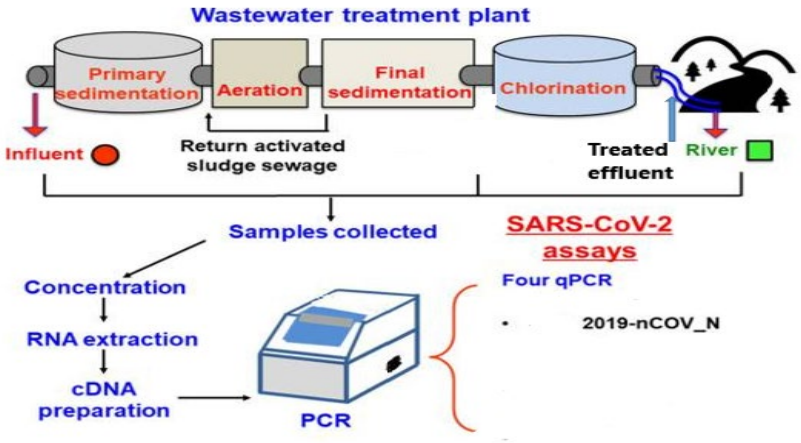

Figure 4.1: Study concept used for sampling.

4.2.3 The concentration of SARS-CoV-2 in Water Samples

Viruses were concentrated from WW and river samples (250-2000 mL based on turbidity) using two different methods, namely, Tangential Flow Filtration (TFF) System and Polyethylene Glycol Precipitation (PEG) methods. The TFF system was applied as described by Rezaeinejad et al. (2014). Figure 4.2 shows an overview of the concentration of SARS-CoV-2 in water samples using the TFF System. Two litres of water samples was prefiltered with 20 µm filter paper and then concentrated to about 250 mL using a TFF System with a 30 kDa membrane cassette (Figure 4.2). The concentrated viruses were backwashed with glycine buffer (Glycine buffer 100 mL, 0.05 mol^{-1}, pH 7.0) to 50 mL for 5 min. Secondary concentration to a volume of 0.5 mL was achieved in an Ultra-15 centrifugal tube with 30 kDa cut-off levels (Amicon Merk, Germany). The concentrated samples were stored at -20°C for RNA extraction and subjected to RT-qPCR analysis within seven days of RNA extraction.

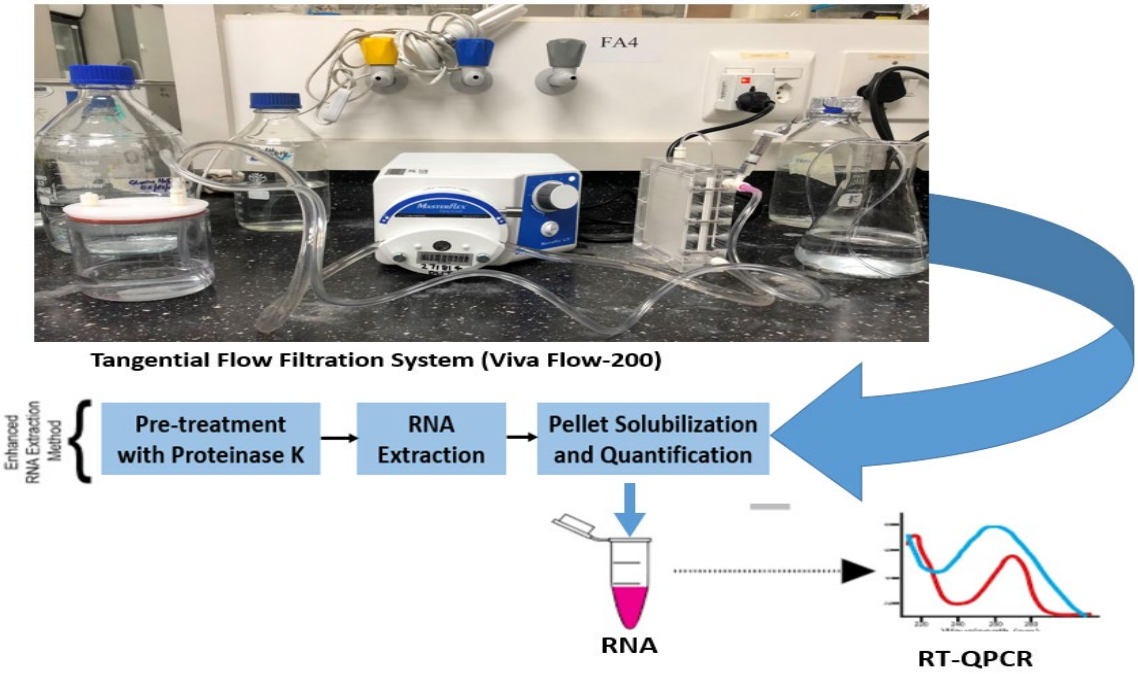

Figure 4.2: Overview of the concentration of SARS-CoV-2 in water samples using the TFF System

Modified polyethylene glycol precipitation technique was used to concentrate all samples, including turbid water (Aw and Gin, 2010). Briefly, water samples with pH adjusted to 7.2 were mixed with 8% PEG 8000 (80 g/L) and NaCl (17.5 g/L). After that, the samples were incubated overnight at 4°C with agitation (150 rpm) to precipitate viruses. The samples were later centrifuged at 6700 x g for 30 min and centrifuged for the second time at 10 000 x g for 30 min to collect pellet then, dissolved in 10-20 mL of phosphate buffer saline (PBS). An equal volume of chloroform was added, centrifuge at 1700 x g for 30 min, and the supernatant was filtered using a 0.22 µm syringe filter to remove bacteria from the concentrate. The concentrated samples were stored at -20°C for RNA extraction and subjected to RT-qPCR analysis within 7 days of extraction.

Safety measures during concentration: Personal protective equipment (PPE) such as coats, face masks, gloves, face shields, and safety glasses were worn during WW sample processing. As per the activity risk assessment prepared for this study, filtered WW samples were treated with 10% bleach and discarded in the sink.

4.2.4 Extraction of Viral Nucleic Acid

NucleoSpin RNA virus Kit (Macherey-Nagel, Düren, Germany) was used for the extraction of RNA from the concentrated samples with modifications. Briefly, 150 µL of the concentrated water sample was mixed with 600 µL of lysis buffer supplied in the NucleoSpin® RNA virus kit. After that, the resulting solution was centrifuged for 5 min at 11,000 x g to pellet the particles. The supernatant was subsequently processed as instructed in the product manual with the 50 µL final elution buffer (provided by kit) added. After that, the concentrations of extracted RNA were determined using a Nanodrop spectrophotometer.

4.2.5 Reverse Transcription of Extracted RNA

Complementary DNA (cDNA) was generated using the ProtoScript II First Strand cDNA Synthesis Kit (Biolabs Inc, New England). The components of each 20 µL reaction were Random Primer Mix, ProtoScript II Reaction Mix, ProtoScript II Enzyme Mix, Nuclease-free water, and template RNA. After that, the reaction mixture was incubated for 5 min at 25°C, 1 hour at 42°C, and 5 min at 85°C. The concentration of the cDNA was measured using a Nanodrop spectrophotometer and stored at -20°C for further analysis.

4.2.6 Quantification of Viral Genomes by qPCR

The concentration of SARS-CoV-2 in the synthesized cDNA was quantified using real-time PCR (qPCR) on QuantStudio 5 Applied Biosystems (ThermoFisher Scientific). The primers probe used in this study targeted the nucleocapsid (N) region of SARS-CoV-2 genome (2019-nCoV_N2); Forward (5′-TTA CAA ACA TTG GCC GCA AA-3′), Reverse (5'-GCG CGA CAT TCC GAA GAA-3′), and Probe (6FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1) (CDC, 2020). The N2 gene was chosen as it is the most widely used target gene in SARS-CoV-2 detection assays. Other researchers have identified N2 as a good target for amplification compared to either N1 or N3 (Randazzo et al., 2020c, Pillay et al., 2021b).

The thermal cycling conditions of the qPCR assays were as follows: preheating at 95°C for 1 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The qPCR reaction was carried out in a 20 µL mixture that contained 4 µL of RNA, 10 µL One Step Primescript III RT-qPCR Mix (Takara), 0.4 µL each of forward and reverse primers, 0.2 µL of the probe and nuclease-free water. When the reverse transcription product (cDNA) was used as a template, the qPCR reaction for the detection of SARS-CoV-2_N2 was carried out in a 20 µL mixture that contained 4 µL of cDNA, 10 µL TB Green Advantage qPCR Premix (Takara), 0.4 µL each of forward and reverse primers, and nuclease-free water. All RT-qPCR reactions were performed in duplicate. For each RT-qPCR run, a series of positive and no-template controls were included. The genome copy number of SARS-CoV-2_N2 was determined based on the standard curve constructed using tenfold serial dilutions of commercially synthesized whole-genome inactivated SARS-CoV-2 RNA USA/WA1/2020 (Microbiologics, USA) of the target viral sequence used as the positive control. PCR grade water was used as the template for negative control instead of the RNA/cDNA template. Both positive and negative control were amplified to determine the efficiency of the qPCR assay.

4.2.7 Data Analysis

Data was imported in Microsoft excel and was used for descriptive statistics such as mean and standard deviation. On the other hand, raw data imputed into excel from laboratory notebooks were crosschecked to ensure data validation.

4.3 RESULTS

4.3.1 Presence of SARS-CoV-2 Genetic Material in Treated and Untreated Wastewater Samples

The prevalence of viral SARS-CoV-2 load in untreated primary influent and treated final tertiary effluent samples of four WWTPs were examined using RT-qPCR. This is to determine the efficiency of the treatment plants in reducing viral load before discharge into the environmental bodies. In this study, samples were considered positive when the average cycle threshold (Ct) value < 40 while values above 40 were considered negative for the SARS-CoV-2_N2 gene. For each qPCR assay, the mean efficiency of the standard curve was selected based on the correlation coefficient (R^2) values between 0.996 and 0.999. In contrast, slope and Y-intercept values of -3.20 and 32.74 were used to show no serious inhibition during the qPCR analysis. In all, 12 (seven untreated and five treated samples) out of 45 wastewater samples from all the surveyed WWTPs were positive (Table 4.1). At least one sample at each site was positive and within the detectable limit (Figure 4.3).

Table 4.1: Prevalence of SARS-CoV-2_N2 in WWTPs Samples

WWTPs	Sample	No of positive samples/no of tested samples)
Amanzimtoti	Influent (AIP)	1/5
	Effluent (AEP)	1/5
Isipingo	Influent (IIP)	2/5
	Effluent (IEP)	2/5
Kingsburgh	Influent (KIP)	2/5
	Effluent (KEP)	2/5
Shallcross	Influent (SIP)	1/5
	Effluent (SEP)	1/5
Marianridge	Influent (MIP)	1/5

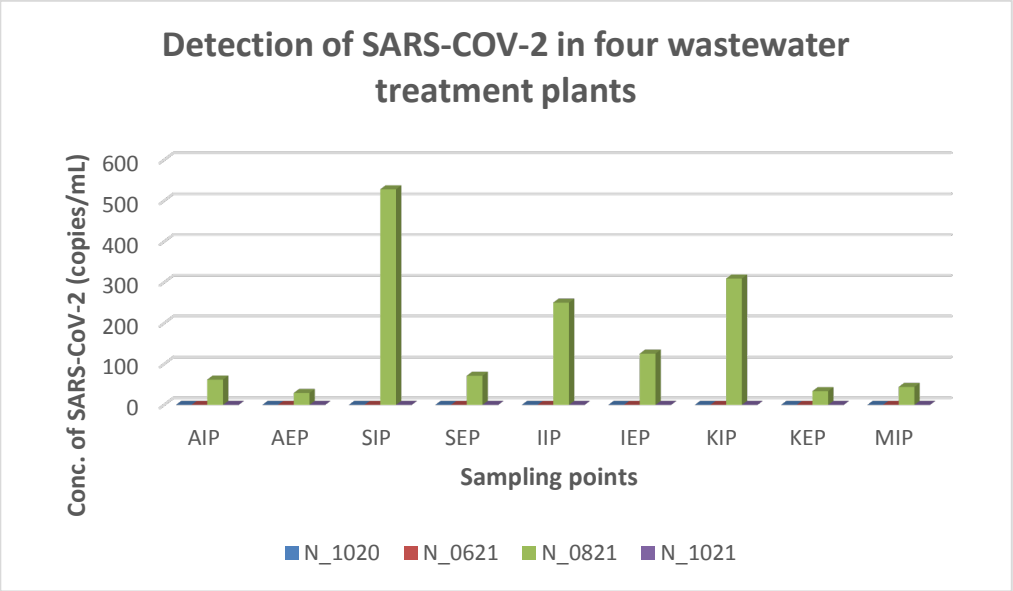

Figure 4.3: Prevalence and varied concentration of SARS-CoV-2_N2 in the surveyed wastewater treatment plants.

On average, the highest viral load of 2.63 x 10^6 ± 1.70 copies/mL was detected in untreated wastewater from Isipingo (IIP) WWTP at the start of the study (7th October 2020), and the lowest concentration of 2.92 x 10^1 ± 0.34 copies/mL was recorded for Amanzimtoti treated effluent (AEP). In October 2020, almost all the samples were below the detectable limit, as shown in Figure 4.0.3. However, the SARS-CoV-2_N2 gene was detected in two WWTPs with positive detection in Kingsburgh influent (KIP) and samples from Isipingo WWTP (IIP and IEP). As further shown in Figure 4.3, all the samples collected from WWTPs in August 2021 tested positive. On average during this period, the highest concentration (5.28 x 10^2 ± 0.42 copies/mL) was at SIP, followed by KIP (3.10 x 10^2 ± 1.71 copies/mL) then, IIP (2.50 x 10^2 ± 1.01 copies/mL), i.e. SIP > KIP > IIP.

4.3.2 The Presence of SARS-CoV-2 in River Water

In terms of percentage viral load removal in the surveyed WWTPs, the removal efficiency shows a reduction in the concentration of SARS-CoV-2_N2 in each of the WWTP when the concentration of the influent (untreated wastewater) was compared with the treated effluent (Figure 4.4). Kingsburgh WWTP is the most efficient with 89.12% removal efficiency of SARS-CoV-2_N2 viral load, while Isipingo is the least efficient with 49.87% removal efficiency. For WWTPs analysis, out of the 45 samples analyzed, 12 were positive for SARS-CoV-2_N2, in which 7 were influent samples while 5 were effluent samples. Conversely, five (5) samples from the river water samples (n = 16) examined for the presence of SARS-CoV-2_N2 using qPCR assay were within the quantifiable concentration. The gene was detected in 31.25% of the sampled river, while 68.75% tested negative (Not detected). Table 4.2 shows that the SARS-CoV-2_N2 genome is detected at least once in each of the four rivers surveyed, while Figure 4.0.5 further presents the mean concentration with a positive viral load between 5.78 x 10^1 and 4.8 x 10^3 copies/mL.

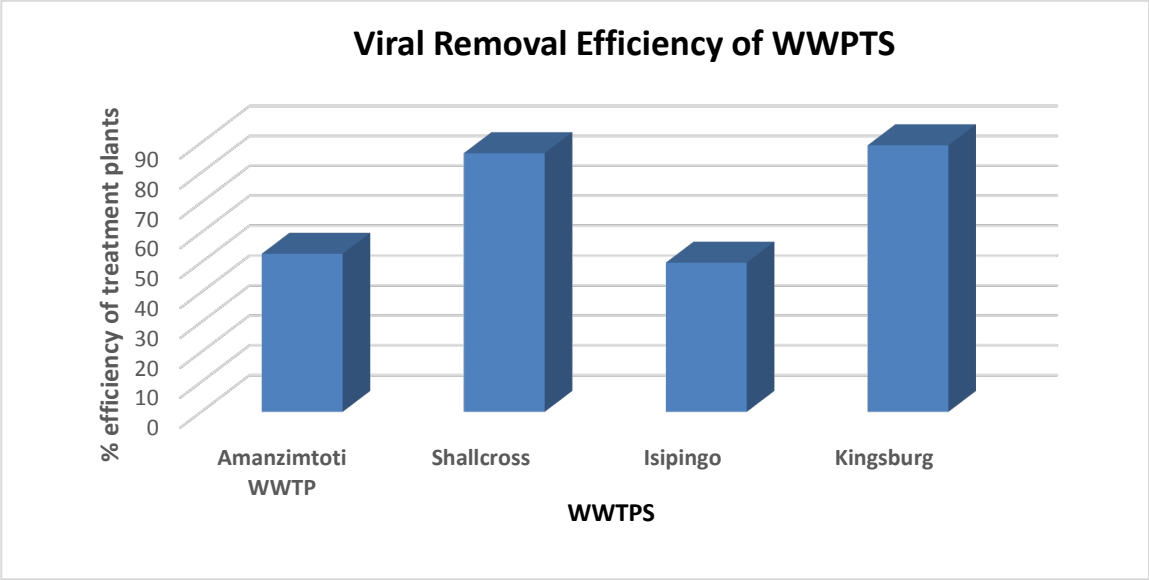

Figure 4.4: The efficiency of wastewater treatment plants in removing SARS-CoV-2_N2 gene from treated waste samples.

The SARS-CoV-2_N2 viral load in the water samples collected from Kingsburgh river in June 2021 was the highest quantifiable concentration (4.80 x 10^3 copies/mL) in all the analyzed river samples in this study (Figure 4.5). However, samples collected in August 2021; Isipingo river (IRP) has the highest viral load (6.48 x10^2 copies/mL), followed by Kingsburgh (KRP, 1.83 x 10^2 copies/mL), Amanzimtoti River (ARP, 7.01 x 10^1 copies/mL). The lowest recorded concentration was in the Shallcross river (SRP, 5.78 x 10^1 copies/mL). Other samples analyzed using qPCR were below the detectable limit during this study period. The non-detection of the viral particles or RNA in some wastewater samples could be due to concentrations below the detection limit for qPCR assay.

Table 4.2: Prevalence of SARS-CoV-2 in River Samples

River	Sample Time	Detection (No of positive samples/no of tested samples)	% Detection
ARP	9/20	0/1	ND
	10/20	0/1	ND
	6/21	0/1	ND
	8/21	1/1	100
SRP	9/20	0/1	ND
	10/20	0/1	ND
	6/21	0/1	ND
	8/21	1/1	100
IRP	9/20	0/1	ND
	10/20	0/1	ND
	6/21	0/1	ND
	8/21	1/1	100
KRP	9/20	0/1	ND
	10/20	0/1	ND
	6/21	1/1	100
	8/21	1/1	100
ND: Not detectable			

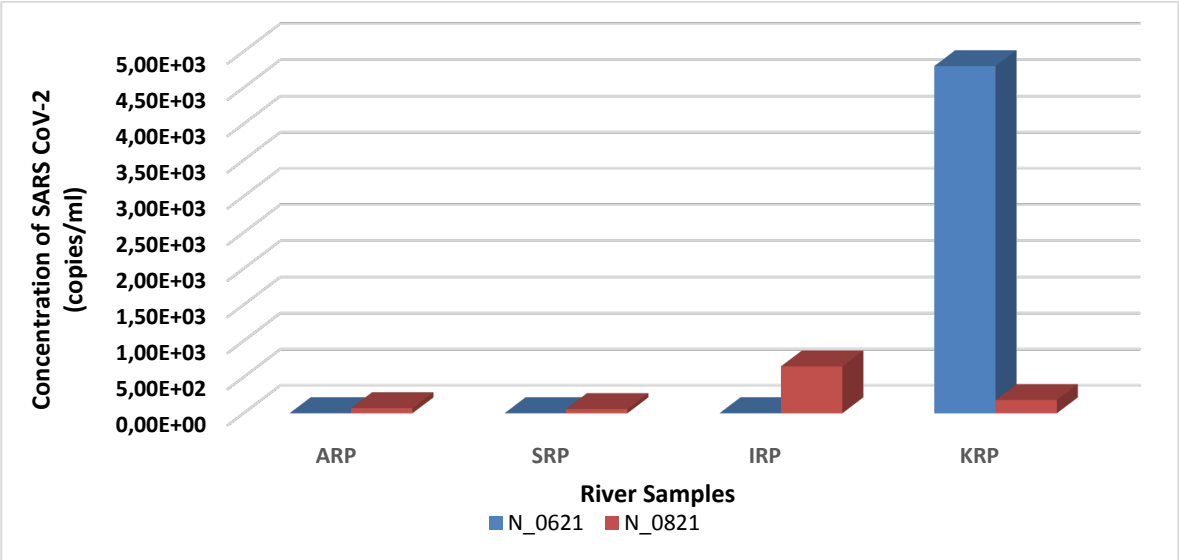

Figure 4.5: Comparison between the detection of SARS-CoV-2 in river samples. The non-detection of the viral particles or RNA in some wastewater samples could be due to concentrations below the detection limit for qPCR assay.

4.4 DISCUSSION

4.4.1 Prevalence of the SARS-CoV-2-N2 gene in water samples

The prevalence of the SARS-CoV-2-N2 gene in raw wastewater has been reported in several studies (Ahmed et al., 2020b, Haramoto et al., 2020, Jmii et al., 2021, Kocamemi et al., 2020, La Rosa et al., 2020, Randazzo et al., 2020b, Carrillo-Reyes et al., 2021). In the same vein, there is a scarcity of knowledge on the persistence of SARS-CoV-2 in treated wastewaters and receiving water bodies (Westhaus et al., 2021). And more importantly, the efficiency of WWTPs in reducing SARS-CoV-2 viral load in eThekwini, Durban has not been thoroughly investigated. Hence, this study presents the efficiency of four WWTPs in removing the viral particles and their persistence in surface water. As observed in this study, 45 wastewater samples were analyzed, 12 samples were positive for the SARS-CoV-2_N2 gene with seven (7) influent and five (5) effluent samples. The results are similar to the study conducted in Paris by Wurtzer et al. (2020b), where six out of the eight untreated effluent samples collected from WWTPs tested positive. Likewise, 35 out of 42 influent samples and 2 out of 18 secondary treated samples were positive (Randazzo et al., 2020c). In comparison, 2 out of 9 samples in raw wastewater Brisbane (Australia) (Ahmed et al., 2020c) were positive, and the other 29 samples as well (Ahmed et al., 2021).

All samples collected on 23rd August 2021 tested positive; this could be linked to the increased cases of Covid-19 in August 2021 and the unrest in the Kwazulu-Natal (KZN) region earlier in July 2021. Prior to the civil unrest, the province had a record of less than 2000 new infections, but this jumped to 2289 on 31st July 2021. The number of new infections increased to 2878 in the region on the 15th of August, and a further increase was reported on 27th August as the number of new cases jumped to 3905 (NICD, 2021). This corroborates the dependence of the prevalence of the virus in wastewater on its shedding in the stool of infected patients, whether symptomatic or asymptomatic (Pillay et al., 2021a). Furthermore, the presence of SARS-CoV-2 in secondary-treated wastewater was reported in both Spain and Japan (Randazzo et al., 2020b, Haramoto et al., 2020). In another study reported by Westhaus et al. (2021), the presence of SARS-CoV-2 in both raw and treated WW samples was confirmed in nine WWTPs. However, Kumar et al. (2021) reported the absence of SARS-CoV-2 in treated WW samples. The concentration of SARS-CoV-2 in the influent samples is in the range of 10^1 to 10^6 copies/mL, with the influent samples of Isipingo being the highest, which was 2.63 x 10^6 ± 1.70 copies/mL. The concentration of SARS-CoV-2 in all the effluent samples examined in this study ranged from 10^1 to 10^3 copies/mL, and this is almost similar to other reported studies (Tanhaei et al., 2021, Westhaus et al., 2021). The non-detection of the viral particles in some samples could be linked to the presence of low concentrations of the RNA fragments in the sample which could be below detectable limit of qPCR method used.

4.4.2 The efficiency of WWTP in reducing the viral load of SARS-CoV-2 and its impact on River water

Isipingo WWTP was the least efficient of the WWTP as it reduced 2.63 x 10^6 copies/mL of SARS-CoV-2 in the influent sample in October 2020 to 8.50 x 10^3 copies/mL in the effluent sample, while 2.50 x 10^2 was reduced to 1.25 x 10^2 in August 2021. A similar high concentration pattern of Isipingo effluent in October 2020 was reported by Pillay et al. (2021b). In addition, this study recorded the highest removal efficiency in Kingsburgh WWTP with 89.12% removal efficiency of SARS-CoV-2 RNA. In October 2020, the concentration of SARS-CoV-2 RNA in the influent sample of Kingsburgh WWTP was about 2.22 x 10^4 copies/mL, while the virus was not detected in the effluent sample. However, in August 2021, 3.09 x 10^2 copies/mL of SARS-CoV-2 RNA in the influent sample was reduced to 3.3 x 10^1 copies/mL of SARS-CoV-2 RNA in the effluent. Furthermore, the presence of SARS-CoV-2 in the river sample could be due to the partial efficiency of the surveyed WWTPs in eThekwini Durban. Also, there was a positive detection of

SARS-CoV-2 in all the river water samples examined in August 2021, and only Kingsburgh river tested positive in June 2021.

Although the viral load in Kingsburgh effluent in June 2021 was below the detectable limit, however, the SARS-CoV-2 load in the analyzed Kingsburgh River sample for this period was high (4.80×10^3) as compared to other samples, a similar pattern was reported by Rimoldi et al. (2020). Likewise, in August 2021, the same WWTP was the most efficient in removing viral particles, but the river receiving the discharged treated effluent had the highest viral concentration. The impact of treated effluent on the surface water was also evidenced in the Isipingo river, with the highest viral concentration during the monitoring period. This could be attributed to the flow of the viral RNA from different sources into the river, human activities (as shown in Figure 4.6), and the discharge of non-treated or inefficiently treated or combined sewage overflows. Figure 4.0.6 shows some of the activities observed around the sampled rivers. The findings of this study on the impact of sewage on river health agree with the report on the detection of SARS-CoV-2 RNA in the secondary-treated WW and river waters in Italy (Rimoldi et al., 2020), where the authors detected SARS-CoV-2 in river waters polluted with sewage without quantification. The viral RNA was detected in river water in Ecuador (Guerrero-Latorre et al., 2020). On the contrary, the virus was not detected in natural streams in Japan (Haramoto et al., 2020), which does not agree with this study. These discrepancies in the result could be traced to disease prevalence in the study area.

Figure 4.6: Activities around the sampled River site during the study.

CHAPTER 5: DETECTION OF NOROVIRUSES IN FOUR SELECTED WASTEWATER TREATMENT PLANTS AND RIVER WATER SAMPLES WITHIN ETHEKWINI DURBAN, SOUTH AFRICA

5.1 INTRODUCTION

Worldwide, viruses are a serious public health concern because of their persistence in the environment. They are shed in high numbers through the faeces of infected individuals and are transmitted via the faecal-oral route (Fong and Lipp, 2005, Sánchez and Bosch, 2016a, Bouseettine et al., 2020). Human enteric viruses are a broad group of viruses that cause waterborne diseases when humans become exposed to them (Fong et al., 2005, Upfold et al., 2021). They are recognized for their low infection dosage, resistance to heat and UV radiation (Haas et al., 1993, Zhu et al., 2018b). Enteric viruses are mostly associated with diarrhoea and gastroenteritis, but they can also cause respiratory infections, conjunctivitis, and even fatal conditions which include aseptic meningitis and encephalitis (Kocwa-Haluch, 2001, La Rosa et al., 2012, Bouseettine et al., 2020).

Noroviruses (NoVs) as an enteric virus cause acute gastroenteritis in children and adults (Patel et al., 2008, Bartnicki et al., 2017). Noroviruses are extremely infectious for a variety of reasons, one of which is that they have a low infectious dosage of fewer than 10 copies of the virus. NoVs are transmitted by the faecal-oral route as well as contaminated environmental media, food, and contaminated surfaces (Patel et al., 2009, Zhou et al., 2020). Research has shown that some viruses are shielded or resistant to disinfectants during wastewater treatment, causing a lower removal rate and their discharge into water bodies (Hewitt et al., 2011). The presence of NoVs in environmental waters was reported in Europe (Lysén et al., 2009, Lodder and de Roda Husman, 2005), the United States of America (Gentry et al., 2009), and Latin America (Fioretti et al., 2018, Prado et al., 2019). The prevalence and diversity of NoVs were reported in African environmental media (surface water, sewage influent, and effluent and shellfish) with significantly high levels of contamination in treated effluent and river water (Mans et al., 2016). Thus, continuous monitoring of circulating norovirus genotypes in environmental media is required to understand their prevalence in the community better. Therefore, early detection of probable pandemic variants is a good attempt for the prevention of an impending outbreak which sends warning signals to the public health sectors for implementing infection measures (Lun et al., 2018). Thus, we examined the influent and effluent samples of selected wastewater samples and river water within eThekwini Durban, South Africa to detect Norovirus GI and GII.

5.2 MATERIALS AND METHODS

5.2.1 Sampling Sites and Collection of Water Samples

Four wastewater treatments plants (WWTPs) were selected and sampled as described in Subsection 4.2.1 and 4.2.1 for this study. Then, the collected samples were transported on ice to the Department of Biotechnology and Food Science, the Durban University of Technology for concentration and further analysis.

5.2.2 Concentration of Water Samples and Extraction of Viral Nucleic Acid

The two earlier described methods (Subsection 4.2.3) were used to concentrate the collected samples' viral particles. NucleoSpin RNA virus Kit (Macherey-Nagel, Düren, Germany) was used to extract the RNA from the concentrated samples with modifications. Briefly, 150 µL of the concentrated water sample was mixed with 600 µL of lysis buffer as supplied in the NucleoSpin® RNA virus kit. After that, the resulting solution was centrifuged for 5 min at 11,000xg to pellet the particles. The supernatant was subsequently processed as instructed in the product manual with the 50 µL final elution buffer (provided by kit) added. Thereafter, the concentrations of extracted RNA were determined using a Nanodrop spectrophotometer.

5.2.3 Reverse Transcription and Detection of Norovirus by Real-time Quantitative PCR

According to manufacturing instruction, complementary DNA (cDNA) was generated using the ProtoScript II First Strand cDNA Synthesis Kit (Biolabs Inc, New England). The cDNA was measured using a Nanodrop spectrophotometer. Then, the amplification reaction for the detection of NoV GI and GII was performed on QuantStudio 5 Applied Biosystems (Thermo Fisher Scientific). The sequences of the primers and probes used for the detection of NoV GI and GII are detailed in Table 5.1 and amplification of both target genes was carried out using the following thermal cycling conditions of 95°C for 15 min and 40 cycles of 95°C for 10 s, 57°C for 30 s and 72°C for 30 s.

Table 5.1: Oligonucleotide sequences used for the amplification of Noroviruses

Norovirus	Code	Primer and Probe Sequence (5'-3')	References
GI	COGIF	CGYTGGATGCGNTTYCATGA	(Kageyama et al., 2003)
	COGIR	CTTAGACGCCATCATCATTYAC	
	JJVIP	FAM-TGTGGACAGGAGATCGCAATCTC-BHQ1	
GII	JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	(Kageyama et al., 2003)
	COG2R	TCGACGCCATCTTCATTCACA	
	RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-BHQ1	

The qPCR reaction was carried out in a 20 µL mixture that contained 4 µL of RNA, 10 µL One Step Primescript III RT-qPCR Mix (Takara), 0.4 µL each of forward and reverse primers, 0.2 µL of the probe and nuclease-free water. When the reverse transcription product (cDNA) was used as the template, the qPCR reaction for the detection of Norovirus GI and GII was carried out in a 20 µL mixture that contained 4 µL of cDNA, 10 µL TB Green Advantage qPCR Premix (Takara), 0.4 µL each of forward and reverse primers, and nuclease-free water. Each sample was analyzed in duplicate and negative control were added to all the qPCR assays. A threshold cycle (Ct) value was determined as the cycle number at which the exponentially increasing fluorescence signal exceeded the threshold value. All samples and standards were duplicated, and an average Ct value was calculated if both wells were positive. A sample was assumed to be negative if the Ct value was higher than 40. A sample was assumed to be positive if the respective virus was detected in one of the two wells. Negative controls were prepared for each qPCR batch using 4 µL of PCR grade water instead of the DNA/cDNA template.

5.3 RESULTS AND DISCUSSION

Worldwide, the prevalence of Norovirus has been reported in different water bodies, which include sewages, rivers, recreational waters, municipal water, and groundwater (La Rosa et al., 2007, La Rosa et al., 2008, La Rosa et al., 2010, Okubo et al., 2019, Upfold et al., 2021). The possible health hazards posed by their discharge into receiving water bodies are all critical issues. Monitoring WWTPs would be a suitable approach to determining if viruses circulate in environmental media. In this study, a total of sixty-five water samples were collected from four WWTPs and four rivers with KwaZulu-Natal, South Africa, then investigated for the presence of NoV GI and GII. Samples were considered positive for Ct values below 40, while values above 40 were considered negative for the viruses. Forty-five wastewater samples were examined for the presence of NoV GI and GII in which 33 samples (74.33%) were positive for NoV GI while 32 samples (71.11%) were positive for NoV GII. A similar study was conducted in six WWTPs in which 55.5% of the wastewater samples were positive for NoV GI while 44.4% of the samples were positive for NoV GII (Shaheen and Elmahdy, 2019). Another study reported approximately 47.9% and 59.0% of NoV GI and GII were detected in the wastewater samples, respectively (Prado et al., 2019). In another study conducted in Spain between September 2016 to September 2017, the authors reported NoV GII as the most prevalent detected strain with 76% and 69.6% NoV GI in the WWTP samples (Santiso-Bellón et al., 2020). Table 5.2 further shows the prevalence of NoV GI and GII in the influent and effluent samples of four WWTPs that were investigated in this study.

Table 5.2: Prevalence of NoV GI and GII in WWTPs samples

WWTPs	Sample	No of positive samples/no of tested samples)	
		NoV GI	NoVGII
Amanzimtoti	Influent (AIP)	4/5	4/5
	Effluent (AEP)	3/5	3/5
Isipingo	Influent (IIP)	3/5	3/5
	Effluent (IEP)	3/5	3/5
Kingsburgh	Influent (KIP)	4/5	4/5
	Effluent (KEP)	4/5	4/5
Shallcrosss	Influent (SIP)	4/5	4/5
	Effluent (SEP)	4/5	3/5
Marianridge	Influent (MIP)	4/5	4/5

The two strains were detected in all the WWTPs throughout the sampling period except in June 2021 and September 2020 in Isipingo WWTP. Out of the 33 samples that were positive for NoV GI, nineteen were influent samples (76%), while the remaining fourteen (70%) were effluent samples. Likewise, for NoV GII, nineteen were influent samples (76%) and the remaining thirteen (65%) were effluent samples. Amanzimtoti WWTP was fully efficient in September 2020 because the influent sample was positive for both strains but below the detectable limit in the effluent samples. NoV GI and GII are widely distributed in influent samples of the surveyed WWTPs, with detection rates varying from 80% in Amanzimtoti, Kingsburgh, Shallcross WWTP, and Marianridge influent, while Isipingo WWTP was 60%. However, relatively lower detection rates were observed in the effluent samples. The detection rates for NoV GI and GII in the effluent samples range from 80% in Kingsburgh WWTP to 60% in Amanzimtoti and Isipingo WWTP. Conversely, in Shallcross WWTP, the detection rate for NoV GI was 80%, while 60% detection rate was recorded for NoV GII.

The effluents' Ct values were relatively higher than the influents in all the WWTPs that were investigated. Figure 5.1 shows the viral load of NoV GI and GII in WWTPs in terms of Ct values. The Ct value that is inversely proportional to the viral load in a sample was used to determine the level of noroviruses' concentration in this study (Alahdal et al., 2021). The Ct values that are less than 29 (High viral load ≤ Ct value of 29) denote strong viral load, while an increase of three in the Ct value denotes a tenfold reduction in the viral load. In addition, Ct values that range between 30-37 indicate a moderate load, while a weak viral load is denoted by values of 38-40 (Alahdal et al., 2021). For both GI and GII, lower Ct values (strong viral load) were recorded in August 2021, while it was consistently higher across the WWTPs in September 2020 (moderate load). In addition, there was no much difference between the Ct values in August 2021, as the values were almost similar across the four WWTPs for both GI and GII except for Isipingo and Kingsburgh effluent which was 30 and 31 respectively. This shows that concentration of GI and GII were higher in the analysed samples. Across the WWTPs, Kingsburgh WWTP was slightly higher compared to other WWTPs with Ct values relatively above 30, representing moderate viral load.

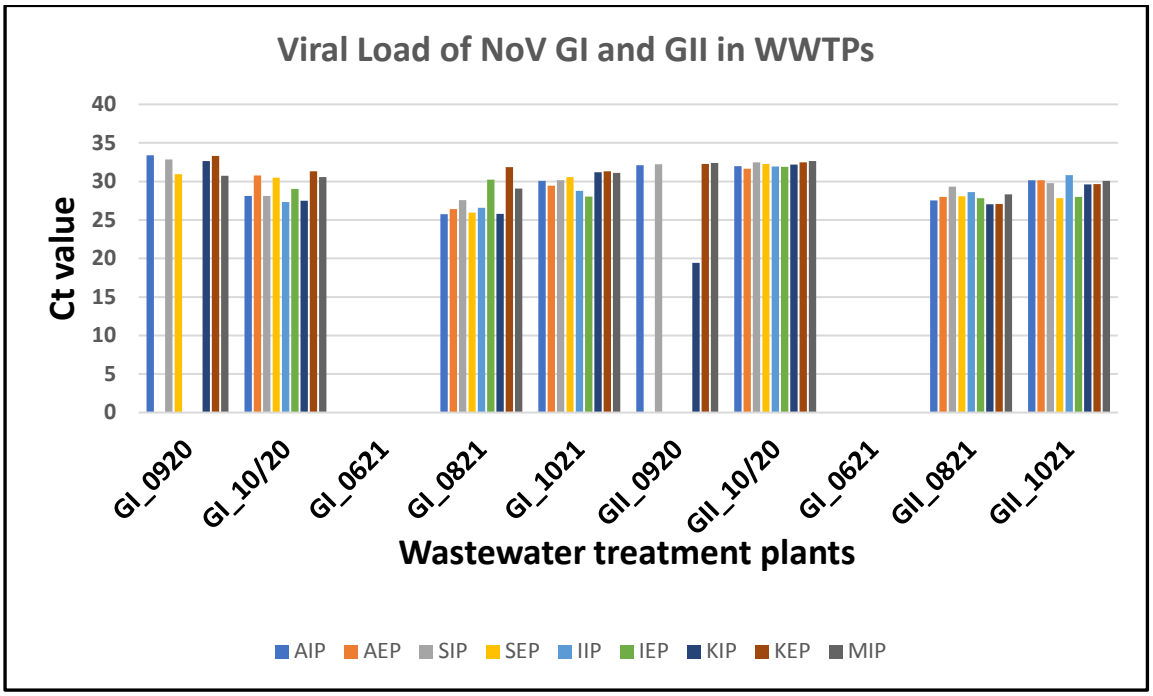

Figure 5.1: Prevalence Noroviruses in the treated and untreated wastewater collected during the monitoring period.

On the other hand, twenty river water samples were investigated to detect NoV GI and GII. Table 5.3 shows the prevalence of NoV GI and GII in the river samples that were examined. Both strains were detected in 14 samples (70%), similar to the study conducted in Egypt (Shaheen and Elmahdy, 2019). In Isipingo and Kingsburg Rivers, both strains of NoV's were not detected in September and October 2020. Likewise, in June 2021, Shallcross and Kingsburgh rivers were negative for NoV GI and GII but positive in the Amanzimtoti river throughout the sampling time (Figure 5.2). For both strains, lower Ct values (26-29) were recorded in September, October 2020, and August 2021 across the investigated four rivers, indicating a strong viral load. However, higher Ct values (29-32) for GI were recorded in the four rivers in June and October 2021. This means there is a moderate viral load in the rivers.

Table 5.3: Prevalence of NoV GI and GII in river samples

River	Sample	No of positive samples/no of tested samples)	
		NoV GI	NoV GII
Amanzimtoti	ARP	5/5	5/5
Isipingo	IRP	3/5	3/5
Kingsburgh	KRP	2/5	2/5
Shallcross	SRP	4/5	4/5

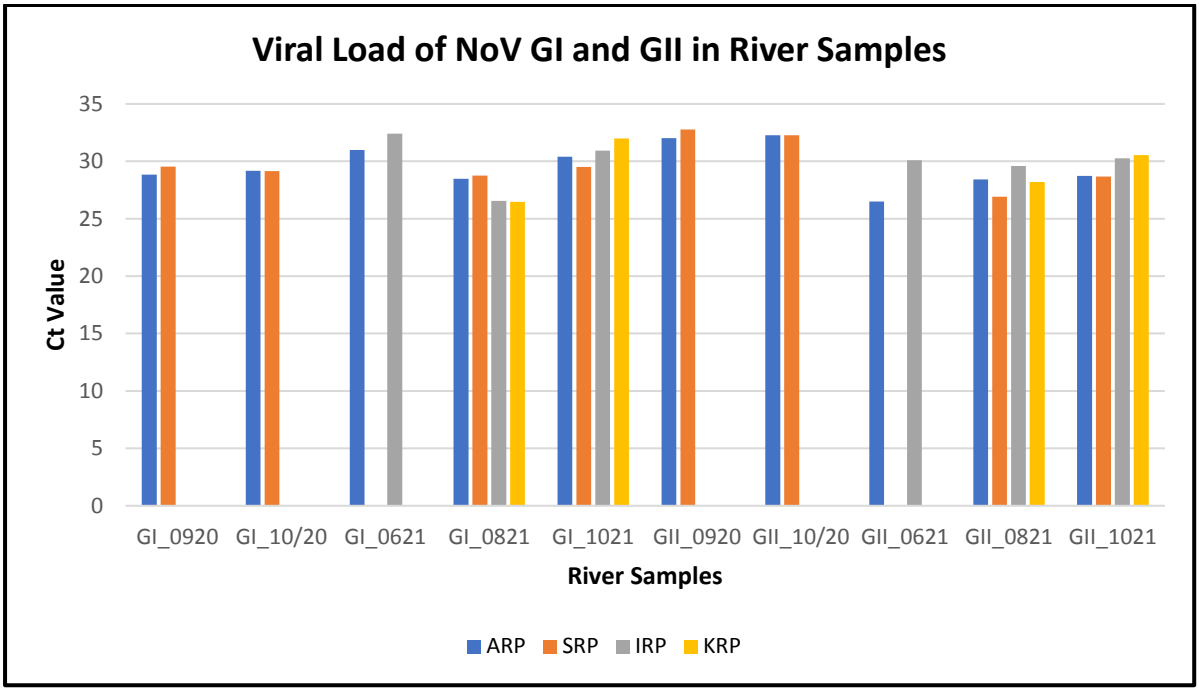

Figure 5.2: Viral Load of NoV GI and GII in river samples.

In the same vein, high Ct values (32) was recorded in September and October 2020 for GII in both Amanzimtoti and Shallcross River, which indicates moderate viral load, while lower Ct values (26-28) were recorded in June, August, and October 2021 hence, a strong viral load. Furthermore, Isipingo and Kingsburgh rivers recorded low Ct values (28-30) for GII in June, August, and October 2021. The virological monitoring of treated effluent samples and river water into which the effluents are discharged could contribute to understanding the effectiveness of the WWTPs in the removal of the viruses. Norovirus, which belongs to the GI strains, is believed to be stable in water compared to the GII strains because they are more likely to be transmitted through water than other transmission routes. Several studies have detected NoV GI and NoV GII in different water bodies such as sewages, rivers, recreational waters, municipal water, and groundwater (La Rosa et al., 2007, La Rosa et al., 2008, La Rosa et al., 2010, Miura et al., 2019).

CHAPTER 6: PHYTOCHEMICALS, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF MEDICINAL PLANTS EXTRACTS AGAINST SELECTED CLINICAL AND WATERBORNE PATHOGENS

6.1 INTRODUCTION

For thousands of years, medicinal plants have been utilized to cure human ailments because they contain broad and diverse organic substances that may have a particular physiological action on the human body (Angiolella et al., 2018). Most of these medicinal plants are locally available and contain diverse structural and bioactive chemical compounds. Phytochemicals, natural chemical bioactive compounds that have biological significance with a wide range of activities include; flavonoids, alkaloids, carbohydrates, diterpenes tannins, glycosides, saponins, phytosterols, protein, phenols, and amino acid, among others. They are known to show medicinal activity as well as exhibit physiological activity (Yadav et al., 2017). These bioactive compounds help build immunity against long-term disease(s) in humans. Generally, they are now thought to be a useful source of unique natural substances utilized in producing anti-diabetic, antioxidant, anti-inflammatory, anti-cancer and antimicrobial medicines with high economic outputs (Fall et al., 2017).

Thus, environmental biotechnologists could tap from plants' rich reservoir for water purification by exploring novel bioactive natural compounds generated from plants to substitute or used in conjunction with conventional treatment reagents. Potable water remains a fundamental human right, with individuals entitled to accessible, affordable, safe and sufficient water (Adeeyo et al., 2020, Megersa et al., 2014a). In order to get safe water, adequate treatment is important to prevent the occurrence of harmful contaminants in water (Hyung and Kim, 2009). It has been recommended that decentralised, and household point-of-use (PoU) water treatment is a way forward in combatting drinking water problems, especially in a remote areas. Hence, one of the new options in treating contaminated water is the use of plant-based water treatment solutions. This has evolved as a feasible cheap and high economic viable material for PoU water treatment technology, **especially in** Africa, where a good percentage of medicinal plants are grown (Megersa et al., 2014a).

The chapter further reports the phytochemical composition and antioxidant activity of all four selected medicinal plants, namely *Ocimum gratissimum, Moringa oleifera, Azadirachta indica (Neem)* and *sesame plant*. Further investigation on the phytochemical composition, potential antibacterial, antibacterial antifungal and antioxidant activities of the above-mentioned four South African medicinal plants against selected waterborne and clinical pathogens were also presented.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of Extracts

Healthy leaves of *Ocimum gratissimum, Moringa oleifera, and* (seed, stem and leaves) of both *Azadirachta indica (Neem)* and *sesame* plant were air-dried and grinded to a fine powder using a blender. The plants were then weighed out in a separate conical flask at 80 g each. 1.5 litres of distilled water, 95% ethanol

and 80% methanol were added to each flask respectively for each plant. Eighty percent (80%) methanol was made by dilution; 400 mL of methanol with 100 mL of distilled water. The flask was shaken and left for three days. The extracts were then filtered and evaporated. The aqueous and methanol extracts were then freeze-dried and the ethanolic samples were left to dry at room temperature. The extracts were stored at -80°C for further analysis.

6.2.2 Phytochemical Screening of Plant Extracts

Phytochemical screening of the compounds in the plant extracts was carried out according to methods outlined by Harris K.K et al. (2014). Phytochemical screening for the presence or absence of tannins, saponins, alkaloids, terpenoids, flavonoids, and phenols tannins were carried out on powdered specimens of the plants.

6.2.2.1 Tannins

For the presence of tannins, 0.5 g of the powdered plant samples were each boiled in 10 mL distilled water and a few drops of 1% ferric chloride was added to each. These were observed for a brownish-green or black colouration to indicate a positive result.

6.2.2.2 Saponins

For the presence of saponins, 5 mL of the aqueous extracts was mixed with 5 mL distilled water and shaken vigorously to form a stable, persistent froth, which shows the presence of saponins. Then five drops of olive oil solution were further added and shaken vigorously again. The formation of an emulsion was observed with a heavy emulsion indicating the presence of saponins.

6.2.2.3 Alkaloids (Mayer's Test)

For the presence of alkaloids, 3 mL of aqueous extracts were mixed with 3 mL of 1% HCl in a steam bath, filtered and then treated with a few drops of Mayer's. Turbidity shows the presence of alkaloids. Further addition of a few drops of olive oil to form an emulsion indicated the presence of alkaloids.

6.2.2.4 Flavonoids

For the presence of flavonoids, 3 mL diluted (1:10) ammonia solution was added to 3 mL of the extract, then 3 mL of the concentrated H_2S0_4 was also added. A formation of yellow colouration indicated a positive result.

6.2.2.5 Phenols

For the presence of phenols, 2 mL of the aqueous extract was dissolved in 4 mL of distilled H_2O and the appearance of blue/green colour showed the presence of phenols.

6.2.2.6 Steroids

Steroids Acetic anhydride (2 mL) was added to 0.5 g powdered leaf material of each plant sample, followed by 2 mL sulphuric acid. Colour changes from violet to blue or green in some plants indicate the presence of steroids [14].

6.2.3 Antibacterial Activity

The following bacterial; S. *aureus, M. luteus, E. coli, B. cereus, K. pneumoniae, P. aeruginosa, C. albicans and C. albicans* were used for antimicrobial activity of the extracts. For the assay, bacterial culture was plated on nutrient agar (Biolab) and kept in the incubator for 24 hrs at 37°C, then subculture onto nutrient broth (Biolab) at the same condition. MacFarland standard of 0.5 absorbance corresponding to 108 cfu/mL was used to standardize the bacterial cell concentration. A suspension (100 µL of 108 cfu/mL) of the test bacteria were placed on Mueller Hinton Agar plates (Fluka, Biochemika). The Whatman No. 1 filter paper was cut into 5 mm disks and dried in an open sterile Petri dishes kept in a biosafety chamber (Labtec Bioflow II, South Africa). The discs were impregnated with 20 µL of each plant at the concentration of 5 mg/mL then placed onto the pre-inoculated bacterial agar plates and kept at 37°C for 24 hrs. The assay was carried out in triplicate. Ciprofloxacin (Fluka, Biochemika) was used as the active control while, DMSO (100%) as a negative control.

6.2.4 Antifungal Activity

The antifungal followed the above similar procedure; the yeast culture was grown using the Sabouraud Dextrose Agar (SDA) and later inoculated in SDA broth. Amphotericin B was used as the positive control. Sterile distilled water containing the fungal spores (10^6 spores/mL) was poured over the SDA base plates (Biolab). 20 µL of each compound at a 5 mg/mL concentration was transferred on each of the three sterile 9 mm discs made from Whatman no. 1 filter paper. 100% DMSO (20 µL) served as the negative control, whilst amphotericin B, 5 µg/mL (Fluka, Biochemika), served as a positive control. Each sample was tested in triplicate. The impregnated discs were allowed to evaporate in an open sterile petri dish in a biological safety cabinet with a vertical laminar flow (Labtec Bioflow II, South Africa) before the impregnated discs were placed onto the agar plates. Agar plates inoculated with the respective yeasts containing the discs with the extracts and controls respectively were incubated ± 37°C. The antifungal activities were recorded as the width (millimetres, diameter of the disc) of the zone of inhibition after incubation.

6.2.5 Antioxidant activity

The decolouration protocol earlier reported by Choi et al. (Choi et al., 2002) for measuring the radical scavenging activity of different plant extracts using the stable free radical scavenger, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used to carry out the antioxidant assay (Choi et al., 2002). Methanol was used to dilute the stock solutions of the plants to the final concentration. A comparison of the result and concentrations of 1, 20, 40, 60, 80, 100, 250, 500, and 1000 µg/mL obtained with Qeucertin-3-rutinoside, that is an effective antioxidant was performed (Choi et al., 2002). The absorbance of each mixture was then measured at a wavelength of 517 nm using a spectrophotometer. The scavenging ability of the plant extracts were then determined as the decolourization percentage of the test sample using equation 6.1.

$$\text{Scavenging capacity } (\%) = \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{Absorbance of negative control}}\ x\ 100 \qquad (6.1)$$

6.3 RESULTS AND DISCUSSION

6.3.1 Phytochemical Analysis of Medicinal Plants

Phytochemical compounds in *O. gratisimum* L. can be of great use in the medical and pharmacological industry (Fall et al., 2017). It can treat/prevent diseases such as cancer, diabetes, hypertension and stroke.

Azadirachtin extracts from the seeds, leaves, and bark trees have been shown to have high biological activity against insect pests while being extremely safe for mammals and the environment. As a result, the neem plant's widespread usage can be attributed to the existence of these bioactive chemicals, which may explain its many traditional applications against a variety of illnesses (Biu et al., 2009). The phytochemical tests in this study revealed the presence of alkaloids, saponins, steroids, flavonoids, phenols and tannins (Table 6.1-Table 6.4) and Figure 6.1.

The chemical contents of the various fractions are classified as secondary metabolite components, which are physiologically active substances. They are responsible for antibacterial, antioxidant, antifungal and anticancer properties (Anyasor et al., 2010, Mistry et al., 2014). Many studies have found that flavonoids and phenolic chemicals in plants have various biological effects, including antioxidant, anti-inflammatory, antibacterial, antiangiogenic, anticancer and antiallergic properties (Fall et al., 2017, Venuprasad et al., 2014, Mistry et al., 2014). In general, flavonoids as secondary metabolites found in plants help to regulate cellular activity and fight free radicals that cause oxidative stress. The presence of flavonoids is shown by forming a yellow precipitate by most methanol and ethanol extracts. The presence of flavonoids implies that the plants can have therapeutic effects (Giri et al., 2019).

Tannins are another complex molecule made by plants; they form a non-absorbable complex with digestive enzymes, proteins, metals, and sugars (Macáková et al., 2014). The presence of tannins in the tested plants corresponds with previous studies for most plant parts (Das et al., 2020). For the parts that tested negative, there might be a probability that the solvent did not extract the tannins in high concentration so there was too little present or within the non-detectable limit to visualise. Saponins have antimicrobial properties against bacteria, fungi, and viruses to improve immune function. The formation of foam in the samples during analysis indicates the presence of saponin. Also, the presence of alkaloids in most plants parts (Table 6.1-6.4) shows the strong pharmacologic effects of the tested plants (Williams et al., 2018). In general, phenolic substances and their derivatives are the main antioxidants or free radical scavengers (Liu et al., 2011) and triterpenoids production by different plant parts indicate high antioxidant and antidiabetic properties (Nazaruk and Borzym-Kluczyk, 2015).

Table 6.1: Phytochemical screening of extracts from different parts of *Azadirachta indica* (Neem)

Phytochemicals	Seed			Stems			Leaves		
	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous
Alkaloids	+	+	-	+	+	-	+	+	+
Flavonoids	-	+	-	-	+	+	+	+	+
Saponins	+	+	+	-	+	+	+	+	+
Phenols	-	+	+	+	+	+	-	+	+
Steroids	-	-	-	-	+	+	+	+	-
Tannins	+	+	+	-	+	+	+	+	+

+: present; –: not detected

Table 6.2: Phytochemical screening of different parts of *Moringa oleifera* plant

Phytochemicals	Seed			Stems young			Stems old		
	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous
Alkaloids	+	+	+	+	+	+	+	+	-
Flavonoids	+	+	-	+	+	+	-	+	+
Saponins	+	+	+	-	+	+	-	+	+
Phenols	-	+	-	+	+	-	+	+	+
Steroids	-	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	-

+: present; –not detected

Table 6.3: Phytochemical screening of *Ocimum gratisimum* leaves extract

Phytochemicals	Leaves		
	Methanol	Ethanol	Aqueous
Alkaloids	+	+	-
Flavonoids	+	+	+
Saponins	-	+	+
Phenols	+	-	-
Steroids	+	+	+
Tannins	+	+	+

+: present; –: not detected

Table 6.4: Phytochemical screening of different parts of Sesame plant. (+: present; –: not detected)

Phytochemicals	Seed			Stems			Leaves		
	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous
Alkaloids	-	+	-	+	+	-	-	-	-
Flavonoids	+	+	+	-	-	-	+	+	+
Saponins	+	+	+	+	+	+	+	+	+
Phenols	+	+	+	-	+	-	+	+	-
Steroids	+	+	-	-	+	+	-	-	-
Tannins	+	+	-	-	+	+	+	+	-

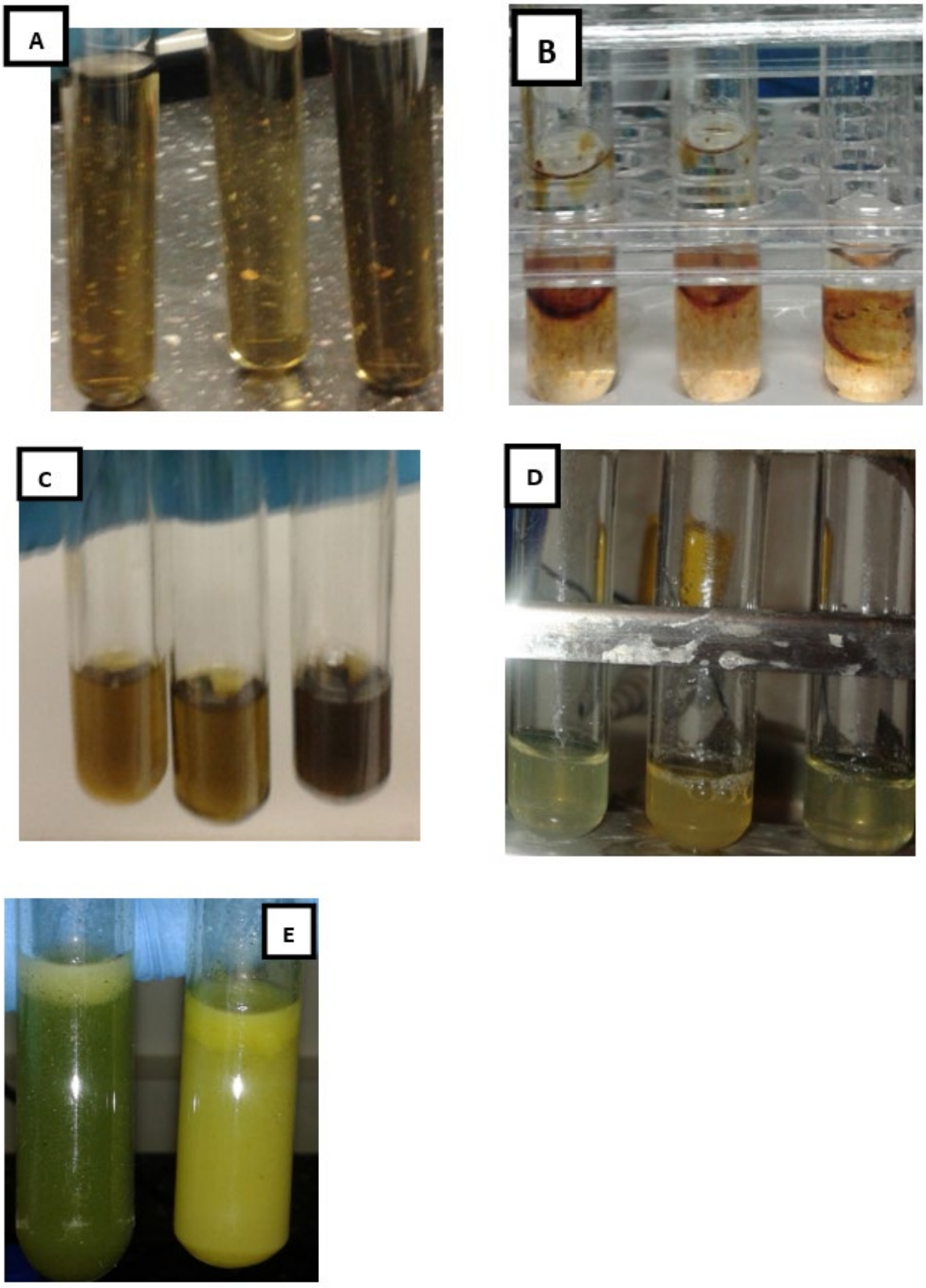

Figure 6.1: Positive colour of tested phytochemical compounds. (A) tannins – brownish-green, (B) terpenoids – reddish-brown colouration of the interface, (C) phenols – green/blue colour, (D) alkaloids – Turbidity and emulsion, and (E) saponins – foamy heavy emulsion.

6.3.2 Antimicrobial Activities of the Plant Extracts

The study conducted to determine the bactericidal activity of *Ocimum gratissimum*, *M. oleifera, Sesame* plant, and *Azadirachta indica* (neem) extracts yielded a range of activities. Ciprofloxacin and DMSO were used as positive and negative control, respectively as all bacterial isolates were inhibited by ciprofloxacin. The yeast isolates were resistant to ciprofloxacin, but both the bacterial and the yeast isolates grew against DMSO, thereby maintaining the validity of the results. *Moringa oleifera* young stem extracts were effective against all test species in ethanol, methanol, and aqueous extracts. The zone of inhibition measured 8 to 12 mm (Tables 6.5-6.7). The maximum bactericidal activity was found to be present in the aqueous extract of *Moringa oleifera* young stem against *M. luteus* with an inhibition of 12 mm. In contrast, the methanol extract inhibited *M. luteus* and *E. coli* with a zone of inhibition of 10 and 11 mm respectively as given in Tables 6.5-6.7. *M. luteus, E. coli*, and *B. cereus* were mildly inhibited by the ethanol extract with a zone of inhibition extending between 8 and 9 mm. The zone of inhibition of *M. oleifera* seed ethanol extracts was found to be moderate against all test species. Ethanol extracts inhibited *Staphylococcus aureus*, *M. luteus, E. coli, K. pneumoniae* and *Pseudomonas aeruginosa.* Likewise, a wider zone of inhibition was recorded for fungi susceptibility to these plant extracts, as shown in Tables 6.5-6.7. Since *M. oleifera* was shown to exhibit antibacterial activity against selected microorganisms, the findings in this study confirm the plant's traditional use and can be recommended for use as an antimicrobial agent and a potential substitute for bio-adsorbent in the biotechnological and medical field.

Table 6.5: Growth inhibition (in mm) of extracts obtained from the young stem of *Moringa oleifera.*

Pathogens	Young stem		
	Aqueous	Methanolic	Ethanolic
S. aureus	0±0.0	6±0.0	7±0.0
M. Luteus	12.0±0.0	10.0±0.0	9.0±0.0
E. coli	9.0±0.0	11.0±0.0	9.0±0.0
B. cereus	7±0.0	8±0.0	8.0±0.0
K. pneumoniae	0±0.0	0±0.0	0±0.0
P. aeruginosa	0±0.0	0±0.0	0±0.0
C. albicans	7.0±0.0	11.0±0.0	8.0±0.0
C. utilus	7.0±0.0	9.0±0.0	8.0±0.0

Table 6.6: Growth inhibition (in mm) of extracts obtained from the seed of *Moringa oleifera.*

Pathogens	Seed		
	Aqueous	Methanolic	Ethanolic
S. aureus	0±0.0	6.0±0.0	6.0±0.0
M. Luteus	0±0.0	0±0.0	6.0±0.0
E. coli	0±0.0	0±0.0	6.0±0.0
B. cereus	0±0.0	0±0.0	0.0 ±0.0
K. pneumoniae	0±0.0	0±0.0	6.0±0.0
P. aeruginosa	0±0.0	0±0.0	6.0±0.0
C. albicans	5.0±0.0	10.0±0.0	8.0±0.0
C. utilus	7.0±0.0	8..0±0.0	8.0±0.0

Table 6.7: Growth inhibition (in mm) of extracts obtained from the old stem of *Moringa oleifera.*

Pathogens	Stem old		
	Aqueous	Ethanolic	Aqueous
S. aureus	0±0.0	0±0.0	0±0.0
M. Luteus	7.0±0.0	6.0±0.0	6.0±0.0
E. coli	6.0 ± 0.0	6.0±0.0	6.0±0.0
B. cereus	6.0±0.0	6.0±0.0	6.0±0.0
K. pneumoniae	0±0.0	0±0.0	0±0.0
P. aeruginosa	0±0.0	0±0.0	0±0.0
C. albicans	5.0±0.0	8.0 ±0.0	7.0±0.0
C. utilus	5.0 ±0.0	7.0 ±0.0	8.0 ±0.0

Table 6.8 depicts the inhibition zones recorded for aqueous, ethanolic and methanolic extracts against each isolate. The antibacterial activity of aqueous, ethanolic and methanolic extracts of *O. gratissimum* was tested against bacterial isolates (Figure 6.2). The zone of inhibition of *O. gratissimum* leaves methanol and ethanol extracts was found to be modest against most tested organisms. The average zone of inhibition recorded ranged from 6 mm to 9 mm in diameter. According to the inhibition zones obtained in this study, *O. gratissimum* displayed greater antibacterial activity against Gram-positive bacteria than Gram-negative. The susceptibility of Gram-positive bacteria to *O. gratissimum* can be accounted to their bacterial membrane structure because they have a lower lipid content when compared to Gram-negative bacteria. Therefore, the membrane's permeability is compromised as it allows the entry of the active compounds responsible for the inhibitory effects such as destruction of the cell wall or genetic material. Therefore, the additional/outer membrane of Gram-negative bacteria enables them to be less susceptible to *O. gratissimum* extracts. This agrees with the study conducted by Airaodion et al. (Airaodion et al., 2020). Therefore, the findings of this study motivate further research of *O. gratissimum* against more strains of multi-drug resistant bacteria, which could eventually promote the use of plant extract as an alternative antibiotic in the medical field and better disinfectant in water treatment. Aqueous extracts of *O. gratissimum* did not exhibit any antibacterial activity against the pathogens except *P. aeruginosa*, which can be attributed to the employment of the disk diffusion method. This is because water molecules have a low viscosity compared to ethanol and methanol molecules.

Table 6.8: Growth inhibition (in mm) of *Ocimum gratissimum* leaves

Pathogens	Extract		
	Aqueous	Methanolic	Ethanol
Staphylococcus aureus	0 ± 0.0	9.0 ± 0.0	6.0±0.0
M. Luteus	0 ± 0.0	6.0 ± 0.0	0±0.0
E. coli	0 ± 0.0	6.0 ± 0.0	6.0±0.0
B. cereus	0 ± 0.0	0 ± 0.0	0±0.0
K. pneumoniae	0 ± 0.0	6.0 ± 0.0	0±0.0
P. aeruginosa	6.0 ± 0.0	6.0 ± 0.0	0±0.0
C. albicans	0 ± 0.0	5.0 ± 0.0	6.0±0.0
C. utilus	0 ± 0.0	0 ± 0.0	0 ± 0.0

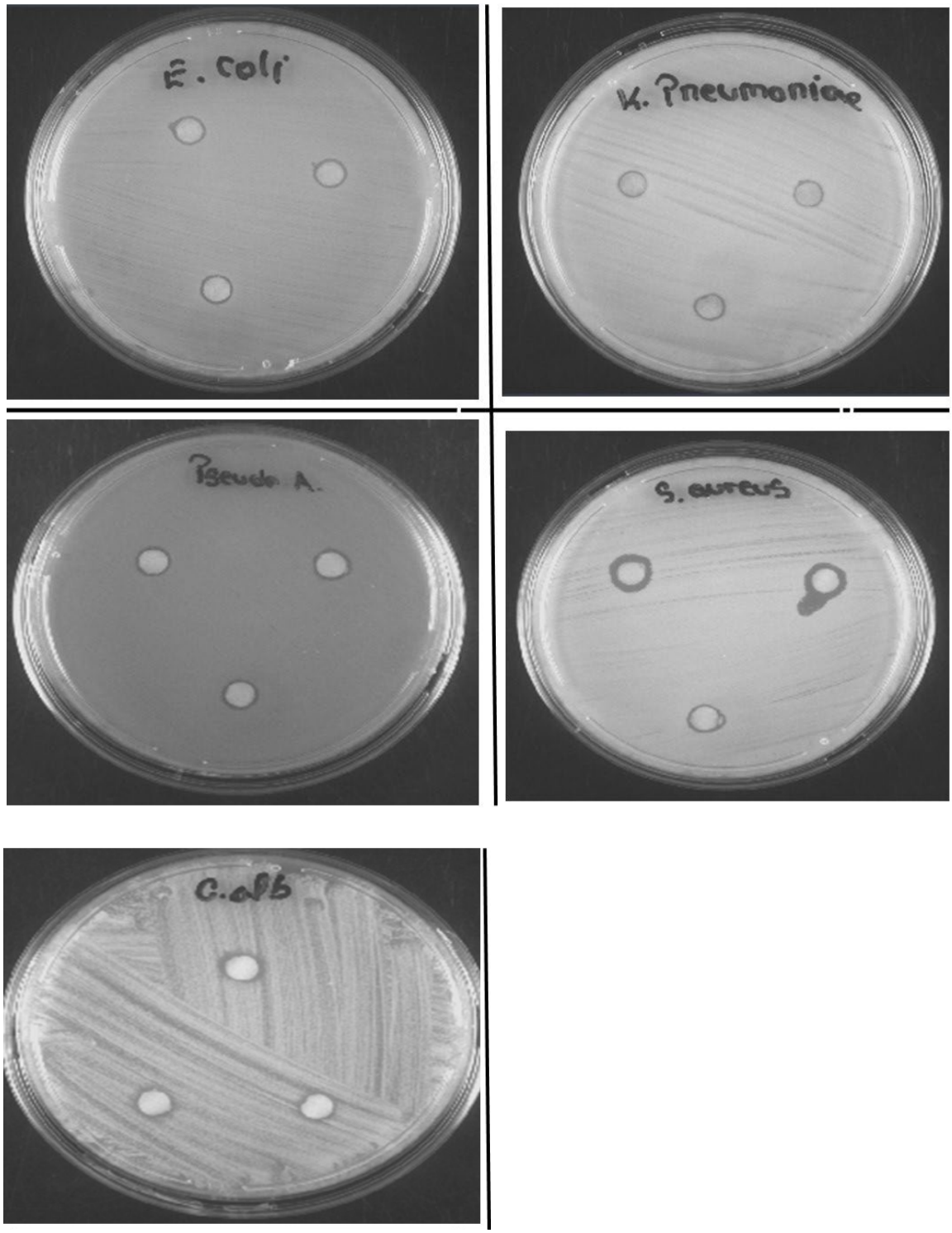

Figure 6.2: Some of the zones of inhibition recorded for *O. gratissimum* leaves extract.

Low viscosity is directly proportional to lower diffusion rates and this might have contributed to the ability of the aqueous extracts not to exhibit any antimicrobial activity (Pattnaik et al., 2021). Methanolic extracts of *O. gratissimum* against *S. aureus* resulted in the inhibiting zone of an average range of 6-9 mm. Therefore, its extract displayed overall greater antibacterial activity than ethanolic and aqueous extract. The antibacterial activity of methanolic extracts can be attributed to the polarity of the solvent and the presence of the phytochemicals that have bioactive compounds.

Although, O. *gratissimum* has been reported to provide effective fungicidal activity against many human pathogens. Antifungal activity of *O. gratissimum* was only obtained against *C. albicans* (Table 6.0.6) for methanol and ethanol extracts while *C. utilus* was resistant to all extracts of *O. gratissimum.* The dried plant is used to treat superficial mycosis in children. It also inhibits *C. albicans* which causes skin infections and protects HIV patients against candidiasis infections. The plant also produces essential oils which are effective against plant pathogens. The presence of eugenol, a phenolic compound, suppresses plant pathogens. The antimicrobial efficiency of *O. gratissimum* is promising and therefore justifies the use of this plant as an alternative to chlorine in water treatment especially multi-drug resistant strains.

All bacterial isolates were resistant to aqueous, ethanolic and methanolic extracts of *A. indica* seeds (5 mg/mL) (See Table 6.9); however, the susceptibility of *S. aureus, E. coli* and *P. aeruginosa* to aqueous and ethanolic extracts of the stem was recorded (Table 6.9). Similarly, *S. aureus, E. coli B. cereus* and *P. aeruginosa* were inhibited by methanol leaves extract, while ethanolic extract also inhibited *M. luteus* and *K. pneumoniae* in addition to the four mentioned strains (Table 6.9). The methanol and ethanol leave extracts exhibited both Gram-positive and negative bacteria. These characteristics can be attributed to the presence of the secondary metabolites in the plants that causes the antibacterial efficiency.

In addition, all the *A. indica* extracts displayed significant antifungal activity against the tested candida strains, especially methanol and ethanol extracts (Table 6.9). Studies have indicated that the ethanolic extract of the *Neem* plant has a greater inhibitory effect than the aqueous extract (Kutawa et al., 2018, Oluwajobi et al., 2019) due to the ethanol serving as a better extractant of the phytochemical constituents present in the neem plant, which is true in the present study. A larger inhibition zone observed against *C. albicans* in this study further supports the use of neem (*A. indica*) use in traditional medicine as an antifungal agent. This study further correlates with previous antimicrobial activities of plant extracts against both clinical and waterborne pathogens (Barua et al., 2017, Maragathavalli et al., 2012). Like other plant extracts, Sesame parts showed more antimicrobial activity (Table 6.10). *Staphylococcus aureus, M. Luteus, E. coli, and P. aeruginosa* were observed to be sensitive to ethanol, methanol and aqueous extracts of sesame leaves at a lower concentration of 3 mg/mL (Table 6.10).

Table 6.9: Growth inhibition (in mm) of extracts obtained from different parts of *Azadirachta indica*

Pathogens	Seed			Stem			Leaves		
	Aqueous	Methanolic	Ethanolic	Aqueous	Methanolic	Ethanolic	Aqueous	Methanolic	Ethanolic
Staphylococcus aureus	0±0.0	0±0.0	0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	0±0.0	7.0±0.0	6.0±0.0
M. Luteus	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	6.0±0.0
E. coli	0±0.0	0±0.0	0±0.0	6.0±0.0	0±0.0	6.0±0.0	0±0.0	6.0±0.0	6.0±0.0
B. cereus	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	6.0±0.0	0±0.0
K. pneumoniae	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	6.0±0.0
P. aeruginosa	0±0.0	0±0.0	0±0.0	6.0±0.0	7.0±0.0	7.0±0.0	0±0.0	7.0±0.0	6.0±0.0
C. albicans	5.0±0.0	8.0±0.0	12.0 ±0.0	6.0±0.0	9.0±0.0	8.0±0.0	6.0±0.0	10.0±0.0	11.0±0.0
C. utilus	5.0±0.0	6.0±0.0	8.0±0.0	5.0±0.0	9.0±0.0	8.0±0.0	6.0±0.0	6.0±0.0	7.0±0.0

Table 6.10: Growth inhibition (in mm) of extracts obtained from different parts of Sesame plant

Pathogens	Seed			Stems			Leaves		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
S. aureus	0 ± 0.0	8.0 ± 0.0	6.0 ± 0.0	7.0±0.0	7.0±0.0	0±0.0	0±0.0	9.0±0.0	7.0±0.0
M. luteus	0 ± 0.0	0±0.0	0±0.0	0±0.0	0±0.0	7.0±0.0	7.0±0.0	0±0.0	8.0±0.0
E. coli	0 ± 0.0	6.0±0.0	8.0±0.0	7.0±0.0	7.0±0.0	6.00±0.0	9.0±0.0	7.0±0.0	8.00±0.0
B. cereus	0±0.0	0±0.0	0±0.0	7.0±0.0	0±0.0	7.0±0.0	0±0.0	6.0±0.0	0±0.0
K. pneumoniae	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
P. aeruginosa	0±0.0	7.0±0.0	7.0±0.0	0±0.0	0±0.0	6.0±0.0	7.0±0.0	7.0±0.0	6.0±0.0
C. albicans	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
C. utilus	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

6.3.3 Antioxidant Activity of the Plant Extracts

Plants have a significant variety of phytochemical compounds with significant antioxidant potential. Research on the antioxidant activity of various plant species may help demonstrate the significance of these species as a source of novel antioxidant molecules (Chaves et al., 2020). Therefore, the abilities of the examined extracts to act as a donor of hydrogen atoms or electrons in the transformation of $DPPH^+$ into the reduced form, DPPH-H, were investigated *in vitro* using the DPPH assay. Tables 6.11-6.14 reveal that the DPPH radical scavenging activity data in terms of percentage inhibition for each of the extracts was compared with Rutin's known antioxidant. Based on the results, we can conclude that the DPPH radical scavenging effects of *O. gratissimum*, *M. oleifera*, *A. indica,* and sesame plant extracts were high.

The results revealed that methanol extract for *Ocimum* leaves (78.4±0.02%), ethanol extract (74.6±0.02%), *Moringa oleifera* seed extract (64.3±0.02%), *Neem* leaves ethanol extract (63.9±0.01%) and *sesame seed* methanol extract (63.2±0.4%) displayed high radical scavenging capacity. For *Neem* extract, moderate activity was seen in *Neem* leaves methanol extract (57.9±0.04%) and water extract (57.04±0.03%), *Neem* seed methanol extract (52.3±0.9%), ethanolic extract (53.9±0.2%), water extract (50.3±0.2%) and, lastly, *sesame* seed water extract was 55.6±1.3%. The remainder of the extract displayed a radical scavenging capacity that is lower than 50%.

According to Pérez-Jiménez et al. (Pérez-Jiménez and Saura-Calixto, 2006), the kind of solvent and polarity can influence the single transfer of electrons and hydrogen atom transfer, both of which are important factors in determining antioxidant activity (Pérez-Jiménez and Saura-Calixto, 2006). The DPPH technique loses colour from deep purple to yellow (Figure 6.3) because the radical reacts immediately with an antioxidant at 517 nm absorbances light (Mashwani et al., 2013). The different levels of discoloration can be used to estimate and reveal the scavenging potential of the extracts. This assay further shows that the extracts are rich sources of antioxidants. The results revealed by the antioxidant assay of the extracts indicate that the antioxidant activity increases with the increasing concentration of plant extracts. The highest scavenging ability was due to high concentrations of the active compounds, especially in the methanolic and ethanolic extracts.

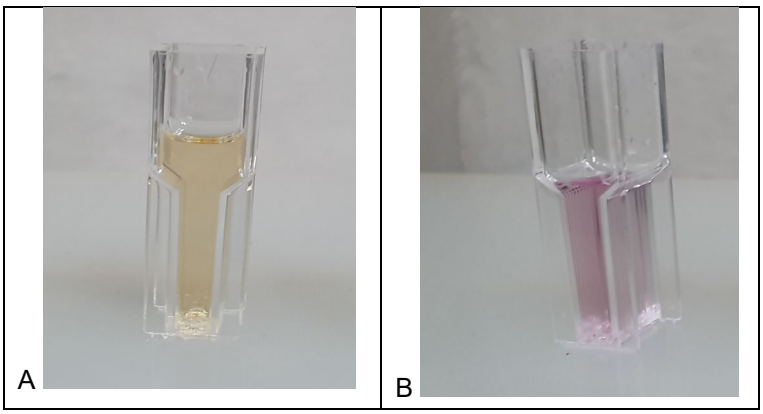

Figure 6.3: Change in colour due to the reduction reaction. (A) The solution was reduced to a lighter yellow colour following the addition of DPPH and incubation; (B) No changes in the colour of the solution due to only a slight reduction in the reaction. Solution retained the purple colour following the addition of DPPH and incubation.

Table 6.11: Percentage scavenging capacity of extracts obtained from *Moringa oleifera*

Concentration (µg/mL)	Rutin	Seed Extracts			Stem Young Extracts			Stem Old Extracts		
		Methanol	Ethanol	Water	Methanol	Ethanol	Water	Methanol	Ethanol	Water
1000	92.4±0.02	39.5±0.05	44.8±0.05	64.3±0.02	48.5±0.01	39.5±0.05	37.8±0.12	35.5±0.5	36.4±0.6	26.5±0.1
500	91.8±0.01	37.1±0.06	41.2±0.02	51.5±0.03	40.9±0.02	37.1±0.06	33.9±0.04	26.9±0.3	32.7±0.8	22.8±0.3
250	87.8±0.05	27.2±0.06	29.4±0.04	45.3±0.02	32.7±0.03	27.2±0.06	27.8±0.12	22.3±0.6	28.1±0.2	19.5±0.5
100	78.7±0.06	24.4±0.10	26.6±0.02	35.5±0.03	25.8±0.05	24.4±0.1	25.1±0.04	20.9±0.5	22.4±0.5	16.1±0.01
80	71.5±0.01	16.8±0.02	25.8±0.03	33.0±0.14	22.3±0.03	16.8±0.02	22.7±0.01	12.1±0.2	15.9±0.4	12.6±0.2
60	68.4±0.06	16.5±0.03	23.3±0.01	27.9±0.05	14.8±0.11	13.4±0.03	14.9±0.15	10.5±0.5	10.45±0.5	8.9±0.12
40	59.8±0.09	13.4±0.01	23.0±0.02	26.7±0.03	12.7±0.08	9.9±0.01	10.7±0.14	7.1±0.06	8.6±0.1	5.5±0.1
20	36.4±0.05	9.9±0.09	19.6±0.1	22.1±0.07	9.3±0.09	2.34±0.09	3.26±0.07	1.2±0.02	1.9±0.9	2.2±0.4
1	8.6±0.08	0.34±0.02	0.7±0.03	5.5±0.05	0.69±0.03	1.1±0.02	0±0.0	0.19±0.01	1.5±0.01	1.2±0.01

Values are represented as mean ±SD (n=3)

Table 6.12: Percentage scavenging capacity of extracts obtained from Neem plant parts

Concentration (µg/mL)	Rutin	Stem Extract			Leaves Extract			*Seed* Extract		
		Methanol	Ethanol	Water	Methanol	Ethanol	Water	Methanol	Ethanol	Water
1000	92.4±0.02	31.9±0.14	24.9±0.13	34.02±0.07	57.9±0.04	63.9±0.01	57.04±0.03	52.3±0.9	53.9±0.2	50.3±0.2
500	91.8±0.01	26.8±0.11	20.4±0.03	30.9±0.08	54.6±0.06	50.9±0.01	52.2±0.02	44.5±0.6	47.6±0.3	42.6±0.12
250	87.8±0.05	23.02±0.05	18.7±0.02	28.5±0.12	51.5±0.04	43.6±0.02	38.7±0.08	41.5±0.14	41.8±0.12	38.1±0.6
100	78.7±0.06	21.7±0.06	14.9±0.04	26.5±0.09	50.2±0.01	40.1±0.05	35.1±0.02	35.2±0.6	39.1±0.6	31.1±0.9
80	71.5±0.01	13.1±0.22	13.8±0.08	23.4±0.12	46.1±0.02	38.3±0.04	32.3±0.02	26.8±0.3	28.4±0.14	22.2±0.7
60	68.4±0.06	11.3±0.11	9.7±0.09	20.3±0.09	37.5±0.08	37.2±0.01	26.5±0.03	21.5±0.8	22.2±0.1	16.3±0.13
40	59.8±0.09	9.6±0.06	9.3±0.05	16.8±0.05	24.1±0.2	35.7±0.02	20.1±0.08	17.1±0.6	15.5±0.5	12.7±0.18
20	36.4±0.05	4.8±0.04	7.9±0.1	13.8±0.08	24.7±0.024	28.9±0.23	17.2±0.16	8.2±0.2	10.6±0.3	6.2±0.1
1	8.6±0.08	1.00±0.03	0.38±0.02	0.79±0.0	2.1±0.03	5.7±0.01	2.4±0.03	2.5±0.5	3.5±0.5	1.9±0.2

Values are represented as mean ±SD (n=3)

Table 6.13: Percentage scavenging capacity of ocimum leaves

Concentration (µg/mL)	Rutin	Extract		
		Methanol	Ethanol	Water
1000	92.4±0.02	78.4±0.02	74.6±0.02	47.1±0.02
500	91.8±0.01	65.3±0.01	68.7±0.02	37.8±0.01
250	87.8±0.05	48.8±0.01	56.5±0.08	43.6±0.13
100	78.7±0.06	37.5±0.1	35.1±0.02	32.6±0.05
80	71.5±0.01	27.8±0.01	30.9±0.03	29.6±0.08
60	68.4±0.06	17.5±0.1	27.5±0.02	22.7±0.12
40	59.8±0.09	12.7±0.13	21.9±0.06	18.9±0.12
20	36.4±0.05	9.9±0.08	23.02±0.01	16.5±0.06
1	8.6±0.08	6.2±0.10	6.9±0.03	1.7±0.04

Table 6.14: Percentage scavenging capacity of extracts obtained from sesame plant parts

Concentration (µg/mL)	Rutin	Seed Extract			Stem Extract			Leaves Extract		
		Methanol	Ethanol	Water	Methanol	Ethanol	Water	Methanol	Ethanol	Water
1000	92.4±0.02	63.2±0.4	48.1±1.2	55.6±1.3	56.7±0.6	36.2±1.1	45.5±1.5	47.2±0.4	46.2±1.2	35.5±1.1
500	91.8±0.01	56.1±0.6	35.6±1.1	43.8±0.8	53.1±0.1	30.9±0.6	39.9±0.7	43.5±0.5	40.9±0.8	29.2±0.7
250	87.8±0.05	47.4±1.2	30.2±0.4	36.9±0.6	41.3±1.0	26.5±0.5	34.1±0.4	31.1±1.1	36.5±0.2	24.6±0.2
100	78.7±0.06	41.5±0.5	25.3±0.8	28.3±1.6	34.5±0.8	21.1±0.1	22.2±1.3	24.2±0.3	20.1±0.1	21.1±1.1
80	71.5±0.01	32.8±0.2	20.6±0.6	22.7±0.7	27.5±0.12	16.7±0.12	11.1±1.4	21.5±0.5	14.6±0.12	10.1±1.1
60	68.4±0.06	22.5±1.5	18.7±0.13	21.1±0.2	21.2±1.2	11.8.5±0.2	8.1±0.2	17.7±1.2	12.5±0.5	6.1±0.2
40	59.8±0.09	13.6±0.3	14.1.0±0.6	16.8±0.2	11.8±0.6	10.1±0.1	5.8±0.2	7.8±0.1	7.1±0.1	3.2±0.4
20	36.4±0.05	8.1±0.2	10.5±0.5	13.2±0.4	5.1±0.4	7.5.0±0.2	3.1±0.1	4.1±0.1	3..1±0.2	2.1±0.1
1	8.6±0.08	4.0±0.0	2.2±0.9	3.1±0.7	2.1±0.01	1.4±0.3	1.2±0.1	1.6±0.01	1.2±0.4	0.5±0.01

Values are represented as mean ±SD (n=3)

Similar to the results obtained in this study, methanol and ethanol has proven to be effective and efficient solvents to extract phenolic compounds from medicinal plants (Siddhuraju and Becker, 2003). Phenolic compounds are one of the main phytochemicals responsible for the variation in antioxidant activity of the plant (Cai et al., 2004). Polyphenols in the extracts are able to transfer a hydrogen atom to the lipid peroxil cycle, forming the aryloxyl molecule, which does not have the ability to act as a chain carrier in conjunction with another radical quenches the radical process. The results obtained directly correlate with a study conducted by Nahak and Sahu (Nahak and Sahu, 2010). The phytochemicals such as alkaloids, phenols, tannins, saponins, triterpenoids, and steroids present in the extracts may be responsible for the antioxidant activities of these plants. It is well known that a wide range of bioactivities depends on these compounds (Pokhrel et al., 2015). The antioxidant assay further revealed that all plant extracts from different parts have varying degrees of antioxidant activity which makes it interesting to know that almost all extracts have antioxidant properties.

CHAPTER 7: EFFICACY OF CRUDE EXTRACTS OF MORINGA OLEIFERA IN TREATING MUNICIPAL WASTEWATER

7.1 INTRODUCTION

Worldwide, physical or chemical removal of pathogens through the use of ultraviolet light or chemical oxidants like chlorine, chloramines, and ozone have been the major standard conventional water treatment methods (Gall et al., 2015). However, application of chlorine disinfection could lead to potential toxic disinfection by-products (DBPs) like bromate and chlorite that may cause major human health risks. Therefore, an urgent need to develop new, safer, sustainable, eco-friendly, and affordable water treatment methods as an add-on product that could serve as means of reducing waterborne pathogens at the point-of-use treatment process. Interestingly, plant-based materials present themselves as a promising alternative in achieving the goal of clean, safer, non-toxic, consistent, biological, and environmentally friendly green treatment processes.

The application of medicinal plant material has increased in various fields as an alternative to chemical and physical reagents even within water sector. One of the most remarkable aspects of medicinal plants is their unique metabolic system (metabolism), which creates extremely complex and bioactive chemicals that influence cellular activity, microbiota, and phenotype in human health and diseases. In view of difficulties and constraints of using chemical reagents, plant materials have gained much attention as effective coagulants and disinfectants for removal of foreign materials from different water sources. Based on the phytochemical and microbial analyses of the investigated plant materials, the extracts from these plants are very rich in bioactive compounds with the potential to remove both chemical and microbial contaminants during water treatment. Hence, this chapter discusses the findings on indigenous use of crude extracts of *Moringa oleifera* young stem as sustainable biomaterial for removal of colloidal particles and microbial contaminants during water treatment.

7.2 MATERIALS AND METHODS

7.2.1 Collection of water samples

Wastewater sample was collected from the Amanzimtoti wastewater treatment plant receiving 50% domestic and 50% industrial influent using the grab sampling method. Samples were collected with the aid of a clean sample collector holder. The collected sample was transported on ice to the laboratory of the Department of Biotechnology and Food Science, Durban University of Technology, for water treatment study.

7.2.2 Preparation of coagulant and disinfectants

Pre-sterilized mortar and pestle was used to crush the aluminum sulphate ($Al_2(SO_4)_3$). Thereafter, the aluminum sulphate was dissolved aseptically in double distilled and sterilized water (2 g per 100 mL). Using sterile distilled water while 7 mL of stock solution was mixed carefully up to 250 mL. The experiment was conducted with 8 drops of alum (coagulants) and 5 drops of 0.5% sodium hypochlorite (disinfectants) (Pritchard et al., 2010a).

7.2.3 Water purification using plant extracts

At the start of the experiment, the wastewater was seeded with a mixture of actual *Escherichia coli* and *Pseudomonas aeruginosa* concentration. Five main phases of conventional water treatment namely; coagulation, flocculation, sedimentation, filtration, and disinfection were used in this study for batch coagulation/disinfection experiments. The positive controls in the jar test studies were alum (coagulant) and 0.5% sodium hypochlorite (disinfectant) purchased from Sigma Aldrich in South Africa. Similarly, the plant crude extracts were used as natural coagulant and disinfectant during the experiments. Water treatment using crude extracts was carried out using JEIO TECH Shaking Water bath (Korea) to maintain a constant temperature.

Duran laboratory glass bottles with 155 mL of influent water sample were used and placed in the slots of the water bath (Figure 7.1). Chemical reagents, alum and sodium hypochlorite were used for the conventional technique as positive control while *Moringa* young stem ethanol (MYSE) and *Moringa* young stem aqueous (MYSA) extracts were used for the experiment. Alum was added to two glass bottles holding 155 mL of influent water sample for positive control. The set-up was agitated at 100 rpm/min for the first 5 minutes and then at 80 rpm/min for the remaining 25 minutes. Thereafter, it was left to settle for 1 hour before being filtered. After filtration, the water in the other bottle was disinfected with 8 drops of 0.5% sodium hypochlorite (disinfectant). The bottle was then sampled three times (Filtered sample and samples taken every 30 mins for an hour) to evaluate the rate of disinfection through the inhibition of *Escherichia coli* and *Pseudomonas aeruginosa*.

Treatment with plant extract (Figure 7.1): Three bottles were filled with 155 mL of influent water sample and dosed with each plant extract (MYSA and MYSE), different concentrations of 2 mg, 4 mg and 6 mg/155 mL equivalent to 0.31, 0.62 and 0.93 mg/L that serve as natural coagulant and disinfectant. The concentrations of *E. coli* and *Pseudomonas aeruginosa* in the raw and treated water samples on Eosin Methylene Blue agar (MBA) and Cetrimide agar (CA) were determined using spread plate method at 35-37℃ for 18-24 hrs, respectively. Then, coagulation activity and the rate of disinfection (inhibition of *E. coli* and *Pseudomonas aeruginosa*) for the treatment were calculated. The performances of test materials for turbidity improvement were measured every 30 mins and values were recorded before and after treatment. Experiments were conducted in duplicates and the percentage turbidity removal (%TR) was calculated using equation 7.1:

$$\%\ \text{Turbidity Removal} = \frac{(Tr_0 - Tf)x100}{T_{r0}} \qquad (7.1)$$

where, T_{r0} and T_f represent the initial and final turbidity (NTU) of water, respectively.

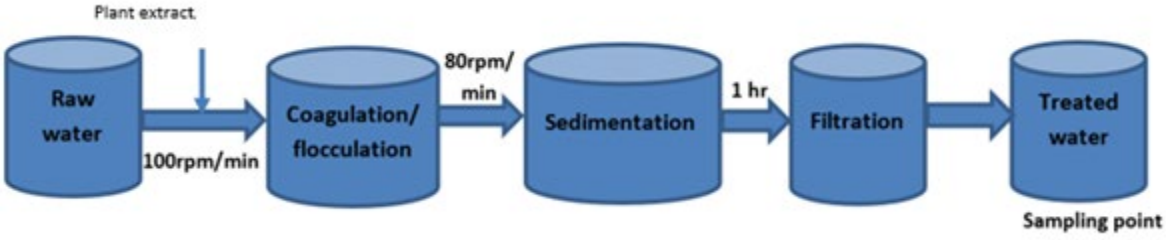

Figure 7.1: Schematic diagram used for water purification process in this study using plant extract.

7.3 RESULTS AND DISCUSSION

Pre-analyses of microbiological and physicochemical characteristics of the sampled water showed the presence of *E. coli* and *P. aeruginosa* as well as water quality characteristics used in the jar test experiment

(Table 7.1). Water treatment and bioremediation potential of MYSE and MYSA extracts were evidence in the rate of turbidity and pathogen removal from the wastewater. The final concentration of used extract in a litre of treated wastewater are 0.31, 0.62 and 0.93 mg/L. This is less than one milligram per litre as well as 0.2 mg/L of alum used for the treatment.

Table 7.1: Characterization of Amanzimtoti wastewater quality

Characteristic	**Wastewater influent**
Turbidity (NTU)	167
Total Suspended solids (TSS in mg/L)	719
Electrical conductivity (EC in mS/cm)	1012
pH	6.4
Temperature (℃)	27
Salinity (ppm)	497
Oxidation-Reduction Potential (mv)	-35.7
E. coli (CFU/mL)	1.30×10^3
P. aeruginosa (CFU/mL)	1.02×10^2

At different doses, the result of this study showed the performance of the plant extracts in causing flocculation and coagulation of suspended solids. It was observed that turbidity removal was directly proportional to settling time. The initial turbidity of the raw wastewater was 167 NTU. However, the recorded results indicated that the alum exhibited maximum turbidity reduction to 2.4 NTU (98.56%) (Figure 7.2). This was evident after an hour of settling time leading to a clear supernatant as shown in Figure 7.3. The coagulation efficiency varies per dose and the plant part used (Figure 7.4). The calculated turbidity removal was greater for ethanol extract at 0.31 mg/L (93.4%) followed by 0.93 mg/L (90.30%) and 0.62 mg/L (86.4%) for MYSE within the settling period. The aqueous extract (MYSA) however, showed less removal efficiency between 81-92% at 60 mins of contact and settling time across the treatment dosage (Figure 7.4). Although, the turbidity concentration of the final treated water is above the limit of 5 NTU by the WHO except the alum concentration. Nonetheless, there was great reduction in turbidity using the individual extract due to their ability to exhibit more turbidity reduction in water treatment.

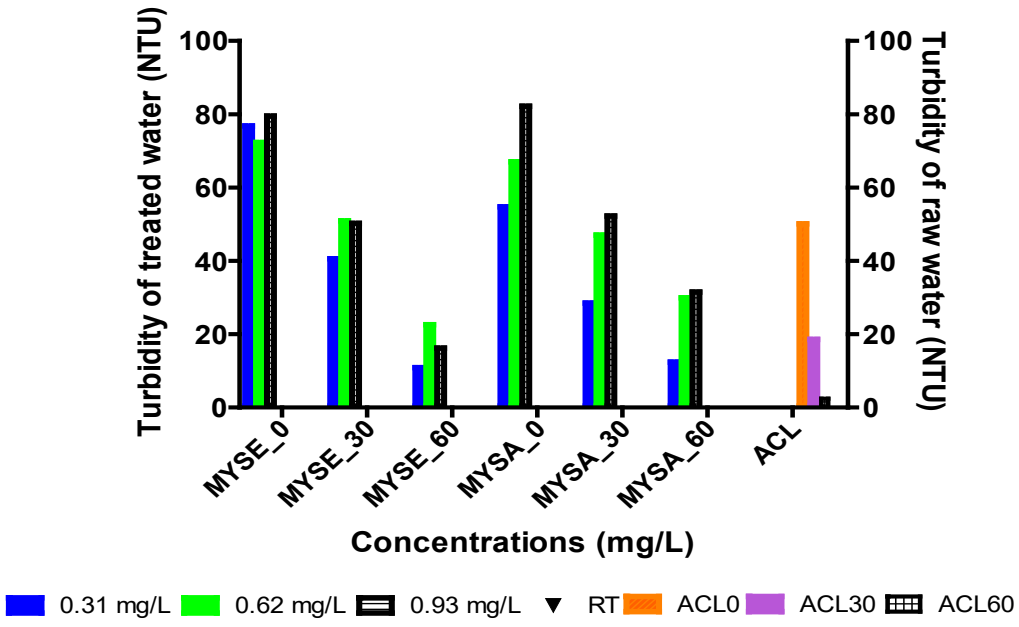

Figure 7.2: Turbidity removal and comparative performance to commercial coagulant used in this study.

In general, turbidity removal rose with increase in settling time throughout the experiment. As such, these results corroborated the earlier findings (Delelegn et al., 2018, Wagai et al., 2011) when the seeds of MO was used for water treatment. Although, there is no study that has explored the medicinal capacity or benefit of young stem of MO as a coagulant and disinfectant, however, the studies of *M. oleifera* leaves and seed powder as antigastritis and antiulcer activity have been reported (Pal et al., 1995, Delelegn et al., 2018). Therefore, these characteristics, in addition to its low cost, make any part of *M. oleifera* a better alternative for water clarification and its ability to reduce the chances of developing or accelerating ulcers when such water is consumed by humans.

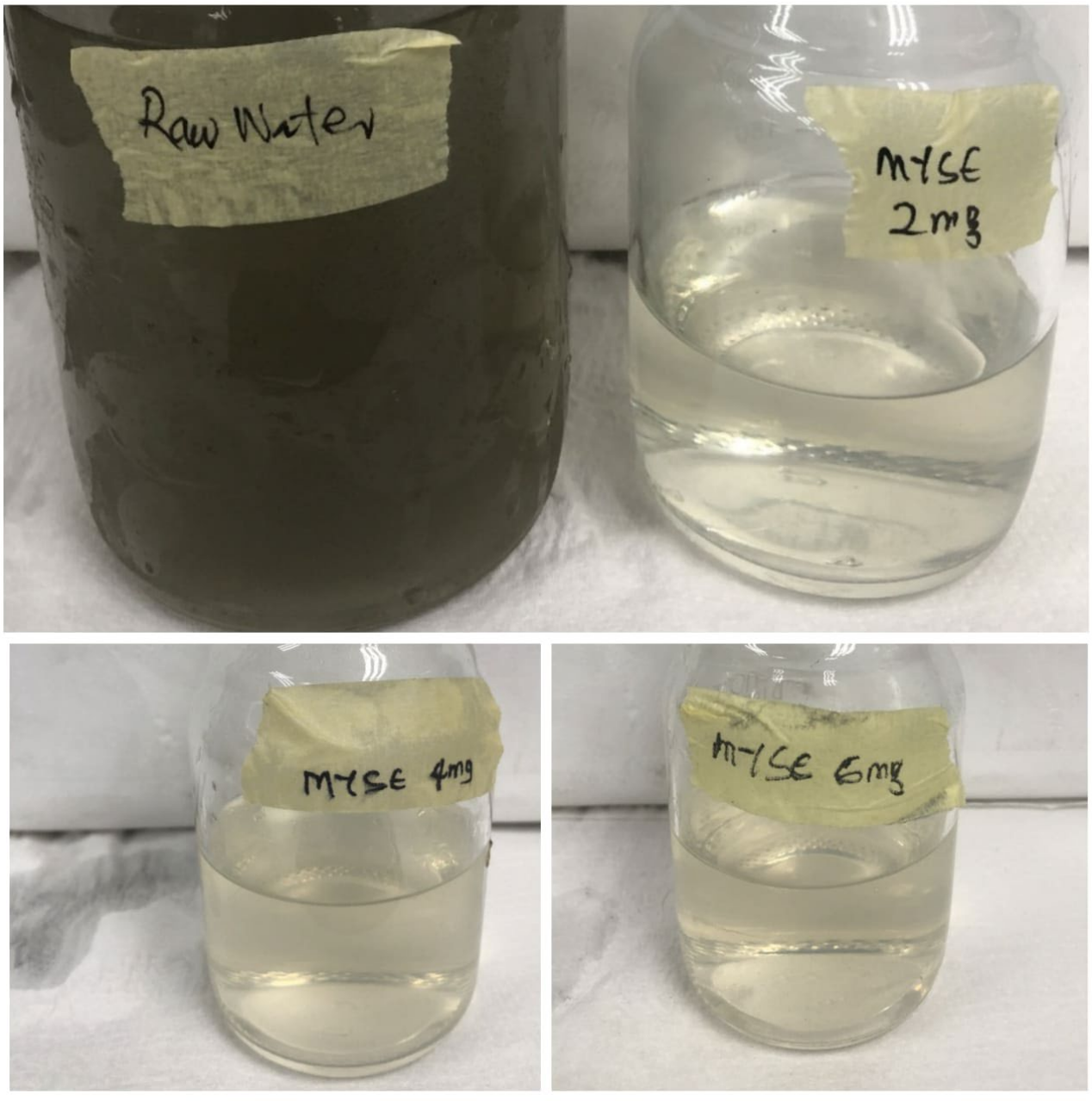

Figure 7.3: Ability of ethanol extract of *Moringa oleifera* young stem in removing turbidity during water treatment after settling and contact time of one hour with the raw wastewater. The final concentration of used extracts was 0.31, 0.62 and 0.93 mg per litre of treated wastewater.

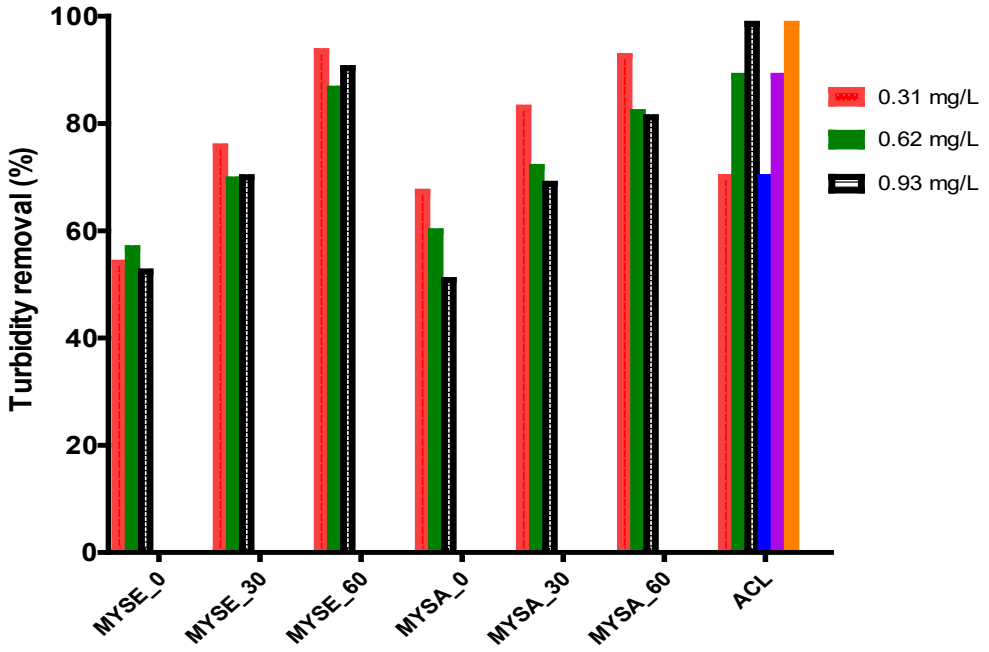

Figure 7.4: Percentage of turbidity reduction and variation from the control as observed after the treatment of wastewater samples with varied dosage of *Moringa oleifera* young stem extracts and alum.

The result of *Escherichia coli* and *Pseudomonas aeruginosa* counts expressed in both raw and treated water are expressed in CFU/mL as given in Figure 7.5 and 7.6. There was a significant reduction of *E. coli and P. aeruginosa* in the treated samples. After 1 hr of settling and contact time when the lowest concentration of MYSA (0.31 mg/L) was used for the treatment, the extract reduced initial *E. coli* concentration of 1.32 x 10^3 to 0 CFU/mL and *P. aeruginosa* of 1.02 x10^2 to 5 CFU/mL. Both extracts (MYSA and MYSE extracts) achieved 98.00-100% bacterial load reduction with percentage treatment variation as shown in Table 7.2 and Table 7.3, respectively for *E. coli* and *P. aeruginosa*. Like chlorine disinfection after an hour of settling time, 100% removal of *E. coli* was recorded at 0.31 and 0.93 mg/L of using MYSA and 0.93 mg/L of MYSE extract. Similar pattern was observed for *P. aeruginosa* at 0.31 mg/L of MYSE at 60 mins. This is in accord with other studies where the efficiency of *M. oleifera* leaf and seed powder at different concentrations was reported to reduce the density of coliform count, *E. coli, Bacillus thuringiensis* and *Pseudomonas aeruginosa* after water treatment (Delelegn et al., 2018, Madsen et al., 1987, Ghebremichael et al., 2005, Pritchard et al., 2009).

We can conclude that small concentration of extract used per litre has high treatment potential when compared with the efficiency of alum (0.2 mg/L). The capacity of the aqueous and ethanol MO young stem extracts in turbidity and bacterial removal increases with increase in settling time throughout the experiment. With bacterial removal efficiency between 99-100% and highest calculated turbidity removal recorded for ethanol extract at 0.31 mg/L (93.4%) followed by 0.93 mg/L (90.30%) and 0.62 mg/L (86.4%) for MYSE within the settling time, however, aqueous extract (MYSA) showed less removal efficiency between 81-92% at 60 mins of contact and settling time across the treatment dosage. This result corroborates the earlier findings (Delelegn et al., 2018, Wagai et al., 2011), where the seeds of MO was used for water treatment. Although, there is no study that has explore the medicinal capacity or benefit of young stem of MO as a coagulant and disinfectant however studies of *M. oleifera* leaves and seed powder as antigastritis and antiulcer activity have been reported (Pal et al., 1995, Delelegn et al., 2018). Therefore, these characteristics in addition to its low cost, makes any part of *M. oleifera* a better alternative for the treatment of drinking water and its ability to reduce the chances of developing or accelerating ulcers when such water is consumed by humans.

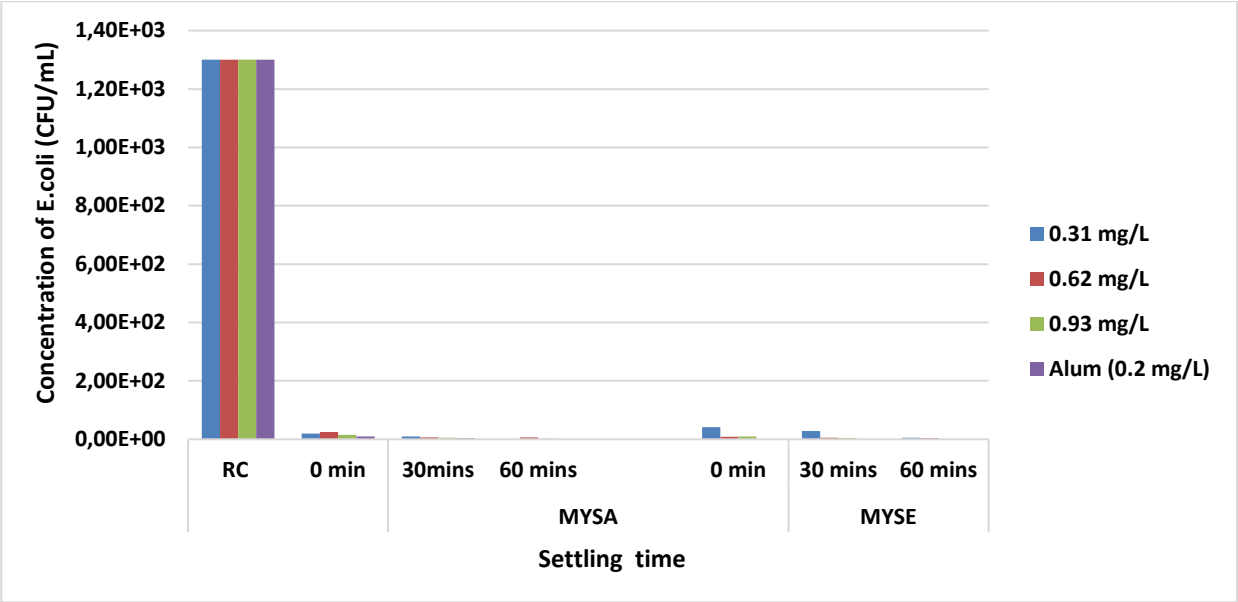

Figure 7.5: Effect of *Moringa oleifera* young stem aqueous and ethanol extracts on bacterial concentration (*E. coli* and *P. aeruginosa)* at different concentrations with no chemical or artificial coagulant.

Table 7.2: Percentage pathogen removal of aqueous *Moringa oleifera* young stem after 1 hr of settling time

Dose (mg/L)	MYSA					
	% E. coli removal			% P. aeruginosa removal		
	0 min	30 mins	60 mins	0 min	30 mins	60 mins
0,31	98,46	99,23	100,00	96,86	98,24	99,51
0,62	98,08	99,54	99,54	93,63	95,78	97,55
0,93	98,85	99,62	99,85	93,82	96,08	99,12
Alum	99,23	99,69	100,00	-	-	-

Table 7.3: Bacterial removal by *Moringa oleifera* young stem ethanol extract treatment after 1 hr of settling time

Dose (mg/L)	MYSE					
	% E. coli removal			% P. aeruginosa removal		
	0 min	30 mins	60 mins	0 min	30 mins	60 mins
0,31	96,85	97,85	99,62	99,22	99,51	100,00
0,62	99,31	99,62	99,69	95,88	97,06	98,53
0,93	99,23	99,69	100,00	99,02	99,51	99,80
Alum	99,23	99,69	100,00	-	-	-

CHAPTER 8: GENERAL CONCLUSION AND RECOMMENDATIONS

8.1 GENERAL CONCLUSIONS

In conclusion, this study detected the presence of SARS-CoV-2 RNA and Noroviruses (GI and GII) in both primary influent and treated effluent of all the four WWTPs as well as the river samples using RT-qPCR. The concentration of SARS-CoV-2 RNA in all four studied WWTPs was at varied concentrations especially in August 2021 when all the collected samples tested positive. The efficiency of the treatment plants in reducing viral load before discharging into the environmental bodies also showed that the WWTPs could reduce the concentration of the SARS-CoV-2 genome to a minimum concentration. However, further treatment to reduce the viral load is required before discharging into environmental media or reusing. Furthermore, the presence of this gene in rivers (the same pattern observed with WWTPs for August 2021 samples) indicated that there could be a partial treatment efficiency of the investigated sewerage system (study sites). The virological monitoring of treated effluent samples and river water into which the effluents are discharged could contribute to understanding the effectiveness of the WWTPs in the removal of the viruses. Therefore, this study shows the potential role of treating effluents released into surface water to contribute to the possible transmission of these viruses. In addition, it highlights the public health risk associated with the reuse of this water for different purposes.

Furthermore, the tested medicinal plants, *Ocimum gratissimum*, *M. oleifera, Sesame* plant and *Azadirachta indica* (neem) extracts showed a diverse range of microbial inhibition against waterborne and clinical pathogens. All extracts, aqueous, methanolic and ethanolic extracts revealed *in-vitro* antioxidant activity. Consequently, the antioxidants in the extracts reacted with DPPH radical; converting it to 1, 1-diphenyl-2-picryl hydrazine due to the extract's hydrogen donating ability. Nearly all the plants displayed phytochemical compounds with observable antioxidant and antimicrobial activities for bactericidal and fungicidal potentials against many pathogens. Water treatment using aqueous and ethanol crude extracts of *Moringa oleifera* young stem shows that small concentration of extract used per litre has high treatment potential when compared with the efficiency of alum. The capacity of the aqueous and ethanol MO young stem extracts in turbidity and bacterial removal rose with increase in settling time throughout the experiment with high bacterial removal efficiency within 60 mins of contact and settling time across the treatment dosage. Although, there is no study that has explored the medicinal capacity or benefit of young stem of MO as a coagulant and disinfectant, however good medicinal properties of *M. oleifera* leaves and seed powder have been mentioned by different researchers.

Great benefits of indigenous plants in water and wastewater treatment has also been noted, for example, the application of medicinal plants in water treatment could help in preventing and controlling diseases due to its rich source of antioxidants, antimicrobials and coagulating properties. This method is an effective technique in enhancing treatment processes without relying on importing chemicals for water purification. These features, in addition to its low cost, make any part of *M. oleifera* a better alternative if the seed or leaves are not locally available for treatment of drinking water. This will further help to reduce the chances of developing or accelerating ulcers when such water is consumed by humans. It can advertently result in creating employment opportunities; and cultivation of new cash crops. This could provide one or more solutions to environmental and health-related risks that chemical-based water treatment technologies could cause. Similarly, it can also provide veritable solution to some of the drawbacks of conventional chemical-based water treatment. Function as the most appropriate alternative and cheap surface and groundwater treatment for rural dwellers with no access to municipality's low provision of fresh and clean water. Growing plants on a large scale for industrial needs is one of the challenges to implementing plant-based natural coagulants and disinfectants. Therefore,

there could be an increase in industrial costs of using plants due to technical adaptation, new tools, machinery and new professionals.

8.2 LIMITATIONS OF THE STUDY

For the antiviral activity of the plant extracts: Inability to secure BSL-2/BSL-3 laboratory with the capacity for cell culture to analyse environmental samples has imposed significant limitation on the possibility of determining the antiviral potential of the analysed medicinal plant extracts. Further problems encountered while conducting the study was the unwillingness of experts in virological studies to collaborate with environmentalist in the virology field due to the anxiety of cross-contamination of samples. Considering the current gap in the potential of studied plants, we recommend further studies to determine the antiviral potential of the extracts and further collaboration in this regard.

8.3 RECOMMENDATIONS

The public health risks of the positive viral samples should be evaluated, and further investigation on the correlation of SARS-CoV-2 loads in wastewater with the number of COVID-19 cases around the study area should be investigated. The development and implementation of the wastewater-based epidemiological concept as complementary measure to survey waterborne SARS-CoV-2 infection and advanced treatment techniques for viral removal are recommended to prevent other outbreaks or infections. Using natural disinfectants and bio-coagulants as an alternative green treatment technique for global water management is cost-efficient and environmentally friendly, especially in developing countries. This study recommends that further investigation on the antimicrobial capacity of the plants against antibiotic-resistant bacteria as well as the ecotoxicology of plant-based treated water should be performed. Further studies on the protein isolation, purification and immobilization of the selected plants especially *M. oleifera* and Sesame seeds are necessary for sustainable and efficient water purification without regrowth or residual organic matters.

Additionally, more studies on the bioactive components of *Moringa stenopetala* in the family of *Moringaceae* should be explored to broaden the scope of this family in water treatment. In general, other medical plants should be promoted to improve water quality and mitigate environmental health-related problems associated with contaminated or partially treated water and wastewater. Based on the current finding, it is therefore recommended that further research should be conducted on the less investigated parts of the plants to determine and characterize their phytochemical benefits, antioxidant and antimicrobial activity against a wider range of microorganisms and multi-drug resistant organisms. Using High Performance Liquid Chromatography (HPLC), proper profiling and isolation of phytochemicals in this plant should be performed. Likewise, it is essential to consider these medicinal plants as natural antibacterial and antiviral materials for medicinal benefits and environmental health.

Another study gap observed in this study is the dearth of laboratories within the country with the facilities for virology research work or laboratories closer to the BSL-2 laboratory. Hence, more virology compliance laboratories and experts should be built and invested for environmentalists in particular. Considering the economic and environmental health nexus of viruses, this study recommends future work on the production of plant-based material to remove viral pathogens from environmental media. Moreover, antiviral analysis remains a huge project requiring a specialized cell culture laboratory, BSL2/3 laboratory and time that cannot be accomplished within a short period of this study. Thus, this study recommends that environmental/wastewater virology, an aspect of Wastewater-based epidemiology, be prioritized as a significant life-transforming project.

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APPENDIX A: ADVANTAGES AND DISADVANTAGES OF CONVENTIONAL AND NATURAL COAGULANTS/DISINFECTANTS FOR WATER TREATMENT

Coagulants				Disinfectants		Natural coagulant and disinfectants	
Aluminum sulfate (Alum)		Ferric salts		Chlorine		Plants	
Advantages	**Disadvantages**	**Disadvantages**	**Advantages**	**Advantages**	**Disadvantages**	**Advantages**	**Disadvantages**
1. Remove color and more efficient in turbidity and suspended solids removal. 2. Removal of high molecular mass fraction organic matter almost completely (>95%). 3. Removal of heavy metals	1. Expensive for dwellers of rural communities. 2. Incomplete removal of the total organic matter present in the water. 3. Less and low removal rate (approximately 35%) for a molecular mass matter of less than 500 g/mol. 4. Production of a large amount of non-biodegradable sludge. 5. Residual aluminum in the treated water leading to an increase in metal concentration. 6. Imposing both health and environmental hazards. These chemicals also affect the nervous system and cause skeletal complications, with possible connections to numerous diseases such as Parkinson, Alzheimer and Lou Gehrig diseases.		Remove (NOM).	Effective in killing pathogenic bacteria, fungi, and viruses.	1. Useful when the water is not turbid (less than 1 NTU), and the pH is below 8.0. 2. It is ineffective against cysts and eggs of protozoa such as Cryptosporidium and Giardia. 3. The use of chlorine influences water quality because Cl_2 residues change the taste of drinking water. 4. Generate harmful disinfection by-products.	1. Cost-effective 2. Biodegradable, lucrative and environmental-friendly material. The process could absorb solid particles, clear the water, and remove up to 90-99.9% of the bacteria from water. 3. Inexpensive and provide an attractive treatment option for resources poor communities, especially in the rural areas. 4. No harmful residual product, low index of residual bio-degradable sludge production and lower sludge volume. 5. Removal of colour. 6. Readily available, production of less hazardous, noncorrosive by-product. 7. Low toxicity and large number of surface charges that increases the efficiency of the coagulation process. 8. Provide sustainable methods of water treatment with no extreme pH. 9. Safer for human health and the environment.	1. Require high concentration as biocoagulant than the required dosage for aluminum sulphate. 2. It could promote the regrowth of pathogens in treated water due to dissolved organic matter (DOM).